PHYSIOLOGICAL TRAITS OF *Saccharomyces cerevisiae* STRAINS ISOLATED FROM TRADITIONAL WINES IN TURKEY

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PHYSIOLOGICAL TRAITS OF *Saccharomyces cerevisiae* STRAINS ISOLATED FROM TRADITIONAL WINES IN TURKEY

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ABSTRACT

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Saccharomyces cerevisiae is the yeast species that is the most important among enological microorganisms. The main characteristic properties that differ *Saccharomyces* from other yeasts are high ethanol and sulfur dioxide tolerance. In this study, Emir and Kalecik Karası grape varieties, harvested in vintage were used in traditional wine production. 37 strains were isolated from washing water of grapes, grape juice and samples taken during wine production. Selected *Saccharomyces cerevisiae* strains were identified by sequence comparison of PCR amplified sequence analysis of the 5.8S internal transcribed spacer (ITS) ribosomal DNA (rDNA) region and carbohydrate fermentation test. Finally isolates were characterized by RAPD-PCR method. The 3 of selected isolates were identified as *Saccharomyces cerevisiae*. Characterized strains were used as starter cultures while wine making. Aromatic compounds of produced wines were analyzed by GC-MS.

Keywords: *Saccharomyces cerevisiae*, wine starter culture, identification, aromatic compounds, rDNA, RAPD-PCR

ÖZ

TÜRKİYEDE GELENEKSEL YÖNTEMLERLE ÜRETİLEN ŞARAPLARDAN İZOLE EDİLEN *Saccharomyces cerevisiae* SUŞLARININ FİZYOLOJİK ÖZELLİKLERİ

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Saccharomyces cerevisiae şarap yapımında rol alanlar arasında en yüksek öneme sahip olan mikroorganizma türüdür. *Saccharomyces cerevisiae* türünü diğer mayalardan ayıran en önemli özelliği etil alkol ve sülfür dioksite olan direncidir. Bu çalışmada, bağ bozumunda hasat edilen Emir ve Kalecik Karası üzüm türleri, geleneksel yöntemlerle şarap üretiminde kullanılmıştır. Üzümlerin yıkama suyundan, üzüm suyundan ve fermantasyon esnasında alınan örneklerden 37 suş izole edilmiştir. İzole edilen suşlar etil alkole dirençlerine göre seçilmiştir. Teknolojik olarak yeterli bulunan suşlar 5.8S internal transcribed spacer (ITS) ribozomal DNA (rDNA) alanlarının karşılaştırmalı sekanslanması ve karbonhidrat fermentasyon testi ile tanılanmıştır. Son olarak *Saccharomyces cerevisiae* suşlar RAPD-PCR yöntemi ile karakterize edilmiştir. İzolatların 3 tanesi *Saccharomyces cerevisiae* olarak tanılanmıştır. Bu suşlar bir sonraki yıl hasat edilen üzümlerden şarap üretiminde starter kültür olarak kullanılmıştır. Üretilen şarapların aromatik bileşenleri GC-MS ile analiz edilmiştir.

Anahtar Kelimeler: *Saccharomyces cerevisiae*, şarap starter kültürü, tanılama, aromatic bileşenler, rDNA, RAPD-PCR

To My Family…

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INTRODUCTION

1.1 Saccharomyces cerevisiae

Fermented foods have been consuming since Neolithic Era by humans. However, understanding phenomena behind the process took long time. Ancients described fermentation as boiling that is caused by reaction between substances of must. Yeast cells were observed in 17th century but the relation between yeasts and fermentation could not be found. In late 18th century and 19th century, firstly relationship between fermentation and a living organism found, then yeasts are defined as a living organism. After describing yeasts as living organism, yeasts were related with fermentative activity of sugar and fermentation conditions were determined. Finally, yeasts, responsible from fermenting beer were named as zuckerpliz which means sugar fungus in English and saccharomyces in Latin.

Saccharomyces belongs to fungi kingdom and is a specie of yeasts. Since *Saccharomyces* is the simplest eukaryote, it has been observed and studied intensively as model organism for higher eukaryotic organisms (Michels, 2002; Replansky, Koufopanou, Greig, & Bell, 2008). *Saccharomyces* is a glucophilic microorganism, so the yeast prefers glucose over fructose as long as it is present in the grape must and when glucose is exhausted utilizes fructose (Tronchoni, Gamero, Arroyo-López, Barrio, & Querol, 2009).

Traditionally, wine production is done by naturally contaminated microorganisms; however, starter culture is used in the mass production in order to provide same quality,

flavor and aroma (Hyma, Saerens, Verstrepen, & Fay, 2011). Traditional wine is produced by spontaneous fermentation of indigenous yeast community present in grape must (Pinna, Budroni, Giordano, Usai, & Farris, 2000). However, most of the wine producers inoculates *Saccharomyces cerevisiae* strain into grape must as starter culture to start fermentation process due to inconsistency and unpredictable wine production changing according to region and year in case of spontaneous fermentation (Holzapfel, 2002).

Recently, strain isolations from traditional fermentation medium and natural flora show increasing trend. References, shown below are the some of the latest studies aimed to determine diversity of yeast flora of wineries and vineyards in different regions.

Table 1.1: Studies indicating diversity of yeast flora of wineries and vineyards.

Source	Region	Source
Winery	Italy	(Cocolin, Pepe, Comitini, Comi, & Ciani, 2004)
Vineyard	France	(Valero, Cambon, Schuller, Casal, & Dequin, 2007)
Winery	South America	(Martínez, Gac, Lavín, & Ganga, 2004)
Vineyard	Italy	(Settanni, Sannino, Francesca, Guarcello, & Moschetti, 2012)
Winery	Spain	(Vilanova & Massneuf-pomarède, 2005)
Vineyard	Portugal	(Schuller, Valero, Dequin, & Casal, 2004)
Vineyard	Italy	(Blanco, Ramilo, Cerdeira, & Orriols, 2006)
Winery	Chili	(Salinas et al., 2010)
Vineyard	Spain	(Clavijo, Calderón, & Paneque, 2010)
Vineyard	China	(Li et al., 2010)
Vineyard	India	(Chavan et al., 2009)
Vineyard	Germany	(Brysch-Herzberg & Seidel, 2015)
Vineyard	Japan	(Takahashi, Ohta, Masaki, Mizuno, & Goto-Yamamoto, 2014)

1.1.1 Cytology

Saccharomyces cerevisiae is one of the simplest member of eukaryotes (Engel et al., 2014; Trivedi, Fantin, & Tustanoff, 1985). The cell consists of two envelopes which are periplasmic space and protoplasm. The cell contains cell wall, plasmic membrane, cytoplasm, organelles and the nucleus. Cell wall is dynamic and multifunctional organelle that provides several functions such as protection against osmotic pressure, organization and rigidity, sites for interactions. Cell wall represents 15-25% of dry weight of the cell.

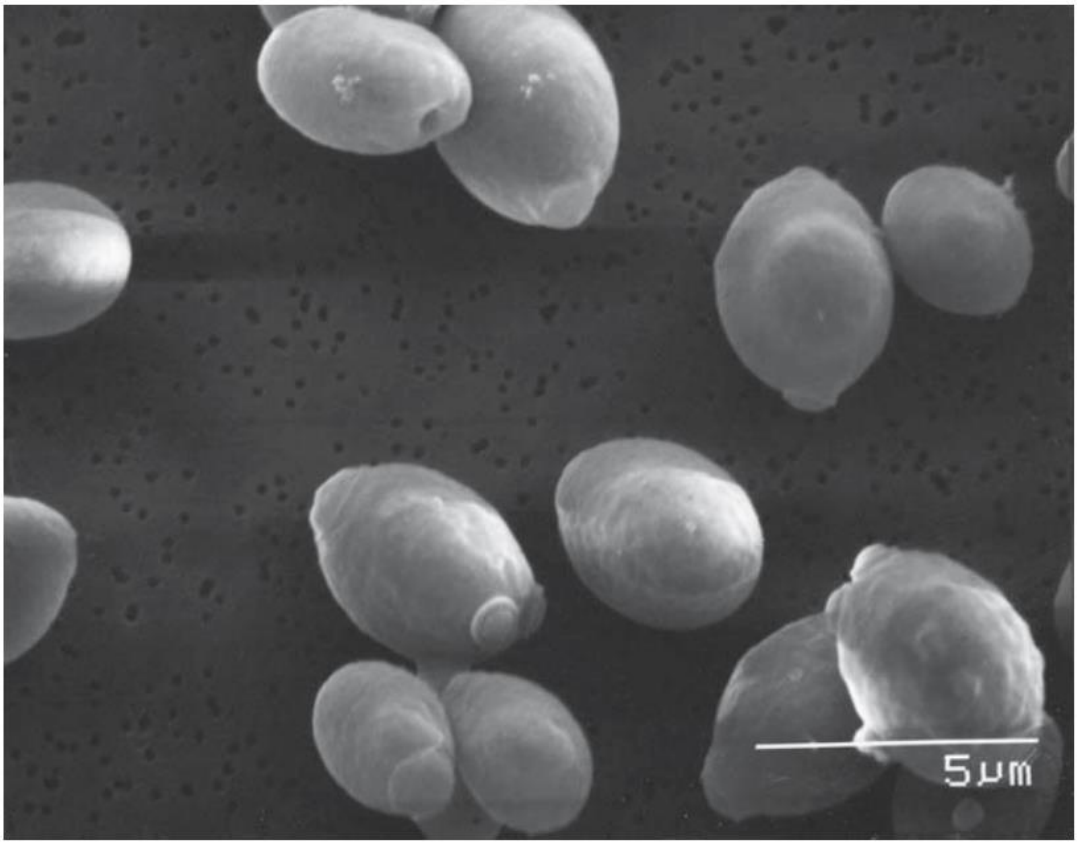

Figure 1.1: Scanning electron microscopy image of *Saccharomyces cerevisiae* (Fugelsang & Edwards, 2007)

The plasmic membrane of yeasts provides exchanges of materials between periplasmic space and surrounding environment of the cell. Glucose and arginine penetration speed into plasmic membrane is slower in the presence of ethanol as a result of decrease in membrane ATPase activity. Temperature and ethanol show synergistic effect on membrane ATPase activity, as temperature increases, effect of ethanol on enzyme activity increases. Plasmic membrane hosts receptor proteins that provide information about environmental stimulants.

Cytoplasm is a buffered solution with pH 5-6 which consists of water and soluble substances such as glycogen, glycolysis and alcoholic fermentation enzymes.

Nucleus is sphere having 1-2 micrometer diameter. The nuclear envelope ephemeral pores with changing locations continuously. Saccharomyces cerevisiae's DNA length is approximately 14 000 kb in haploid strain. DNA in the nucleus is organized into chromosomes. Saccharomyces cerevisiae has 16 chromosomes with sizes ranging from 200 to 2000 kb.

Table 1.2: Groups of *Saccharomyces* Species

Group	Species
Group I Saccharomyces sensu stricto	Saccharomyces cerevisiae
	Saccharomyces bayanus
	Saccharomyces paradoxus
	Saccharomyces pastorianus
Group II Saccharomyces sensu lato	Saccharomyces dairensis
	Saccharomyces exiguus
	Saccharomyces unisporus
	Saccharomyces servazzi
	Saccharomyces castelli
Group III	Saccharomyces kluyveri

Some of the yeast strains are capable of producing proteinic toxins which have the ability to kill sensitive strains. These producing strains are called as killer strains and strains which are not resistant to toxins are called as sensitive strains. Another type of yeasts is resistant but not producing these toxins.

1.1.2 Taxonomy

Classifications made by Barnett (2000) is the most valid among taxonomic studies on Saccharomyces. The classifications according to phenotypic similarities are;

- cell morphology,
- ability of spore formation,
- assimilation of different carbon sources,
- usage of nitrates,
- growth-factor needs,
- tolerance to cycloheximide.

The use of these classifications on *Saccharomyces cerevisiae* were studied in detail by Ribéreau-Gayon (1986). In the light of Ribéreau-Gayon's studies; the API 20 C system was designed by Lafon-Lafourcade and Joyeux and Cuinier and Leveau for the identification of wine yeasts (Dubourdieu & Gayon, 2006). It contains eight carbon source fermentation tests, 10 assimilation tests and a cycloheximide tolerance test (Hayford & Jespersen, 1999). Then, the API 50 CH system was advanced for a more complete identification.

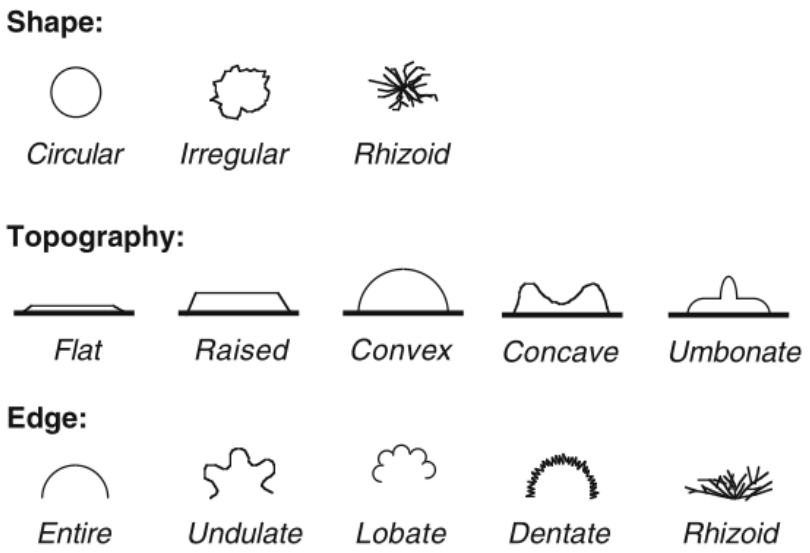

Figure 1.2: Types of colony shape (Fugelsang & Edwards, 2007).

Saccharomyces appear globose or ovoidal cells under microscope images (Sheu, Barral, & Snyder, 2000). Colonies are smooth, usually flat, occasionally raised and opaque (Voordeckers et al., 2012). *Saccharomyces cerevisiae* is able to ferment glucose, sucrose, and raffinose and assimilate glucose, sucrose, maltose, raffinose, and ethanol but not nitrate. *Saccharomyces* cannot use five-carbon sugars as carbon source (Wickerham & Burton, 1948).

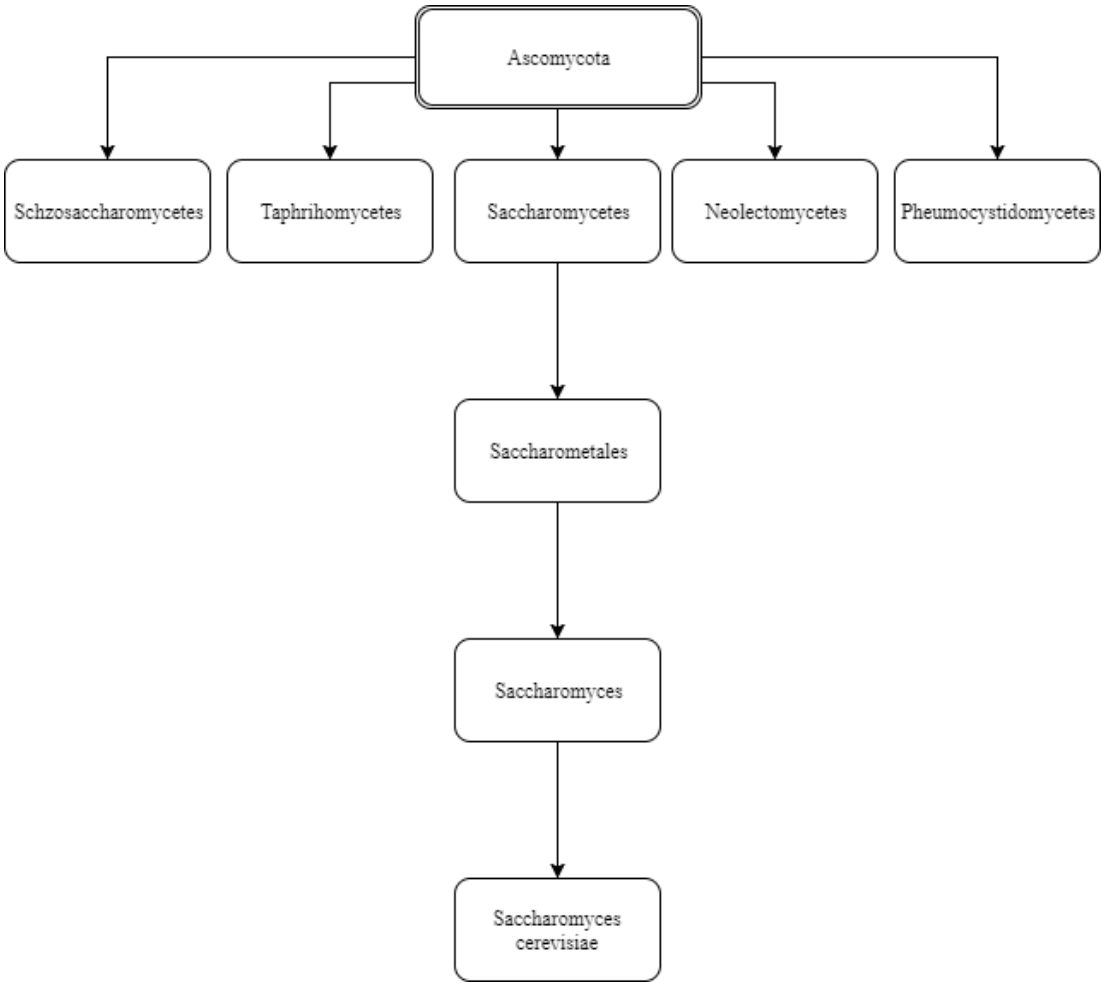

Figure 1.3: Classification of *Saccharomyces cerevisiae* (Fugelsang & Edwards, 2007).

Yeasts that are related with wine making are also divided into pragmatic informal groups. Yeasts that initiate the fermentation are informally named as “fermentative

yeasts". After fermentation process, some yeasts grow on the top layer of wine that contacts with oxygen and forms film layer, these yeasts are mentioned as "film yeasts" (Fleet, 1992).

1.1.3 Ecology

Saccharomyces is one of indigenous microorganism of vineyard flora, but the populations are often lesser comparing with other yeasts (Fleet, 1993). Several studies show that *Saccharomyces* on grapes constitutes below 0.1% of naturally occurring yeast flora population (Mercado & Combina, 2010). On the other hand, during the beginning of alcoholic fermentation, *non-Saccharomyces* yeasts increase in number and reach peak population. Although *Hanseniaspora/Kloeckera* are normally dominant on grapes at harvest, they are inhibited after the beginning of alcoholic fermentation. In agreement and additionally; comparing with *Saccharomyces*, many *non-Saccharomyces* show lower tolerance to ethanol. This causes those yeasts' inhibition when the ethanol amount reaches 5%v/v in medium (Deak, 2008).

Only 15 yeast species, exist on grapes, are determined as relevant to alcoholic fermentation, and diseases in wine. The yeast present in grape musts, such as *Candida*, *Cryptococcus*, *Debaryomyces*, *Hansenula*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, and *Rhodotorula* are called "native," "natural," or "wild" yeasts (Zott et al., 2010). These yeasts originate in the vineyard or winery. Recently, the microorganisms those commonly present in grape must and do not belong to the genus *Saccharomyces* are described as "*non-Saccharomyces* yeasts". After fermentation stage, addition to *Saccharomyces*, must include *Dekkera*/ *Brettanomyces*, film yeasts, *Saccharomycodes*, and *Zygosaccharomyces*, which are the cause of wine spoilage (Bezerra-Bussoli, Baffi, Gomes, & Da-Silva, 2013).

As numbers of *non-Saccharomyces* yeasts decreases with increasing ethanol concentration, *Saccharomyces* surpasses and finally alcoholic fermentation is

completed by *Saccharomyces* (Granchi, Bosco, Messini, & Vincenzini, 1999). As a result, in commercial wine production, must is inoculated with commercial cultures of *Saccharomyces* in order to prevent from microbiological spoilage and residual sugar (Capece et al., 2010).

When the culture reaches stationary phase, it is expected that at least half of the fermentable sugar has been utilized. *Saccharomyces* is capable of utilizing remaining sugar until dryness is reached.

1.2 Identification of Saccharomyces cerevisiae

Conventional morphological, cultural, biochemical and molecular techniques are methods used to identify different yeast species (Chavan et al., 2009). Phenotypic characterization is not sufficient for identification of yeast species having similar characteristics (Bernardi, de Melo Pereira, Cardoso, Dias, & Schwan, 2008). Molecular techniques are alternatives to biochemical methods for the identification and characterization of microorganisms (Bernardi et al., 2008).

Spontaneous fermentation occurs by participation of several yeast species native of the grape skin. Yeasts from *Kloeckera*, *Hanseniaspora* and *Candida* genera are dominant at the beginning of the fermentation. It is followed by other species such as *Metschnikowia* and *Pichia* in the middle of fermentation, when the ethanol concentration is approximately to 3–4%. The alcohol-resistant strains of the Saccharomyces sensu stricto group of yeasts dominate the last part of fermentation (Redžepović, Orlić, Sikora, Majdak, & Pretorius, 2002).

Examination of the cellular carbohydrate source fermentation ability in liquid medium is one of the tests used to identify microorganisms (De Araújo Vicente et al., 2006).

Common distinguishing physiological characteristic of *Saccharomyces* is its ability for vigorous anaerobic or semi-anaerobic fermentation, and ethanol production. Sugars including D-glucose, D-fructose, D-mannose, and D-maltose can be utilized by this genus; however, lactose, pentose, alditols, and citrate as carbon sources cannot be utilized, nitrate cannot be assimilated as a nitrogen source, exogenous urea can be hydrolyzed by any of them (Wickerham & Burton, 1948).

1.2.1. Phenotypic Characteristics

Phenotypic identification methods are typically based on reactions to different chemicals. Commercial test kits are superior after advances on the classical methods of biochemical identification. Commercial test kits consist of miniaturized and multi test units. In test kit systems, the single cell isolates grow in a group of growth media, reflecting alterations in medium. Comparison of results with microorganisms' known patterns can be used to identify unknown cultures with the aid of bioinformatics.

The Analytical Profile Index (API; bioMérieux, Hazelwood, MO) is widely known system for identification of microorganisms. The API 20E system is a miniaturized microtube system that contains 20 small wells to perform 23 standards utilization tests to isolated colonies of microorganism on growth medium. Yeast Identification can be done by using API 20C AUX in 48–72 h.

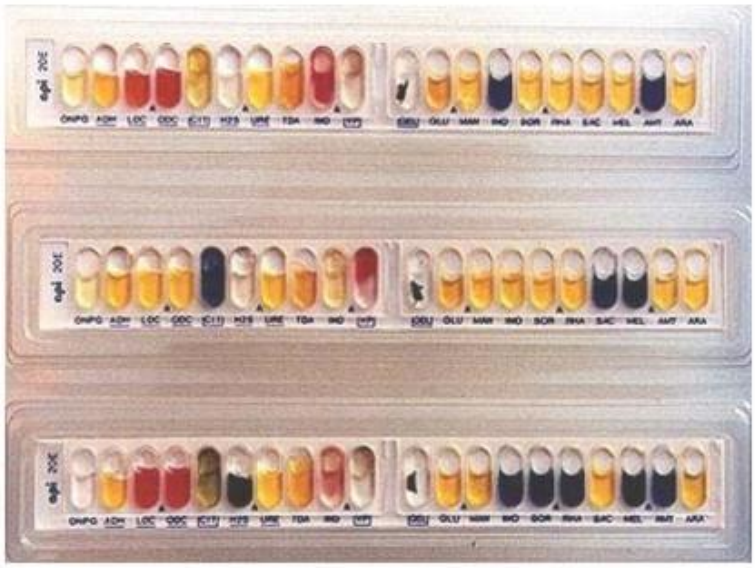

Figure 1.4: API 20E system (bioMérieux, Lyon, France)

The main characteristics of the *Saccharomyces*, as they are mentioned above, ellipsoidal or cylindrical cells, and the formation of smooth walls. Vigorous fermentation capabilities of carbon sources sucrose, raffinose and trehalose. Reproducible and reliable identification results can be obtained by several commercially available kits such as API 20C and ID 32C systems (bioMérieux, Lyon, France) and the Biolog YT plate system (Biolog Inc., California, USA).

1.2.2 Genomic Characteristics

Frequent exchange of genetic material between Saccharomyces sensu stricto and other members of Saccharomyces group, high genetic variability and the limited ribosomal RNA divergence cause difficulties in discrimination of these two groups using classical microbiological methods (Chang et al., 2007). Therefore, several molecular techniques based on replication of whole or some fragments of genomic material. Some of the molecular methods used for identification of yeasts are;

- Mitochondrial DNA Restriction Profiling
- DNA–DNA Reassociation
- Pulsed-Field Electrophoresis Karyotyping
- Mitochondrial DNA Restriction Endonuclease Profiling
- Randomly Amplified Polymorphic DNAs (RAPDs)
- PCR amplification using primers based on intron splicing sites
- The analysis of the 18S rRNA gene
- The analysis of the internal transcribed spacers (ITS) region, PCR-RFLP
- The analysis of the D1/D2 region of the 26S rRNA gene
- Amplified Fragment Length Polymorphism based analysis
- Microsatellite-based techniques.

Molecular methods used in combination with phenotypic methods are common in recent studies. Some of the studies on the identification of yeasts in wine making are listed below.

Table 1.3: Molecular methods used to identify microorganisms in different studies.

Molecular Based Identification Tests	**References**
RAPD-PCR, PFGE, SAU-PCR	(Cocolin et al., 2004)
RAPD-PCR, PFGE, mtDNA	(Fernández-Espinar, López, Ramón, Bartra, & Querol, 2001)
RAPD-PCR, PCR-TTGE	(Giusto et al., 2006)
PCR-RFLP, ITS	(Redžepović et al., 2002)
PCR-RFLP, ITS	(De Araújo Vicente et al., 2006)
PFGE, PCR-PFLP, mtDNA	(Bernardi et al., 2008)
PCR-RFLP, ITS	(Valero et al., 2007)
PFGE, PCR-PFLP	(Christine et al., 2007)
RAPD-PCR, PFGE	(Daran-Lapujade et al., 2003)
PCR-RFLP, mtDNA	(Martínez et al., 2004)
PCR-TTGE	(Manzano, Cocolin, Iacumin, Cantoni, & Comi, 2005)
PFGE	(Ryu, Mikata, Murooka, & Kaneko, 1998)
PCR-RFLP, mtDNA, ITS	(Pramateftaki, Lanaridis, & Typas, 2000)
PFGE, PCR-PFLP, SAU-PCR	(Gil-Lamaignere, Roilides, Hacker, & Müller, 2003)

1.2.2.1 Real Time PCR

In recent years, real-time quantitative PCR (QPCR) have been used to detect and quantify microorganisms in different ecosystems. The main advantage of QPCR is the ability of detection at lower microbial load levels (Martorell, Querol, & Ferna, 2005). This method can detect as low as one cell per milliliter. It is one of the used methods to detect the yeast populations in must and fermentation samples.

Method is based on monitoring formation of PCR products continuously during the reaction. Changes in the intensity of fluorogenic reporter give proportional concentration information about sample. This method requires prior optimization for obtaining standard curve (Granchi et al., 1999).

The TaqMan method uses a fluorogenic probe to monitor formation of polymerase chain reaction products. This probe is an oligonucleotide, and it contains a reporter dye covalently attached at the 5' end and a quencher dye covalently attached at the 3' end (Savazzini & Martinelli, 2006). The quencher dye absorbs the emission wavelength of the reporter dye and as long as the probe is intact, reporter dye's light emission is inhibited by quencher dye. During PCR reaction, the probe is hydrolysed by the TaqMan DNA polymerase enzyme. The quencher dye is separated from the reporter dye. As a result, fluorescence emission of the reporter dye increases, which is quantitative for the initial amount of template (Zott et al., 2010).

Since QPCR combines microorganism identification in sample and initial amount of template in one single step, it is advantageous comparing with other methods.

1.2.2.2 RAPD PCR

The RAPD method has been initially used to detect polymorphism in genetic mapping, taxonomy and phylogenetic studies (Capece, Salzano, and Romano 2003).

Analysis by RAPD-PCR contains the use of small random primers such as M13 to amplify fragments of template DNA belonging to related microorganism. The single primer will anneal at any point on the genome where a near-complementary sequence exists, and if two priming sites are sufficiently close, then PCR amplifies the fragment between them. Fragments of numerous sizes may be produced according to number of priming sites (Cocolin et al., 2004). Formed patterns on electrophoresis gel are specific

for the microorganisms. This technique is rapid, easy and suitable for typing and identification of microorganisms, but several problems are present. While RAPD have been used extensively for diverse studies, difficulties of its reproducibility causes criticisms on this method. Main reason of this is lacking of proper optimization and validation of the technique in different strains and species (Atienzar & Jha, 2006). However, some scientists claim that it is a reliable method .

Firstly, the method is facing reproducibility issues. The whole patterns of DNA template on electrophoresis gel are not always the same in independent experiments. Secondly, the results are affected by the nucleotide sequence of the primer used. After the PCR products have been resolved, genetic distance is calculated manually as the number of different bands between two patterns divided by the sum of all bands in the same patterns. The dice matrix obtained from relation level is used to create an unrooted dendrogram (Capece et al. 2010).

1.2.2.3 PFGE

Pulsed field gel electrophoresis is widely used in determination of eukaryote genomics. PFGE is a technique, used to generate a DNA fingerprint for isolates and a derivative of conventional gel electrophoresis. Physiologically similar strains can be evaluated in rapid, relatively easy and inexpensive way by electrophoretic karyotyping.

Localization of genomic material, physical genome mapping in various organisms are performed by PFGE with combination of other molecular methods. Electrophoresis is run in the pulsed electrical field based on the fractionation of high-molecular-weight fragments of DNA digested such as chromosomes. This technique provides satisfying separation of DNA fragments of up to 10 Mb. Chromosomal DNA of prokaryotes and lower eukaryotes are considered as in this range (Basim, 2001).

Genomic chromosome patterns obtained by PFGE are called electrophoretic karyotypes. By comparing the results from this method and DNA sequencing, it has been confirmed that chromosomal patterns of the two strains are identical when DNA sequence similarity is over 85%, on the other hand, lower DNA similarity causes completely different patterns on electrophoresis gel. Pulsed Field Gel Electrophoresis is not reliable comparing with DNA base sequence comparisons, but it provides important complementary results (Vilanova & Massneuf-pomarède, 2005).

In differentiation of wine yeasts, karyotyping provides several practical solutions. The electrophoresis gel images of Saccharomyces sensu stricto species are similar and are different from other species. A cluster analysis has showed that the Saccharomyces sensu stricto strains could be divided into four group that represent the four species.

1.2.2.4 ITS 5.8S Sequencing

For *Saccharomyces cerevisiae*, several methods have been proven as suitable to identify members of this species (Chavan et al., 2009). One of them is the restriction analysis of the rRNA region spanning the 5.8S gene and the two internal transcribed spacers (ITSs) (5.8S-ITS region). The amplified DNA electrophoresis patterns of *Saccharomyces cerevisiae* when its DNA is digested with the endonuclease *Cfo*I, *Hae*III, or *Hinf*I identify this species accurately (Martorell et al., 2005).

Sequence comparisons of the rRNA genes have shown a relatively high degree of evolutionary conservation. The region spanning the internal transcribed spacers (ITSs) and the entire 5.8S rRNA gene is amplified by PCR using pITS1 and pITS4 which are derived from conserved regions of the 18S and 28S rRNA genes, respectively (Bezerra-Bussoli et al., 2013).

ITS1 TCCGTAGGTGAACCTGCGG (White, Bruns, Lee, & Taylor, 1990)

ITS4 TCCTCCGCTTATTGATATGC (White et al., 1990)

For Saccharomyces sensu stricto species, the size of amplicons is over 800 base pairs and less than 800 base pairs for the other Saccharomyces species.

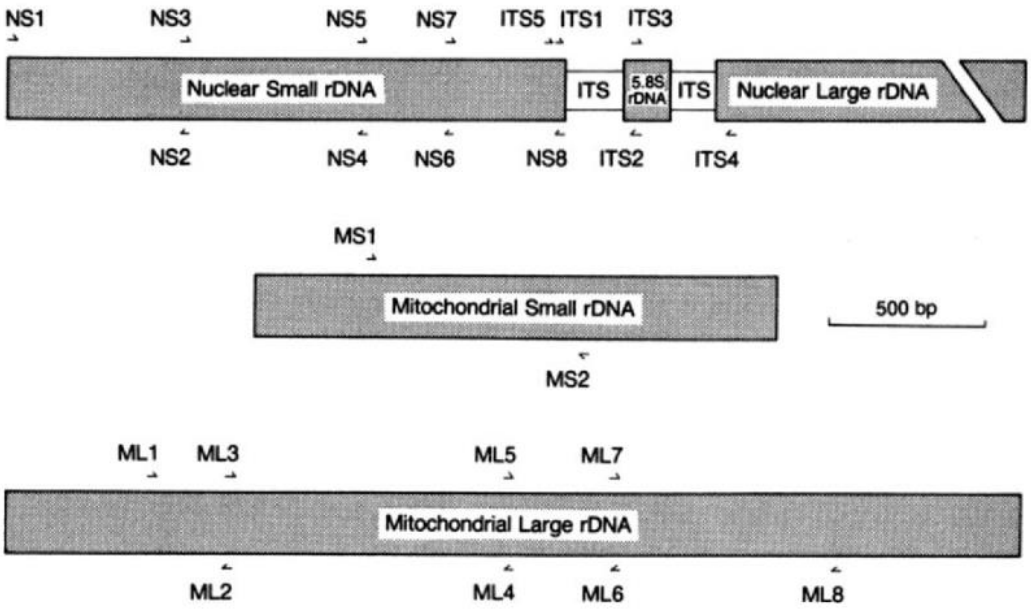

Figure 1.5: ITS regions in mitochondrial rDNA (T. White, Bruns, Lee, & Taylor, 1990)

1.3 Wine and Wine Production

1.3.1 Destemming and Crushing

Crushing grapes breaks the skins of the berries and allows the juice to flow. Stems and seeds contain high amount of phenolic materials. Phenolic compounds provide

bitterness and astringency to wine. Therefore, destemming is done to remove stems and crushing is done gently not to harm seeds (Fugelsang & Edwards, 2007).

1.3.2 Maceration

Maceration of musts before initiation of fermentation is a technique in red wine processing that increases diffused amount of desirable grape flavor compounds and pigments from grape skins to must. Normally, musts are held at temperatures between 15ºC and 20ºC for 12 to 24 h. Maceration duration can be prolonged up to 1 to 2 weeks. During maceration, *non-Saccharomyces* yeasts dominate flora, since they are able to grow faster than *Saccharomyces* at lower temperatures. Must temperature should be lowered rapidly. Dry ice, or liquid CO_2 is two of materials used to decrease temperature of must. Warmer maceration temperatures encourage growth of spoilage microorganisms such as *Lactobacillus*. Sulfur dioxide is used for its antioxidative and antimicrobial properties in wine making. Addition of 50 to 75 mg/L total SO_2 is adequate for inhibition of spoilage microorganisms. Lysozyme addition to must is also used to reduce initial populations of Gram-positive bacteria. Even if the musts are maintained at cool temperatures, other undesirable microorganisms such as acetic acid bacteria or some *non-Saccharomyces* yeasts can still grow. Du Toit and Lambrechts (2002) observed acetic acid bacteria to increase from 10^3 up to 10^5 CFU/mL in one Cabernet Sauvignon must, held at 15ºC to 18ºC for 3 days with 40 to 50 mg/kg SO_2.

Significant quantities of pigment and colors are extracted from the grape solids during the prolonged, cold skin contact time. However, little tannin is extracted during the cold soak because the juice contains no alcohol.

1.3.2 Alcoholic Fermentation

Initiation of fermentation by indigenous *Saccharomyces cerevisiae* takes longer time comparing with commercial starter cultures. Prolonged initiation time causes several problems such as increased activity of spoilage microorganisms. In order to prevent from such defects, commercial wine starter cultures are used. Main aim of using a starter culture is to initiate fermentation as quickly as possible while suppressing the spoilage microorganisms by creating competition for nutrients.

1.3.2 Malolactic Fermentation

During malolactic fermentation, L-malic acid is metabolized to L-lactic acid and CO_2. Malolactic fermentation commercially carried out by *Oenococcus oeni*. Desirable effect of malolactic fermentation is reduced acidity of wine and slightly increase in pH.

1.3.3 Aging and Storage

After completion of fermentation process, wines may preferably be aged in wooden, mostly oak barrels to enhance aromatic compounds. Since wood is permeable for ethanol, water, and oxygen; these molecules diffuse into or outside to the barrel. Aging in the barrel may cause growth of many spoilage microorganisms. Activity of *Acetobacter, Brettanomyces, Saccharomycodes, Zygosaccharomyces,* film yeasts are the common reason for wine faults. Relative humidity of ambient air has an effect on water and ethanol diffusion. Headspace in the barrel is another factor effects quality of wine. Headspace provide space for oxygen to which promotes growth of oxidative microorganisms.

1.3.4 Temperature

During fermentation, heat generation causes increase in temperature. Temperature control during fermentation is essential for maximizing activity of microorganisms. White wine fermentation temperature is suggested as low temperatures; 10ºC to 18ºC for better volatile compounds retention, however red wines are fermented at higher temperatures 18ºC to 29ºC for increased pigment and tannin extraction.

Fermentation rates of *Saccharomyces* vary with temperature. Temperature also affects the population balance between *Saccharomyces* and *non-Saccharomyces* yeasts. While at lower fermentation temperatures, *non-Saccharomyces* yeasts can consist of most of the must population, *Saccharomyces cerevisiae* represents the dominant species at warmer temperatures (Sharf and Margalith, 1983).

1.3.5 Natural Fermentations

Natural fermentation occurs by indigenous yeast population present in grape must. In recent years, for the purpose of providing flavor complexity and diversity, which is present in spontaneously fermented wines, natural fermentation using indigenous cultures is popular (Soden et al. 2000). Additional benefits to diversity are fuller, rounder palate structure and increased amount of sensory impact metabolites. *Non-Saccharomyces* yeasts increase aromatic molecule variety that increases wine acceptability.

Candida, *Pichia*, *Kloeckera*, *Kluyveromyces*, and *Torulaspora* are yeast species commercially used to enhance wine aroma. (Ciani and Maccarelli, 1998).

One negative attitude to natural fermentation is that some *non-Saccharomyces* yeasts can spoil wine by synthesis of unwanted volatile odor and flavor compounds; and may cause nutrient depletion before *Saccharomyces* initiates fermentation.

1.4 Volatile Compounds

Volatile molecular compounds are called odors. Considering, human's sense of smell is better than their sense of taste, volatile compounds have significant effect on acceptability of wine (Shepherd, 2004).

Wine volatile compounds are divided into two categories; aroma and bouquet. While aroma is arising from the grape fruits; bouquet is generated during fermentation and aging. Flavor includes the tastes, odors, and mouth sensation (Rapp & Mandery, 1986).

The diversity of wine odors is caused by the variety of the processes involved in their production; (1) compounds coming from grapes, depending on the variety, soil, climate, and viticulture applications, (2) biochemical reactions before fermentation, during maceration of must, (3) biochemical reactions during alcoholic and malolactic fermentations, (4) chemical reactions during maturation of the wine in barrel (Rapp & Mandery, 1986).

Monoterpenes represent the floral aroma. Esters, aldehydes, alcohols, and ketones constitutes monoterpenes group (Carrau et al. 2005; Mateo and Jimenez 2000).

Pyrazines are the second group of aromatic compounds, sourced from grape (Rajini, Aparna, Sasikala, & Ramana, 2011). Nonflavonoid phenols sourced from grapes and diffused from oak barrels during aging also contributes aroma to wine.

Ethyl acetate mostly represents the volatile character. This metabolite is one of the indicators of wine spoilage and its odor resembles fingernail polish remover (Plata, Mauricio, & Ortega, 2003; Rojas, Gil, Piñaga, & Manzanares, 2001).

Table 1.4: Aromas and their descriptors (Fugelsang & Edwards, 2007)

Origin	Descriptor
Bacterial	Vinegar, sauerkraut, sweaty, buttery, acetone, mousey, vegetal
Yeast	Yeasty, mousey, horsey, barnyard, wet dog, rotten eggs, mushroom
Molds	Mildew, musty
Oxidation	Acetaldehyde, sherry, overripe apples
Alcohol	Hot, burning, sweet
CO_2	Spritzy, prickly
SO_2	Burnt match, wet wool, skunk, cooked cabbage, sharp
Sorbate	Soapy, fishy
Acetaldehyde	Oxidized, sherry, nuts, overripe apples
Acetic Acid	Vinegar
Brettanomyces	Horsey, barnyard, wet wool, mousey
Diacetyl	Buttery
Ethyl acetate	Barn-aid, acetone
Ethyl mercaptan	Skunk, burn rubber
H_2S	Rotten eggs
Lactic acid	Sauerkraut, sweaty, milky
Lactobacilli	Vegetal
Oenococcus	Buttery
Pediococci	Vegetal
Sorbate and Oenococcus	Geranium
TCA	Mildew, musty

The aim of this study is isolation and characterization of indigenous *Saccharomyces cerevisiae* strains from Kapadokya/Nevşehir and Kalecik/Ankara regions. Strains, having desirable starter culture properties are used and evaluated as potential starter culture.

CHAPTER 2

MATERIALS AND METHODS

Potassium metabisulfite, ethanol (≥95%), yeast extract, lactose, sucrose, mannitol, glucose, maltose, agar-agar, agarose, peptone from casein, Tris-Borate-EDTA buffer, amoxicillin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycerol was purchased from Merck KGaA (Germany). Distilled water was used for the preparation of all growth medias. DNA extraction was purchased from Qiagen (Germany). PCR master kit and ethidium bromide was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Ultra-pure water was used for the preparation of molecular tests' mediums. ERBSLÖH Hefix® 2000 is used as commercial starter culture.

2.1 Wine Production

2.1.1 Grape Varieties

Emir and Kalecik Karası grape varieties, harvested in vintage were used in traditional wine production. Since the aim was isolating indigenous yeast strains, contamination between grape varieties was prevented. Grapes were held at 4^{o}C in cold room between arrival and initiation of wine production (Fugelsang & Edwards, 2007).

2.2 Destemming, Crushing, Pressing and Must Adjustment

Grapes were destemmed and crushed by hand gently. Since tannin amount in the seeds has negative effect on wine taste, seeds should not be damaged. All equipment, that will be used in wine making were disinfected by sulphur dioxide containing solution.

Grape juice with crushed grape pulp were transferred to disinfected vessels. In order to eliminate any possible risk of contamination, there were two parallel batch prepared.10%w/v potassium metabisulfite stock solution was prepared and added until its concentration reaches 30 mg SO_2/L must (Fugelsang & Edwards, 2007).

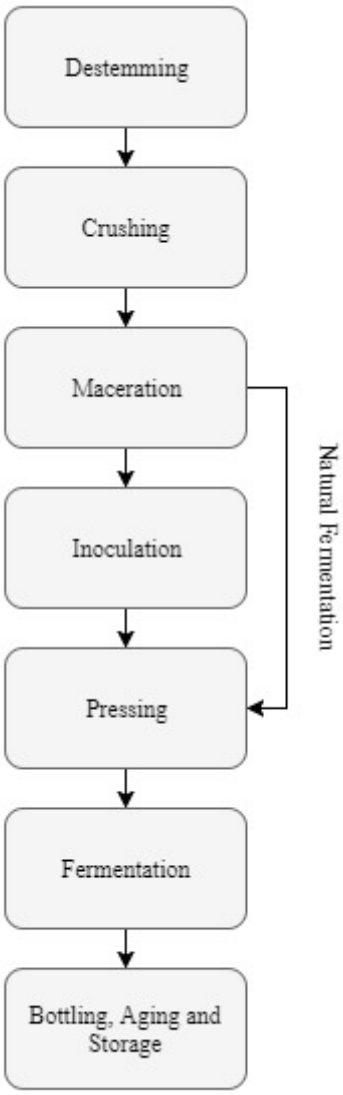

Figure 2.1: Red wine production steps (Fugelsang & Edwards, 2007).

2.2.1 Maceration

Crushed and destemmed must was transferred and leaved at 4^{o}C temperature for one week. After this period, must was allowed to warm up, up to 18^{o}C. Traditional and commercial wines differ at inoculation step. Vessels were not inoculated, and indigenous yeasts were allowed to ferment must in traditionally produced wines. However, wines produced at the stage of comparison of effect of isolated yeasts on aromatic components, were inoculated with selected strains. During fermentation, by increasing temperature and ethanol concentration, phenolic compounds are transferred from grape skin to must. At this step, grape must and pulp are mixed twice a day by punch down method, in order to prevent from drying of the top side, contacting with air, and aeration of must. Drying may result in mold growth.

2.2.2 Yeast Inoculation and Fermentation

Musts, that will be used to determine inoculum effect on aromatic compounds, were inoculated with assigned strains. Strains were grown YPD broth media than centrifugated and washed with distilled water twice. Obtained yeasts were inoculated at 10^{6}-10^{7} cfu yeast/ml must concentration to vessels. It was paid attention to equal the temperature of receiving must and inoculum. Fermentation was begun at 20^{o}C as it is optimal.

2.3 Sampling and Colony Isolation

There are three types of sample; obtained from washing water of grapes, crushed grape juice and from must at the different stages of fermentation. Grapes were washed with distilled water before crushing. Washing water were collected under aseptic conditions for further colony isolation. After washing of grapes, batches were separated and crushed; juice from vessels were transferred to sterile tubes. Samples were collected

in small amount from all batches daily, during fermentation until 16^{th} day of fermentation. Collected samples were stored at -20^{o}C temperature.

Collected samples were diluted up to 10^{-6} in 1% peptone water solution, in order to isolate single cell colonies. Diluted samples were inoculated by spread plate inoculation technique onto YPD agar growth media containing 0.015%w/v amoxicillin, to inhibit bacterial growth. Isolated colonies were inoculated by streak plate and single cell colonies were obtained. Obtained colonies were transferred to slant agar and kept at 4^{o}C.

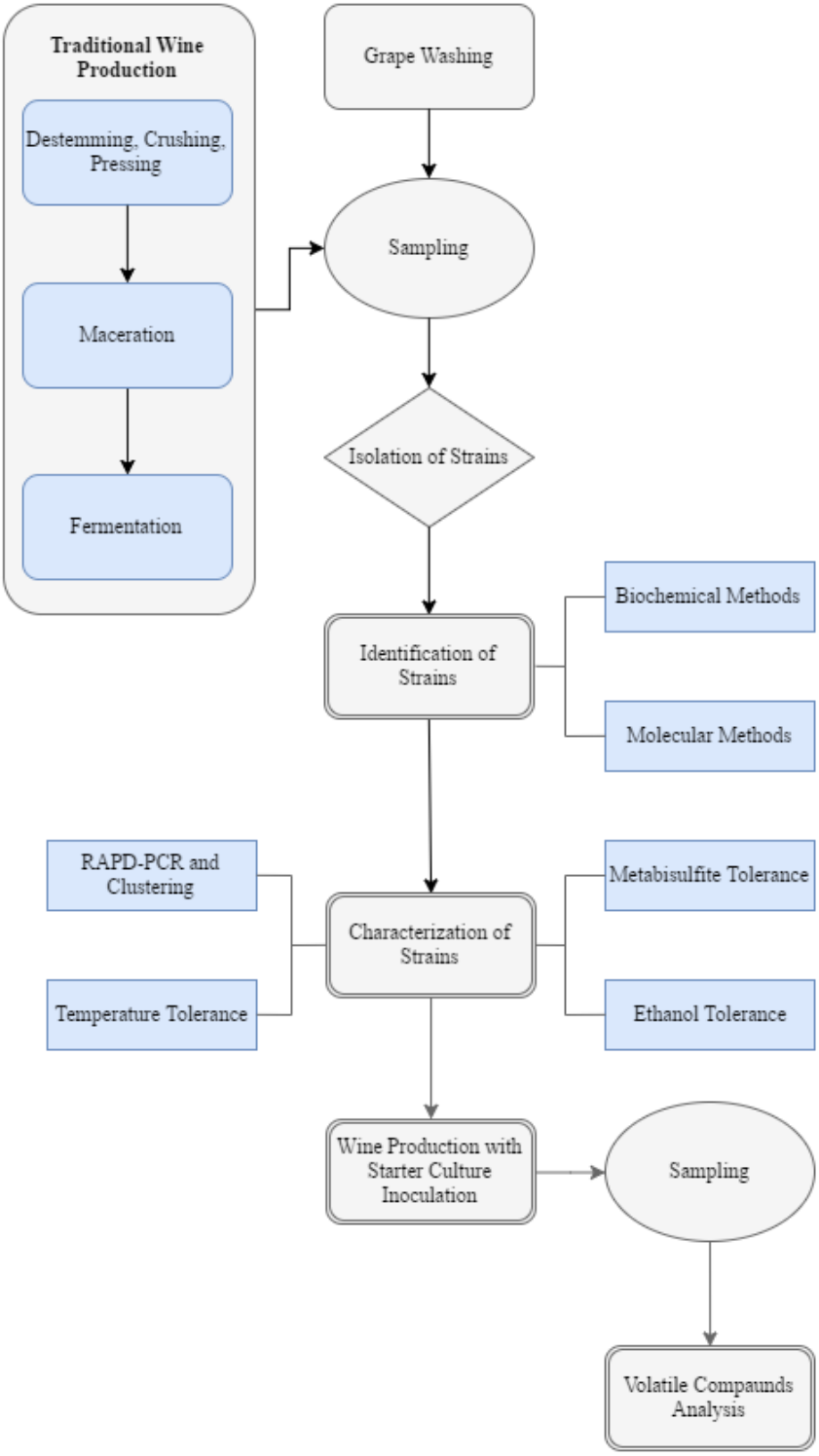

Figure 2.2: Schematic representation of wine making, classification, identification and volatile compounds analysis.

2.4 Identification of Strains by Biochemical and Molecular Tests

Identification of strains was done by examining carbohydrate fermentation abilities and sequencing ITS regions.

2.4.1 Biochemical Tests

Fermentation abilities of isolates were examined. Liquid growth mediums containing 5% (w/v) lactose, sucrose, mannitol, glucose, maltose were inoculated with isolates. Gas formation in the Durham tubes was controlled (Guimarães, Moriel, Machado, Picheth, & Bonfim, 2006).

2.4.2 DNA Extraction

DNA's of isolates were extracted according to Qiagen Blood & Tissue product manual. Isolates were grown in liquid YPD growth medium at 37ºC for 24 hours. From each strain, approximately 5 x 10^6 cfu were centrifugated for 5 min at 190 rpm. Precipitated pellets were resuspended in 200 µl phosphate-buffered saline solution. 20 µl proteinase K enzyme and 200 µl Buffer AL were added to all tubes. Mixtures were mixed thoroughly by vortexing. Samples were incubated at 56°C for 10 min. and 200 µl ethanol was added to samples. Mixtures were mixed thoroughly by vortexing. Obtained mixtures were pipetted into DNeasy Mini spin column placed in a 2-ml collection tube. Collection tubes, filled with mini spin column were centrifugated at 8000 rpm for 1 min. The flow-through and collection tube parts were discarded, spin columns were placed in a new 2-ml collection tube. 500 µl Buffer AW1 was added to new collection tubes and they were centrifugated for 1 min at 8000 rpm. The flow-through and collection tube parts were discarded once again. The spin columns were placed in a new 2-ml collection tube one more time. 500 µl Buffer AW2 was added to all collection tubes, and mixtures were centrifugated for 3 min at 14,000 rpm. The spin columns in the collection tubes were transferred to a new 1.5 ml or 2 ml micro-

centrifuge tube. Elution of DNA was achieved by adding 200 µl Buffer AE to the membrane of spin column. Final mixtures were incubated for 1 min at room temperature 15–25°C and centrifugated for 1 min at 8000 rpm.

2.4.3 ITS Region Sequencing

Sequencing of ITS regions belonging to selected isolated strains' DNAs was done by Bigdye Cycle Sequencing Kit v3.1 and ABI 3100 Genetic Analyzer. Patterns was aligned by clustering than, phylogenetic tree based on 5.8S rDNA was drawn (Capece et al., 2010).

2.5 Characterization

2.5.1 Ethanol Tolerance Test

Isolated strains were applied to ethanol tolerance test. 24-hour single cell colonies were inoculated in YPD broth medium containing 10%, 13%, 15%v/v ethanol and incubated at 30ºC for 72 hours. Gas accumulation in Durham tubes was controlled (Guimarães et al., 2006).

2.5.2 Sulfur Dioxide Tolerance Test

Isolated strains' tolerance to sulfur dioxide was tested. 24-hour single cell colonies were inoculated YPD broth medium containing 50, 100, 150, 200 mg SO_2/L. Gas accumulation in Durham tubes was controlled (Guimarães et al., 2006).

2.5.3 Temperature Tolerance Test

Obtained strains' temperature tolerances were observed. 24-hour single cell colonies were inoculated in YPD broth medium and incubated at 28°C, 37°C, 45°C for 72 hours. Gas accumulation in Durham tubes was controlled (Guimarães et al., 2006).

2.5.4 RAPD PCR

Extracted DNA's from each *Saccharomyces cerevisiae* were subjected to RAPD-PCR using primer M13. Reactions were carried out in a mixture containing Tris–HCl, KCl, $MgCl_2$, dNTPs, primer and Taq-polymerase. Amplifications were carried out with an initial step at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 38°C for 1 min and ramp to 72°C with 0.6°C/s, 72°C for 2 min. RAPD-PCR products were analyzed by electrophoresis on 1.5% (w/ v) agarose gels in 0.5x Tris Borate EDTA at 120 V for 4 h. Agarose gels were stained in 0.5x TBE buffer containing 0.5 μg/ml ethidium bromide for 30 minutes. Pictures of the gels are digitally captured (Capece et al., 2010).

2.6 Determination of Volatile Components

Öküzgözü grape variety was chosen as fermentation medium. Considering ethanol tolerance property of strains, S14, S15, S16 and S17 were selected as starter cultures. Five different batch of fermentation vessel have been prepared as mentioned above except these musts inoculated with wine starter culture. During fermentation, 200 ml samples were taken once in seven days into glass sample bottles. Samples were analyzed with GC-MS (Capece et al., 2010).

2.6.1 Statistical Analysis

Volatile compounds data from the GC-MS results were analyzed using the MiniTab statistical software program (Minitab, Inc., Pennsylvania, U.S.A) for Windows. One-Way ANOVA and Tukey's multiple range test were used to the volatile data to determine significant differences between the wines for volatile compounds at $P < 0.05$ significant level.

CHAPTER 3

RESULTS AND DISCUSSION

Ethanol tolerance of strains plays significant role in the selection of starter cultures. In this study, randomly selected 37 strains were subjected to ethanol tolerance test to eliminate technically ineffective ones. 16 of isolates were observed as tolerant to at least 10%v/v ethanol concentration. Strains, provided from Fermicru AR2 Saccharomyces cerevisiae No. LO122, Nederland and German Hauswein Starter 38409LM; 77767 Appenweier, Frankenweg 52, 64725 Bensheim, Zeppelinstr, 11A were added to samples in order to compare.

Table 3.1 Sources of isolated and reference strains

Strain Name	Source	Ethanol Tolerance		
		10%	**13%**	**15%**
S1	Emir must	+	-	-
S2	Emir must	+	-	-
S3	Emir washing water	+	-	-
S4	Kalecik Karası washing water	+	-	-
S5	Kalecik Karası washing water	+	-	-
S6	Emir 4th day of fermentation	+	-	-
S7	Emir 5th day of fermentation	+	-	-
S8	Emir 5th day of fermentation	+	-	-
S9	Emir 5th day of fermentation	+	+	-
S10	Emir 5th day of fermentation	+	+	-
S11	Kalecik Karası 10th day of fermentation	+	+	-
S12	Kalecik Karası 10th day of fermentation	+	+	-
S13	Kalecik Karası 12th day of fermentation	+	+	-
S14	Emir 9th day of fermentation	+	+	+
S15	Kalecik Karası 16th day of fermentation	+	+	+
S16	Kalecik Karası 16th day of fermentation	+	+	-
S17	Fermicru AR2 Saccharomyces cerevisiae No. LO122, Nederland			
S18	German Hauswein Starter 38409LM; 77767 Appenweier, Frankenweg 52			

3.1 Identification of Strains by ITS Region Sequencing

As the result of molecular identification, based on ITS region sequencing, 18 isolates, having ethanol tolerance higher than 10%(v/v) were classified. Phylogenetic tree was obtained by cluster alignment and neighbor-joining method as shown in the figure.

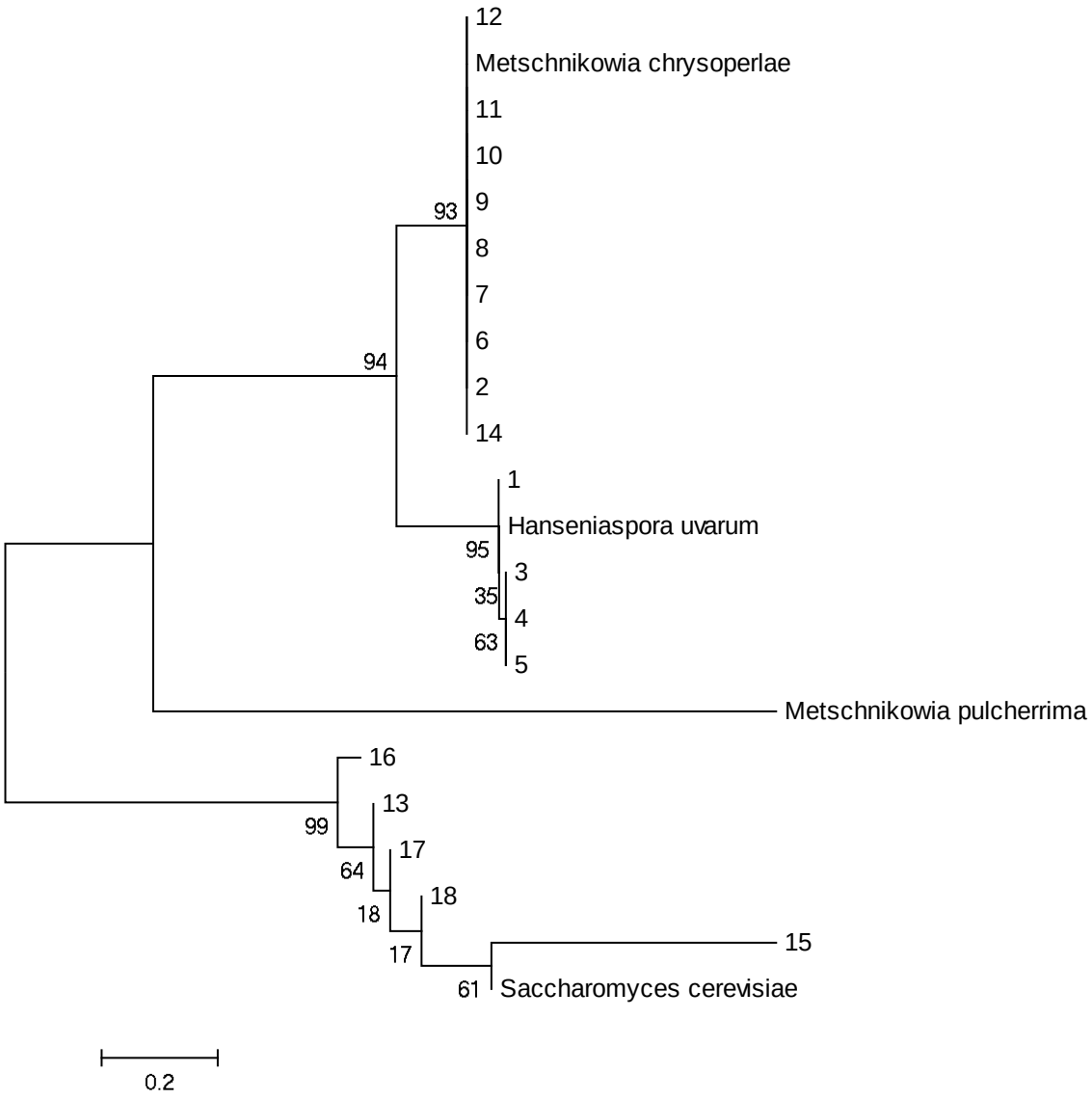

Figure 3.1: Cluster analysis results of rDNA ITS Region Sequencing

While S2, S6, S7, S8, S9, S10, S11, S12, S14 were identified as *Metschnikowia chrysoperlae*; S3, S4, S5 showed significant similarity with *Hanseniaspora uvarum*; S13, S15, S16, S17, S18 were identified as *Saccharomyces cerevisiae*.

3.2 RAPD PCR

Saccharomyces cerevisiae strains were characterized by RAPD-PCR in order to observe strains' genotypic diversity. Randomly amplified polymorphic DNA (RAPD) fingerprinting of yeasts by amplifying with M13 primer gave the best results in the profiling of *S. cerevisiae* sensu stricto strains (Giusto et al., 2006). Obtained results from RAPD-PCR shows similarity with literature (Cocolin et al., 2004; Giusto et al., 2006; Guiamal & Hedreyda, 2011).

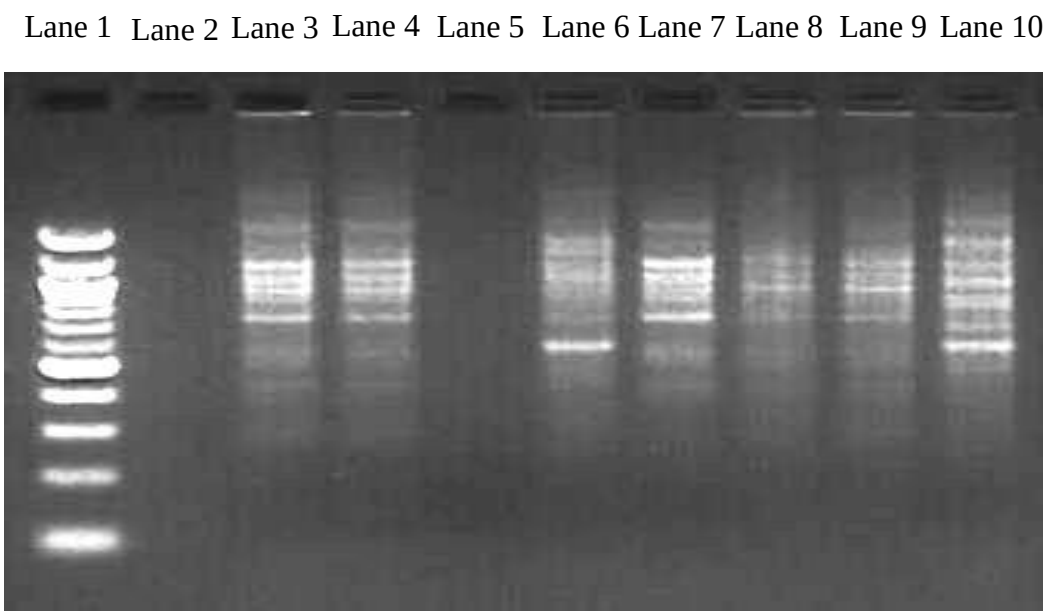

Figure 3.2: Gel electrophoresis image of RAPD PCR. Lane 1, 1 kb Thermo Fisher ladder; lane 2, and lane 5, negative control; Lane 3, S13; Lane 4, S15; Lane 6, S16; Lane 7, S18; Lane 8 and 9, reference strain from Ankara University culture collection; Lane 10, S14.

S13, S15 and S18 had same number of band on agarose gel and pattern, while S16 and reference strain showed missing and extra bands. These results were compatible with ITS region sequencing results.

3.3 Carbohydrate Fermentation Test

Strains, identified as *Saccharomyces cerevisiae* were applied to carbohydrate test. None of the isolates fermented lactose while all of isolates could utilize glucose and maltose. Only S16 could not ferment sucrose while it was the only strain that can utilized mannitol as carbon source. *Saccharomyces cerevisiae* can utilize glucose; cannot ferment lactose. Sucrose, mannitol and maltose fermentation varies from strain to strain (Barnett et al., 2000). Results also shows similarity with similar study done by Guimarães et al. (2006).

Table 3.2: Carbohydrate fermentation test

Sample	Carbohydrate Fermentation Test				
	Lactose	**Sucrose**	**Mannitol**	**Glucose**	**Maltose**
S13	-	+	-	+	+
S15	-	+	-	+	+
S16	-	-	+	+	+
S17	-	+	-	+	+
S18	-	+	-	+	+

3.4 Temperature Tolerance Test

All of the isolates survive at 28ºC and 37ºC temperature while none of them could grow at 45ºC. Optimal temperature for growth of *Saccharomyces cerevisiae* is 37ºC

(Liu & Shen, 2008). Temperature tolerance of strains showed parallel results with studies done by van Uden et al. (2007) and Guimarães et al.(2006).

Table 3.3: Temperature tolerance test

Sample	Temperature Tolerance (°C)		
	28	**37**	**45**
S13	+	+	-
S15	+	+	-
S16	+	+	-
S17	+	+	-
S18	+	+	-

3.5 Ethanol Tolerance Test

Sustaining ability of a starter culture to grow at the presence of high concentration of ethanol is very desirable in wine industry. Isolated strains were applied to ethanol tolerance test. Strains were inoculated in YPD broth medium containing %10, %13, 15%v/v ethanol and incubated at 30°C for 72 hours in order to analyze strains' ethanol resistance. Isolates, applied to ethanol tolerance test, showed high survivability to increasing ethanol concentrations 10%, 13% and 15% (v/v). Among the all isolates, only S13 cannot survive at 15% (v/v) ethanol concentration. Salvado et al. (2011) and Guimarães et al.(2006) also obtained similar results. In these studies, isolated *Saccharomyces cerevisiae* strains were shown ethanol tolerance up to 15% (v/v).

Table 3.4: Ethanol tolerance test

Sample	Ethanol Tolerance		
	10%	13%	15%
S13	+	+	-
S15	+	+	+
S16	+	+	+
S17	+	+	+
S18	+	+	+

3.6 Sulfur Dioxide Tolerance Test

Potassium metabisulfite is used to eliminate spoilage microorganisms and limit oxidation of biochemicals in winemaking. Growing ability in the presence of high sulfur dioxide concentration provides significant advantage in competition with indigenous yeast strains. All isolates that applied to sulfur tolerance showed high concentration of sulfur dioxide; 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L. These results were supported by Bağder et. al. (2014).

Table 3.5: Sulfur dioxide tolerance test

Sample	Sulfur Dioxide Tolerance (mg/L)			
	50	100	150	200
S13	+	+	+	+
S15	+	+	+	+
S16	+	+	+	+
S17	+	+	+	+
S18	+	+	+	+

3.7 Aromatic Compound Analysis

Usage of different starter cultures (Commercial starter culture, S14, S15, S16, S17) has made considerable influence on some aromatic components of the wine samples. All the isolated starter cultures have shown higher amount and diversity in aldehydes and ketones, higher alcohols, fatty acids and esters. Some of the isolated strains showed ability to produce several aromatic compounds that commercial starter culture cannot, such as butyrate, vanillin thiazole.

Table 3.6: Aromatic compounds (μg/L wine)

	Control	**S14**	**S15**	**S16**	**S17**
Acetoin	nd	8.67 ± 0,15[a]	nd	nd	4.37 ± 0.06[b]
Acetaldehyde	nd	nd	nd	2.31 ± 0.32[a]	67.65 ± 0.79[b]
1-Hexanol	8.16 ± 0.05[c]	7.79 ± 0.15[c]	14.16 ± 1.13[a]	12.2 ± 0.3[b]	10.83 ± 1.07[b]
Z-3-Hexezenol	nd	2.29 ± 0.01	nd	nd	nd
Citronellol	nd	13.53 ± 0.26[c]	16.59 ± 1.09[a]	3.34 ± 0.18[b]	nd
2-Methyl-1-propanol	105.82 ± 8.24[b]	82.83 ± 1.73[c]	151.61 ± 8.73[a]	91.76 ± 11.19[c]	55.03 ± 9.07[d]
1-Butanol	nd	8.86 ± 6.79[a]	18.9 ± 0.67[a]	nd	6.45 ± 4.82[a]
1-Butanol, 3-methyl-, acetate	nd	11.92 ± 0.01[b]	10.43 ± 0.25[b]	21.54 ± 4.55[a]	nd
2,3-Butanediol	nd	24,24 ± 0,54	22,9 ± 1,02	21,34 ± 0,22	6,78 ± 0,28
Z-2-Pentenol	5,83 ± 0,07	6,32 ± 0,69	nd	2,84 ± 0,35	2,84 ± 0,35

Table 3.6 (continued)

	Control	S14	S15	S16	S17
2-Pentanone, 4-hydroxy-4-methyl	nd	3.18 ± 0.07[a]	2.9 ± 0.38[a]	nd	nd
6-Methyl-1-heptanol	nd	9.61 ± 1.48	nd	nd	nd
Benzyl alcohol	nd	2.83 ± 0.02[c]	3.71 ± 0.05[a]	2.94 ± 0.05[b]	nd
Phenethyl alcohol	nd	74.07 ± 2.52[c]	nd	246.42 ± 23.52[a]	142.05 ± 12.29[b]
Behenic alcohol	nd	5.09 ± 0.19[b]	9.32 ± 0.49[a]	nd	nd
4-Hexane	7.62 ± 0.07[a]	5.6 ± 0.07[b]	4.77 ± 0.08[d]	5.00 ± 0.11[c]	nd
Pentaethylen glycol	nd	15.42 ± 0.38[b]	27.48 ± 13.61[a]	48.55 ± 7.16[a]	26.1 ± 13.51[b]
Heptadecylalcol	nd	6.72 ± 0.37[a]	5.77 ± 0.16[b]	3.5 ± 0.03[c]	nd

Table 3.6 (continued)

	Control	S14	S15	S16	S17
Pentadeconaic acid	20.6 ± 0.99^{b}	7.89 ± 0.67^{c}	35.63 ± 0.18^{a}	3.1 ± 0.24^{d}	34.98 ± 0.69^{a}
Hexadeconaic acid	nd	49.58 ± 1.88^{a}	nd	13.37 ± 0.18^{c}	18.59 ± 0.68^{b}
Propionic acid	nd	5.16 ± 0.09^{b}	nd	nd	6.78 ± 0.13^{a}
Octadecanoic acid	45.36 ± 0.38^{a}	31.05 ± 0.38^{b}	21.59 ± 3.15^{c}	14.54 ± 0.41^{d}	29.46 ± 1.55^{b}
Isoamyl acetate	5.98 ± 0.11^{d}	11.92 ± 0.03^{c}	10.43 ± 0.24^{c}	15.19 ± 1.14^{b}	17.6 ± 0.46^{a}
1,2-Benzenedicarboxylic acid	17.63 ± 0.18^{a}	4.9 ± 0.54^{d}	12.27 ± 0.78^{c}	4.29 ± 0.39^{d}	14.66 ± 0.13^{b}
1,2-Benzenedicarboxylic acid	nd	81.25 ± 3.45^{c}	480.4 ± 20.06^{b}	1224.01 ± 64.06^{a}	nd
Ethylene diglycol	nd	24.3 ± 3.44^{a}	nd	30.05 ± 7.74^{a}	42.77 ± 17.37^{a}
Hexaehtylene glycol	nd	13,71 ± 3.65^{b}	36,58 ± 3.67^{b}	38.99 ± 2.97^{b}	119.46 ± 30.44^{a}

Table 3.6 (continued)

	Control	S14	S15	S16	S17
7-Hydroxy-Octa-2,4-Dienoic Acid	nd	6.86 ± 1.03[a]	8.03 ± 0.34[a]	nd	nd
Butanoic acid	7.02 ± 0.15[a]	5.65 ± 2.16[a]	nd	7.62 ± 0.15[a]	6.21 ± 0.11[a]
Hexanoic acid	17.94 ± 4.43[a]	4.5 ± 0.02[c]	10.09 ± 0.06[b]	10.33 ± 0.06[b]	5.82 ± 0.63[b]
Palmitic acid	nd	74.18 ± 3.03[b]	26.73 ± 0.97[d]	49.74 ± 6.02[c]	103.54 ± 4.58[a]
Octanoic Acid	48.8 ± 13.88[a]	11.81 ± 0.09[c]	12.83 ± 0.18[b]	26.34 ± 0.52[b]	6.73 ± 0.08[c]
Decanoic acid (capric acid)	nd	5.89 ± 0.34[d]	11.62 ± 0.02[c]	34,41 ± 0.39[a]	22.74 ± 0.18[b]
Sorbic Acid	702.87 ± 6.84[e]	830.27 ± 11.15[d]	1023.32 ± 9.42[b]	959.68 ± 8.49[c]	1807.21 ± 16.64[a]
Tetradeconaic acid	34.79 ± 1.32[a]	11.17 ± 1.13[b]	0.34 ± 0.08[c]	12.09 ± 0.4[b]	nd
Vanilin	nd	5.26 ± 2.02	nd	nd	nd
2,6,10,14,18,22-Tetracosahexaen	52.65 ± 14.54[a]	11.82 ± 0.73[b]	4.1 ± 3.83[b]	22.28 ± 4.16[b]	17.98 ± 0.03[b]

Table 3.6 (continued)

	Control	S14	S15	S16	S17
Ethyl linoleate	nd	63.51 ± 2.43[a]	6.15 ± 1.14[d]	11.47 ± 0.27[c]	21.95 ± 1.15[b]
Ethyl acetate	nd	7.5 ± 0.06[b]	13.61 ± 0.03[a]	nd	nd
Ethyl 4-hydroxybutanoate	nd	58.6 ± 0.7[a]	25.17 ± 2.58[c]	32.51 ± 2.11[b]	13.95 ± 0.11[d]
Ethyl 9-hexadecanoate	nd	22.57 ± 0.82	nd	nd	nd
Diethyl Phthalate	93.81 ± 10.54[c]	nd	nd	157.22 ± 17.7[b]	316.42 ± 19.16[a]
D-Limonene	5.68 ± 0.84[a]	4.74 ± 0.15[a]	nd	nd	nd
Elemicin	nd	nd	6.22 ± 0.29[c]	89.47 ± 13.69[b]	192.66 ± 27.44[a]
Thiazole	nd	16.15 ± 3.76	nd	nd	nd
Xylenol	nd	4.72 ± 0.73[b]	30.05 ± 9.48[a]	nd	22.35 ± 0.92[a]

Table 3.6 (continued)

	Control	S14	S15	S16	S17
-7-Hydroxyocta-2,4 dienoic acid	nd	6.79 ± 0.93[a]	8.02 ± 1.34[a]	nd	nd
Phenol, 2,3 dimethyl	nd	8.42 ± 1.48[c]	23.3 ± 1.07[a]	13.77 ± 1.88[b]	6.45 ± 0.58[c]
Tetrapentacosan	nd	37.2 ± 12.6[a]	18.81 ± 0.06[a]	nd	29.26 ± 9.39[a]
Mercaptan	nd	nd	nd	6.87 ± 2.78	nd
Tetracosane	nd	6.21 ± 0.49[a]	nd	3.2 ± 0.5[b]	nd
Pentacosane	nd	11.8 ± 0.76[b]	23.85 ± 7.88[a]	nd	4.51 ± 2.14[b]
Hexzatriacontane	nd	108.91 ± 29.33[a]	nd	119.25 ± 23.13[a]	129.79 ± 10.79[a]
Butyrate	nd	14.64 ± 4.28[a]	14.1 ± 3.21[a]	9.87 ± 3.59[a]	18.29 ± 1.52[a]
2(3H)-Furanone	nd	10.95 ± 0.14[b]	11.64 ± 0.04[a]	nd	7.56 ± 0.11[c]

ND: not determined. In the same row, means with different letters significantly differed in ANOVA test ($P < 0.05$).

Alcohols that contains more than two carbon atoms are called as higher alcohols. Several of these are produced during fermentation such as propanol, butanol, butanediol (Ribéreau-Gayon, 1986). Higher alcohols and their derivatives reacting with other components have strong odors that affects wine aromas. The main higher fermentation alcohols are methyl-2-propanol-1 and the mixture of methyl-2-butanol-1 and methyl-3-butanol-1. These components provide to wine aromatic complexity. Test results show that isolated strains have potential to produce higher alcohols comparing with commercial *Saccharomyces cerevisiae* starter culture.

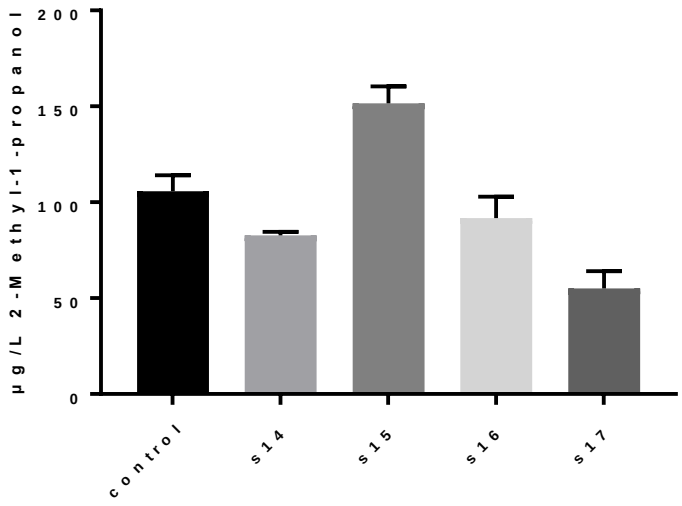

Figure 3.3: 2-methyl-1-propanol amount in wines.

The C6, C8 and C10 fatty acids are produced during fermentation by yeast. They are known as fermentation inhibitors. Unsaturated long-chain fatty acids (C18, C20) are fermentation promoters, under anaerobic conditions. According to One-Way ANOVA results, each starter culture strains have produced significantly different amount of fatty acids. While commercial starter culture has produced higher amount of hexanoic acid, octanoic acid, tetradeconaic acid and octadecanoic acid; S17 strain has produced the highest total amount of fatty acids. However, most of the source of fatty acids was sorbic acid, that may causes rancid flavor in wine.

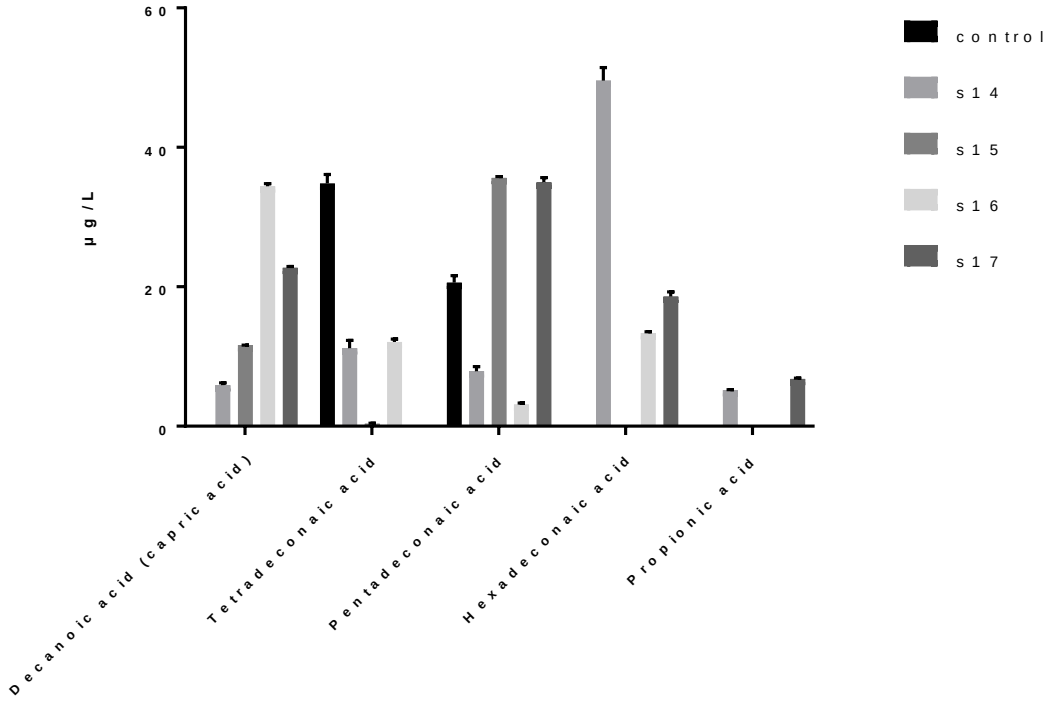

Figure 3.4: Decanoic acid, tetradeconaic acid, pentadeconaic acid, hexadeconaic acid and propionic acid amounts in wines.

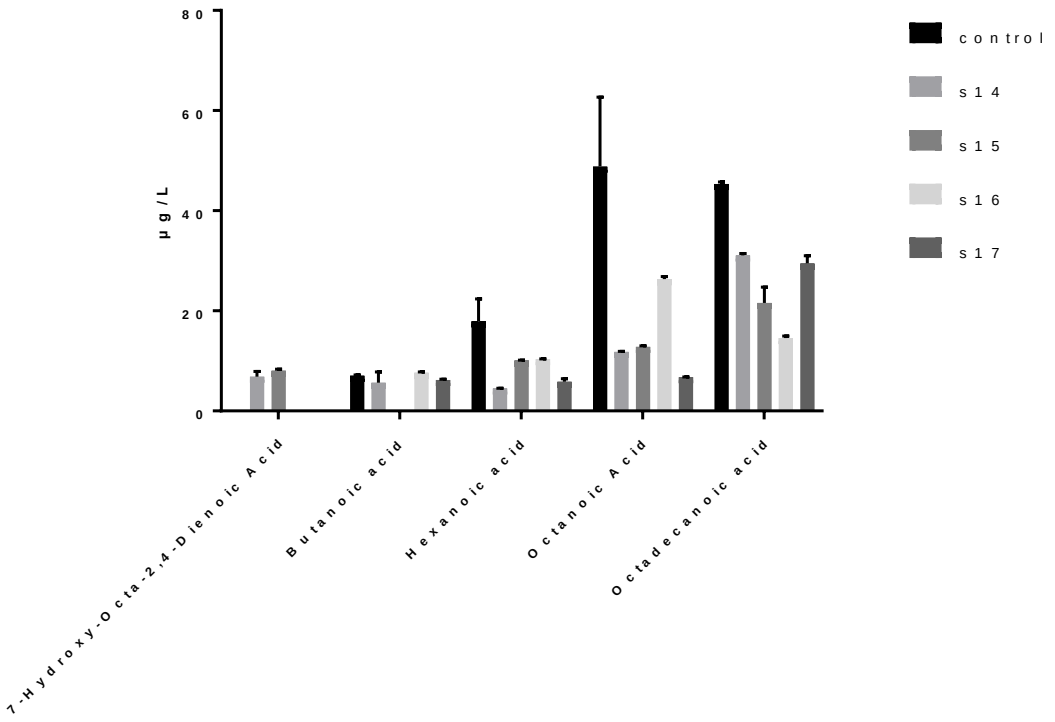

Figure 3.5: 7-hydroxy-octa-2,4-dienoic acid, butanoic acid, hexanoic acid, octanoic acid and octadecanoic acid amounts in wines.

Grapes contain few aldehydes. Hexenal and hexanol have been responsible from contributing to the herbaceous odors of C6 compounds.

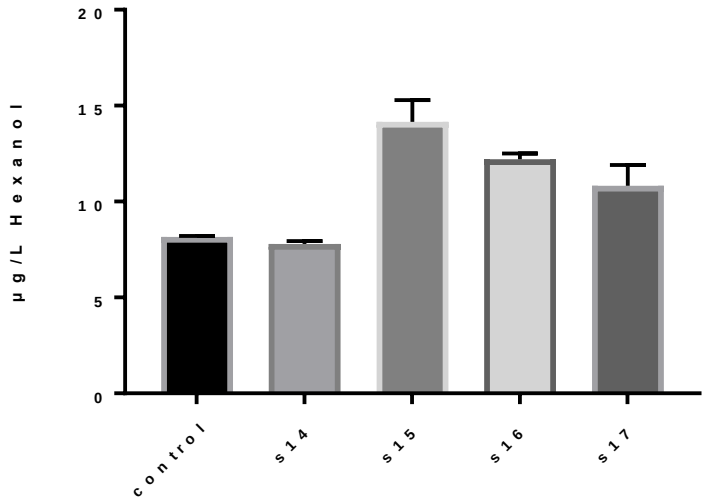

Figure 3.6: Hexanol amount in wines.

Wines, inoculated with S15, S16, S17 showed higher amount of hexanol while rest of samples, inoculated with commercial starter culture and S14 contains less amount.

Acetal is formed an aldehyde reacts with an alcohol. About twenty compounds of acetals have been detected in wine. Acetals have also herbaceous odor that may add to the aromatic complexity. The best-known acetal is γ-butyrolactone.

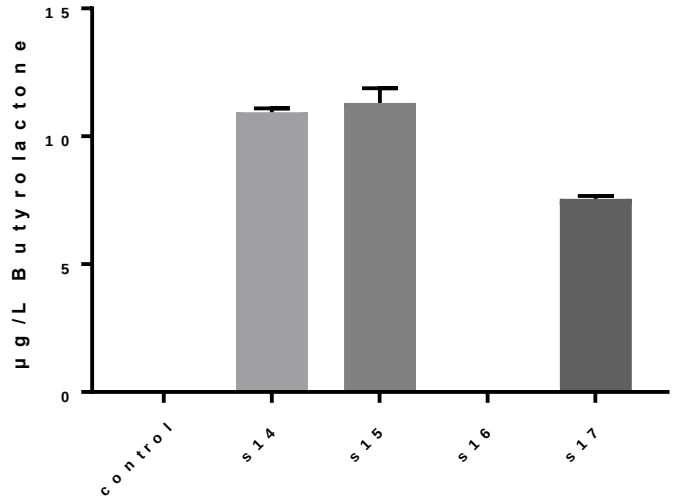

Figure 3.7: Butyrolactone amount in wines.

The several acids are formed by the substitution of their benzene ring. Free forms are more prevalent, mainly in red wine.

Phenolic acids do not contribute color in alcohol solution; however, oxidation causes yellowish color in their solutions. From an organoleptic perspective, phenolic acids have no specific flavor or odor. However, they are sign of action of certain microorganisms that produce volatile phenols. In white wines, vinyl phenols, with an odor reminiscent of gouache paint, are accompanied by vinyl gaiacols.

Another acid that develops during fermentation due to the action of yeast is succinic or 1-4- butanedioic acid. This acid is produced by all living organisms and is involved in the lipid metabolism and the Krebs cycle, in conjunction with fumaric acid.

CHAPTER 4

CONCLUSION

In this study, from Emir and Kalecik Karası grape varieties, potential starter culture strains were isolated, identified and characterized. All of the isolated strains were classified as *Metschnikowia chrysoperlae*, *Hanseniaspora uvarum* or *Saccharomyces cerevisiae*. All yeast samples were showed 99% similarity with reference strains.

In the scope of this study, strains, that have been identified as *Saccharomyces cerevisiae*, were examined for fermenting ability of different carbohydrate sources. S13, S15, S17 and S18 could ferment all carbohydrate sources except lactose and mannitol. All of isolates could utilize glucose and maltose. Only S16 could not ferment sucrose while it is the only strain that can utilize mannitol as carbon source.

During fermentation, temperature of growth medium requires to be controlled. Wine starter cultures are to be expected as resistant to changes in fermentation temperature. Isolated strains were applied to temperature resistance test at 28°C, 37°C and 45°C. While all the isolated strains grown at 28°C and 37°C temperatures, none of them could survive at 45°C.

Increasing ethanol concentration in fermentation medium inhibits activity of starter cultures. This may cause residual sugar left in must and promotes spoilage microorganisms. All isolated *Saccharomyces cerevisiae* strains shown 100-150 ml/L (v/v) ethanol concentration in growth medium.

Another characteristic of starter cultures is resistance to sulfur dioxide. All of the strains were recognized as sulfur dioxide resistant since they kept the ability to grow in growth media containing sulfur dioxide up to 200 mg/L (w/v).

Results of these tests showed S13, S15 and S16 had potential to be used as starter culture. In order to analyze effect of strains to aromatic complexity of wines, four vessels of Öküzgözü grape must were inoculated with these strains. One of the vessels was inoculated with commercial starter culture to compare with isolated strains.

Wines, inoculated with isolated strains showed wider aromatic complexity with respect to inoculated with commercial starter culture. Especially isolated strains contributed significant amount of fatty acids levels of wine. Total aromatic compounds of wines were obtained as 1180.56 for commercial culture, 1876.34 for S13 strain, 2177.03 for S15 strain, 3371.40 for S16 strain and 3353.28 for S17 strain.

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APPENDICE A

STATISTICAL ANALYSIS

Table A.1 One-way ANOVA: Acetoin versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	1	27,7350	27,7350	2125,29	0,000
Error	4	0,0522	0,0131		
Total	5	27,7872			

S = 0,1142 R-Sq = 99,81% R-Sq(adj) = 99,77%

Pooled StDev = 0,1142

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S14	3	8,6700	A
S17	3	4,3700	B

Table A.2 One-way ANOVA: Acetaldehyde versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	1	6403,973	6403,973	17629,66	0,000
Error	4	1,453	0,363		
Total	5	6405,426			

S = 0,6027 R-Sq = 99,98% R-Sq(adj) = 99,97%

Pooled StDev = 0,603

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S17	3	67,650	A
S16	3	2,310	B

Table A.3 One-way ANOVA: 1-Hexanol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	4	87,397	21,849	43,06	0,000
Error	10	5,074	0,507		
Total	14	92,470			

S = 0,7123 R-Sq = 94,51% R-Sq(adj) = 92,32%

Pooled StDev = 0,712

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	14,1600	A
S16	3	12,2000	B
S17	3	10,8300	B
Control	3	8,1600	C
S14	3	7,7900	C

Table A.4 One-way ANOVA: Citronellol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	288,762	144,381	336,27	0,000
Error	6	2,576	0,429		
Total	8	291,338			

S = 0,6553 R-Sq = 99,12% R-Sq(adj) = 98,82%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	16,590	A
S14	3	13,530	B
S16	3	3,340	C

Table A.5 One-way ANOVA: 2-Methyl-1-propanol (Isobutyl a versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	4	15146,8	3786,7	53,40	0,000
Error	10	709,2	70,9		
Total	14	15856,0			

S = 8,421 R-Sq = 95,53% R-Sq(adj) = 93,74%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	151,61	A
Control	3	105,82	B
S16	3	91,76	B C
S14	3	82,83	C
S17	3	55,03	D

Table A.6 One-way ANOVA: 1-Butanol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	234,1	117,1	5,03	0,063
Error	5	116,3	23,3		
Total	7	350,4			

S = 4,823 R-Sq = 66,81% R-Sq(adj) = 53,54%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	18,900	A
S14	3	8,860	A
S17	2	6,445	A

Table A.7 One-way ANOVA: 1-Butanol, 3-methyl-, acetate (versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	218,20	109,10	15,76	0,004
Error	6	41,53	6,92		
Total	8	259,73			

S = 2,631 R-Sq = 84,01% R-Sq(adj) = 78,68%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S16	3	21,540	A
S14	3	11,920	B
S15	3	10,430	B

Table A.8 One-way ANOVA: 2,3-Butanediol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	592,004	197,335	541,09	0,000
Error	8	2,918	0,365		
Total	11	594,922			

S = 0,6039 R-Sq = 99,51% R-Sq(adj) = 99,33%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S14	3	24,240	A
S15	3	22,900	A B
S16	3	21,340	B
S17	3	6,780	C

Table A.9 One-way ANOVA: Z-2-Pentenol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	31,756	10,585	58,32	0,000
Error	8	1,452	0,181		
Total	11	33,208			

S = 0,4260 R-Sq = 95,63% R-Sq(adj) = 93,99%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S14	3	6,3200	A
Control	3	5,8300	A
S17	3	2,8400	B
S16	3	2,8400	B

Table A.10 One-way ANOVA: 2-Pentanone, 4-hydroxy-4-methyl versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	1	0,1176	0,1176	1,58	0,278
Error	4	0,2986	0,0746		
Total	5	0,4162			

S = 0,2732 R-Sq = 28,26% R-Sq(adj) = 10,32%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S14	3	3,1800	A
S15	3	2,9000	A

Table A.11 One-way ANOVA: Benzyl alcohol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	1,37940	0,68970	383,17	0,000
Error	6	0,01080	0,00180		
Total	8	1,39020			

S = 0,04243 R-Sq = 99,22% R-Sq(adj) = 98,96%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	3,7100	A
S16	3	2,9400	B
S14	3	2,8300	C

Table A.12 One-way ANOVA: Phenethyl alcohol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	45219	22609	95,45	0,000
Error	6	1421	237		
Total	8	46640			

S = 15,39 R-Sq = 96,95% R-Sq(adj) = 95,94%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S16	3	246,42	A
S17	3	142,05	B
S14	3	74,07	C

Table A.13 One-way ANOVA: Behenic alcohol (1-Docasonal) versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	1	26,839	26,839	194,35	0,000
Error	4	0,552	0,138		
Total	5	27,392			

S = 0,3716 R-Sq = 97,98% R-Sq(adj) = 97,48%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	9,3200	A
S14	3	5,0900	B

Table A.14 One-way ANOVA: 4-Hexane versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	15,12683	5,04228	712,69	0,000
Error	8	0,05660	0,00708		
Total	11	15,18343			

S = 0,08411 R-Sq = 99,63% R-Sq(adj) = 99,49%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
Control	3	7,6200	A
S14	3	5,6000	B
S16	3	5,0000	C
S15	3	4,7700	D

Table A.15 One-way ANOVA: Pentaethylen glycol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	1730	577	5,50	0,024
Error	8	838	105		
Total	11	2569			

S = 10,24 R-Sq = 67,36% R-Sq(adj) = 55,12%

Pooled StDev = 10,24

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S16	3	48,55	A
S15	3	27,48	A B
S17	3	26,10	A B
S14	3	15,42	B

Table A.16 One-way ANOVA: Heptadecylalcol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	16,4238	8,2119	150,77	0,000
Error	6	0,3268	0,0545		
Total	8	16,7506			

S = 0,2334 R-Sq = 98,05% R-Sq(adj) = 97,40%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S14	3	6,7200	A
S15	3	5,7700	B
S16	3	3,5000	C

Table A.17 One-way ANOVA: Ethylene diglycol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	536	268	2,15	0,197
Error	6	747	124		
Total	8	1283			

S = 11,16 R-Sq = 41,78% R-Sq(adj) = 22,37%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S17	3	42,77	A
S16	3	30,05	A
S14	3	24,30	A

Table A.18 One-way ANOVA: Hexaehtylene glycol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	19272	6424	26,70	0,000
Error	8	1924	241		
Total	11	21196			

S = 15,51 R-Sq = 90,92% R-Sq(adj) = 87,52%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S17	3	119,46	A
S16	3	38,99	B
S15	3	36,58	B
S14	3	13,71	B

Table A.19 One-way ANOVA: 7-Hydroxy-Octa-2,4-Dienoic Acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	1	2,065	2,065	3,50	0,135
Error	4	2,360	0,590		
Total	5	4,425			

S = 0,7681 R-Sq = 46,67% R-Sq(adj) = 33,34%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	8,0300	A
S14	3	6,8567	A

Table A.20 One-way ANOVA: Butanoic acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	6,83	2,28	1,93	0,204
Error	8	9,45	1,18		
Total	11	16,27			

S = 1,087 R-Sq = 41,95% R-Sq(adj) = 20,18%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S16	3	7,620	A
Control	3	7,020	A
S17	3	6,210	A
S14	3	5,647	A

Table A.21 One-way ANOVA: Hexanoic acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	4	331,60	82,90	20,69	0,000
Error	10	40,06	4,01		
Total	14	371,66			

S = 2,001 R-Sq = 89,22% R-Sq(adj) = 84,91%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
Control	3	17,940	A
S16	3	10,330	B
S15	3	10,090	B
S17	3	5,820	B C
S14	3	4,500	C

Table A.22 One-way ANOVA: Palmitic acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	9777,4	3259,1	193,59	0,000
Error	8	134,7	16,8		
Total	11	9912,1			

S = 4,103 R-Sq = 98,64% R-Sq(adj) = 98,13%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S17	3	103,543	A
S14	3	74,180	B
S16	3	49,740	C
S15	3	26,727	D

Table A.23 One-way ANOVA: Octanoic Acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	4	3467,3	866,8	22,46	0,000
Error	10	385,9	38,6		
Total	14	3853,3			

S = 6,212 R-Sq = 89,98% R-Sq(adj) = 85,98%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
Control	3	48,800	A
S16	3	26,343	B
S15	3	12,830	B C
S14	3	11,810	B C
S17	3	6,730	C

Table A.24 One-way ANOVA: Decanoic acid (capric acid) versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	1432,030	477,343	6353,99	0,000
Error	8	0,601	0,075		
Total	11	1432,631			

S = 0,2741 R-Sq = 99,96% R-Sq(adj) = 99,94%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S16	3	34,410	A
S17	3	22,740	B
S15	3	11,620	C
S14	3	5,890	D

Table A.25 One-way ANOVA: Sorbic Acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	4	2249823	562456	4619,27	0,000
Error	10	1218	122		
Total	14	2251041			

S = 11,03 R-Sq = 99,95% R-Sq(adj) = 99,92%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S17	3	1807,2	A
S15	3	1023,3	B
S16	3	959,7	C
S14	3	830,3	D
Control	3	702,9	E

Table A.26 One-way ANOVA: Tetradeconaic acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	1887,146	629,049	789,84	0,000
Error	8	6,371	0,796		
Total	11	1893,517			

S = 0,8924 R-Sq = 99,66% R-Sq(adj) = 99,54%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
Control	3	34,790	A
S16	3	12,090	B
S14	3	11,170	B
S15	3	0,340	C

Table A.27 One-way ANOVA: Pentadeconaic acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	4	2701,054	675,264	1692,31	0,000
Error	10	3,990	0,399		
Total	14	2705,044			

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	35,630	A
S17	3	34,980	A
Control	3	20,600	B
S14	3	7,890	C
S16	3	3,100	D

Table A.28 One-way ANOVA: Hexadeconaic acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	2298,96	1149,48	855,28	0,000
Error	6	8,06	1,34		
Total	8	2307,03			

S = 1,159 R-Sq = 99,65% R-Sq(adj) = 99,53%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S14	3	49,580	A
S17	3	18,587	B
S16	3	13,370	C

Table A.29 One-way ANOVA: Propionic acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	1	3,9366	3,9366	314,93	0,000
Error	4	0,0500	0,0125		
Total	5	3,9866			

S = 0,1118 R-Sq = 98,75% R-Sq(adj) = 98,43%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S17	3	6,7800	A
S14	3	5,1600	B

Table A.30 One-way ANOVA: Octadecanoic acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	4	1602,79	400,70	156,74	0,000
Error	10	25,56	2,56		
Total	14	1628,35			

S = 1,599 R-Sq = 98,43% R-Sq(adj) = 97,80%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
Control	3	45,360	A
S14	3	31,050	B
S17	3	29,460	B
S15	3	21,590	C
S16	3	14,540	D

Table A.31 One-way ANOVA: Isoamyl acetate versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	4	239,991	59,998	189,65	0,000
Error	10	3,164	0,316		
Total	14	243,154			

S = 0,5625 R-Sq = 98,70% R-Sq(adj) = 98,18%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S17	3	17,600	A
S16	3	15,190	B
S14	3	11,920	C
S15	3	10,430	C
Control	3	5,980	D

Table A.32 One-way ANOVA: Ethyl lactate versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	72,99260	36,49630	12441,92	0,000
Error	6	0,01760	0,00293		
Total	8	73,01020			

S = 0,05416 R-Sq = 99,98% R-Sq(adj) = 99,97%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	13,6100	A
S17	3	13,4700	B
S14	3	7,5000	C

Table A.33 One-way ANOVA: 1,2-Benzenedicarboxylic acid, , versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	4	422,661	105,665	479,69	0,000
Error	10	2,203	0,220		
Total	14	424,864			

S = 0,4693 R-Sq = 99,48% R-Sq(adj) = 99,27%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
Control	3	17,630	A
S17	3	14,660	B
S15	3	12,270	C
S14	3	4,900	D
S16	3	4,290	D

Table A.34 One-way ANOVA: 1,2-Benzenedicarboxylic acid,_1 versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	2018177	1009088	670,05	0,000
Error	6	9036	1506		
Total	8	2027213			

S = 38,81 R-Sq = 99,55% R-Sq(adj) = 99,41%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S16	3	1224,0	A
S15	3	480,4	B
S14	3	81,3	C

Table A.35 One-way ANOVA: Ethy linoleate versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	6085,00	2028,33	943,42	0,000
Error	8	17,20	2,15		
Total	11	6102,20			

S = 1,466 R-Sq = 99,72% R-Sq(adj) = 99,61%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S14	3	63,510	A
S17	3	21,950	B
S16	3	11,470	C
S15	3	6,150	D

Table A.36 One-way ANOVA: Ethyl acetate versus Volatile Compounds

```
Source              DF        SS        MS         F      P
Volatile Compounds   1  55,99815  55,99815  24888,07  0,000
Error                4   0,00900   0,00225
Total                5  56,00715

S = 0,04743   R-Sq = 99,98%   R-Sq(adj) = 99,98%

Grouping Information Using Tukey Method

Volatile
Compounds  N     Mean  Grouping
S15        3  13,6100  A
S14        3   7,5000    B
```

Table A.37 One-way ANOVA: Ethyl 4-hydroxybutanoate versus Volatile Compounds

```
Source              DF       SS       MS       F      P
Volatile Compounds   3  3237,08  1079,03  371,74  0,000
Error                8    23,22     2,90
Total               11  3260,31

S = 1,704   R-Sq = 99,29%   R-Sq(adj) = 99,02%


Grouping Information Using Tukey Method

Volatile
Compounds  N    Mean  Grouping
S14        3  58,600  A
S16        3  32,510    B
S15        3  25,170      C
S17        3  13,950        D
```

Table A.38 One-way ANOVA: Diethyl Phthalate versus Volatile Compounds

```
Source              DF     SS     MS       F      P
Volatile Compounds   2  78921  39460  149,57  0,000
Error                6   1583    264
Total                8  80504

S = 16,24   R-Sq = 98,03%   R-Sq(adj) = 97,38%


Grouping Information Using Tukey Method

Volatile
Compounds  N    Mean  Grouping
S17        3  316,42  A
S16        3  157,22    B
Control    3   93,81      C
```

Table A.39 One-way ANOVA: D-Limonene versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	1	1,325	1,325	3,64	0,129
Error	4	1,456	0,364		
Total	5	2,782			

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
Control	3	5,6800	A
S14	3	4,7400	A

Table A.40 One-way ANOVA: Elemicin versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	52339	26169	83,48	0,000
Error	6	1881	313		
Total	8	54220			

S = 17,71 R-Sq = 96,53% R-Sq(adj) = 95,37%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S17	3	192,66	A
S16	3	89,47	B
S15	3	6,22	C

Table A.41 One-way ANOVA: Xylenol versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	1011,7	505,9	16,63	0,004
Error	6	182,5	30,4		
Total	8	1194,2			

S = 5,515 R-Sq = 84,72% R-Sq(adj) = 79,62%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	30,050	A
S17	3	22,350	A
S14	3	4,720	B

Table A.42 One-way ANOVA: -7-Hydroxyocta-2,4 dienoic acid versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	1	2,27	2,27	1,71	0,262
Error	4	5,32	1,33		
Total	5	7,59			

S = 1,153 R-Sq = 29,90% R-Sq(adj) = 12,37%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	8,020	A
S14	3	6,790	A

Table A.43 One-way ANOVA: Phenol, 2,3 dimethyl versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	511,68	170,56	94,68	0,000
Error	8	14,41	1,80		
Total	11	526,09			

S = 1,342 R-Sq = 97,26% R-Sq(adj) = 96,23%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	23,300	A
S16	3	13,770	B
S14	3	8,420	C
S17	3	6,450	C

Table A.44 One-way ANOVA: Tetrapentacosan versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	510,4	255,2	3,10	0,119
Error	6	493,9	82,3		
Total	8	1004,3			

S = 9,073 R-Sq = 50,82% R-Sq(adj) = 34,43%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S14	3	37,200	A
S17	3	29,260	A
S15	3	18,810	A

Table A.45 One-way ANOVA: Tetracosane versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	1	13,590	13,590	55,46	0,002
Error	4	0,980	0,245		
Total	5	14,570			

S = 0,4950 R-Sq = 93,27% R-Sq(adj) = 91,59%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S14	3	6,2100	A
S16	3	3,2000	B

Table A.46 One-way ANOVA: Pentacosane versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	572,4	286,2	12,77	0,007
Error	6	134,5	22,4		
Total	8	706,9			

S = 4,735 R-Sq = 80,97% R-Sq(adj) = 74,63%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	23,850	A
S14	3	11,800	B
S17	3	4,510	B

Table A.47 One-way ANOVA: Hexzatriacontane versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	654	327	0,65	0,556
Error	6	3023	504		
Total	8	3677			

S = 22,45 R-Sq = 17,78% R-Sq(adj) = 0,00%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S17	3	129,79	A
S16	3	119,25	A
S14	3	108,91	A

Table A.48 One-way ANOVA: Butyrate versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	3	107,0	35,7	3,26	0,081
Error	8	87,6	11,0		
Total	11	194,7			

S = 3,310 R-Sq = 54,98% R-Sq(adj) = 38,10%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S17	3	18,290	A
S14	3	14,640	A
S15	3	14,100	A
S16	3	9,870	A

Table A.49 One-way ANOVA: 2,6,10,14,18,22-Tetracosahexaen versus Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	4	4138,3	1034,6	21,21	0,000
Error	10	487,8	48,8		
Total	14	4626,1			

S = 6,985 R-Sq = 89,45% R-Sq(adj) = 85,24%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
Control	3	52,650	A
S16	3	22,280	B
S17	3	17,980	B
S14	3	11,820	B
S15	3	4,100	B

Table A.50 One-way ANOVA: 2(3H)-Furanone, Volatile Compounds

Source	DF	SS	MS	F	P
Volatile Compounds	2	28,6146	14,3073	1288,95	0,000
Error	6	0,0666	0,0111		
Total	8	28,6812			

S = 0,1054 R-Sq = 99,77% R-Sq(adj) = 99,69%

Grouping Information Using Tukey Method

Volatile Compounds	N	Mean	Grouping
S15	3	11,6400	A
S14	3	10,9500	B
S17	3	7,5600	C

APPENDICES B

Microorganism Sequences

S1 ITS1-ITS4 Region Sequence

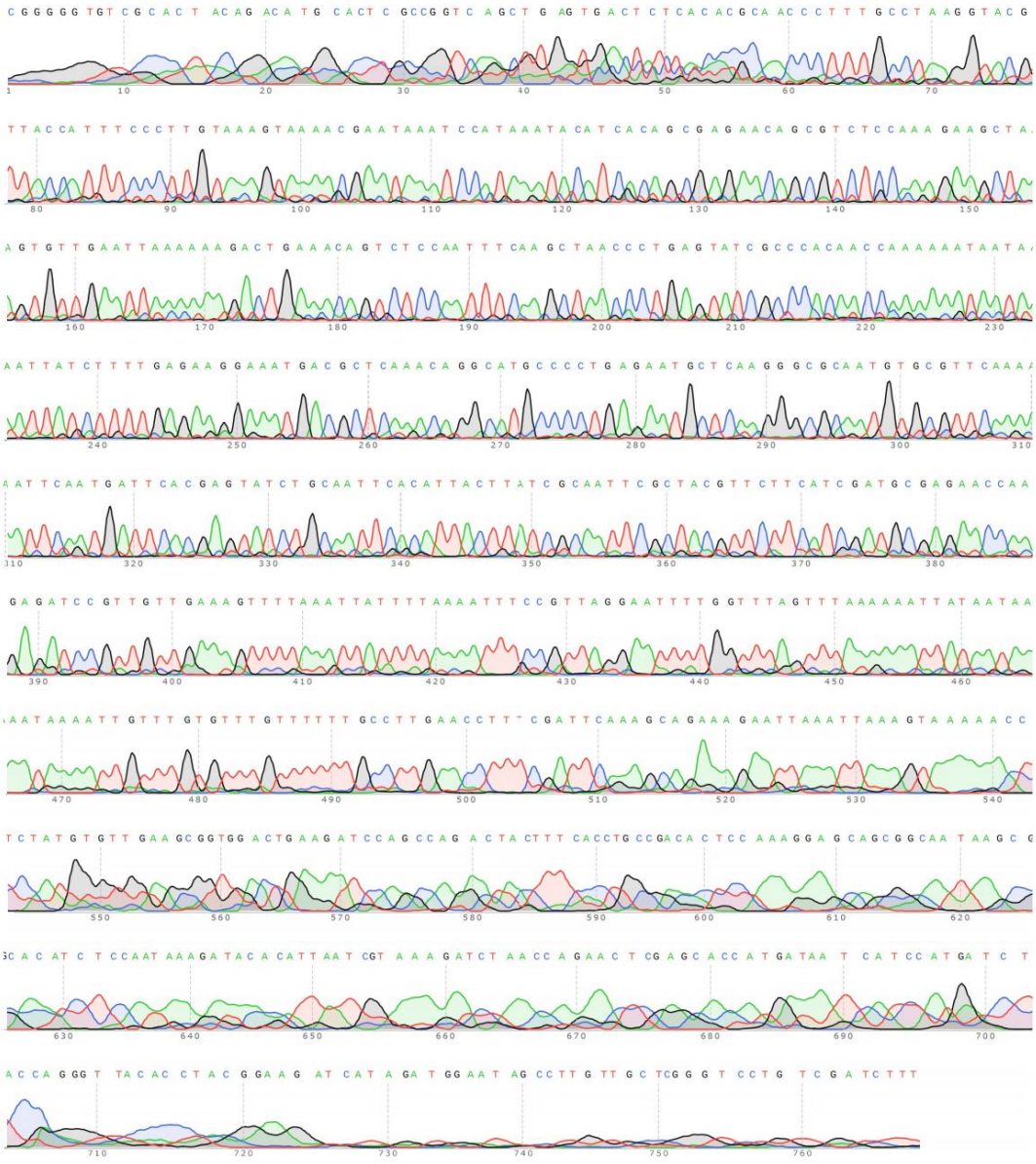

Figure B.1: S1 ITS1-ITS4 Region Sequencing

S2 ITS1-ITS4 Region Sequence

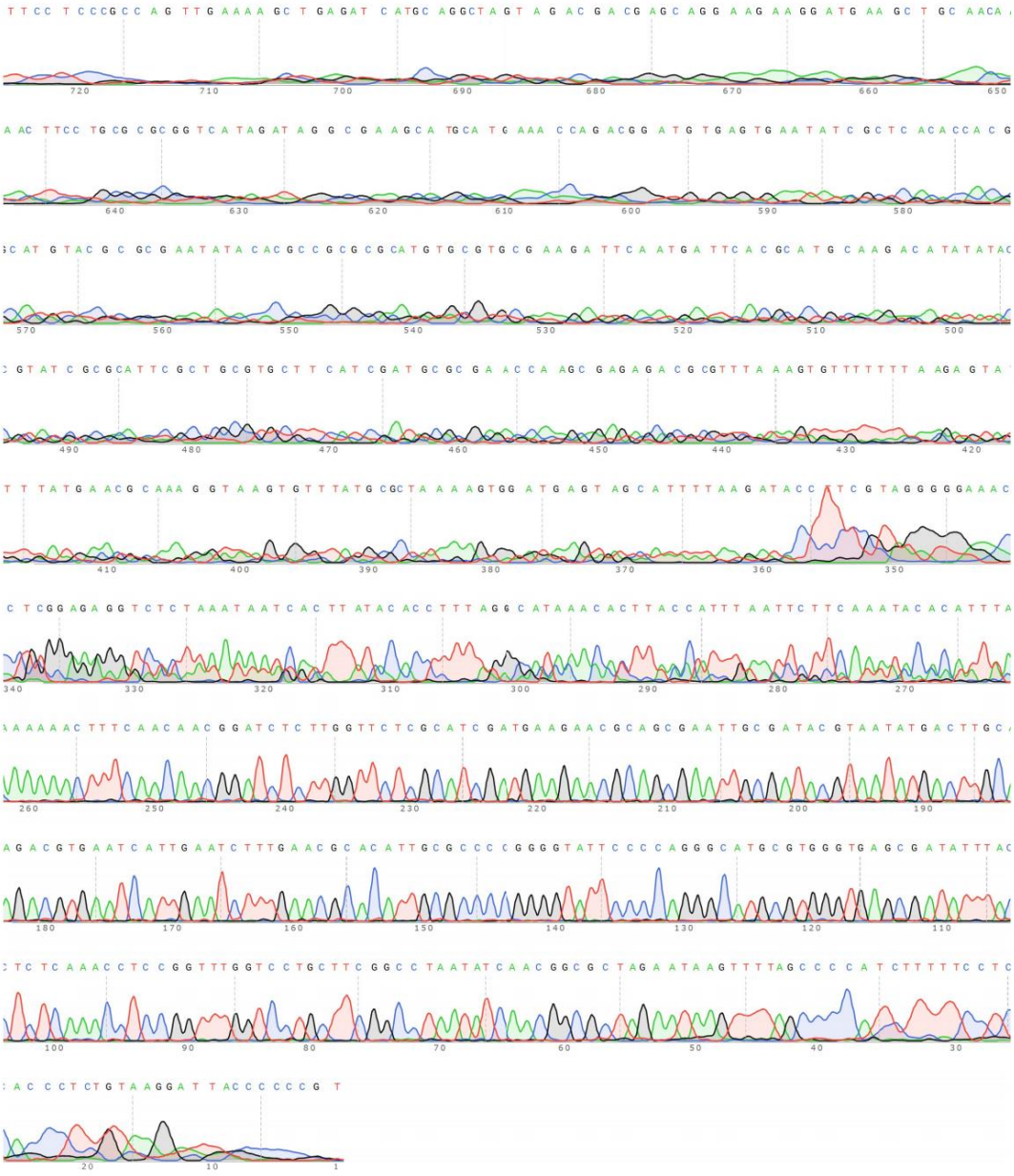

Figure B.2: S2 ITS1-ITS4 Region Sequence

S3 ITS1-ITS4 Region Sequence

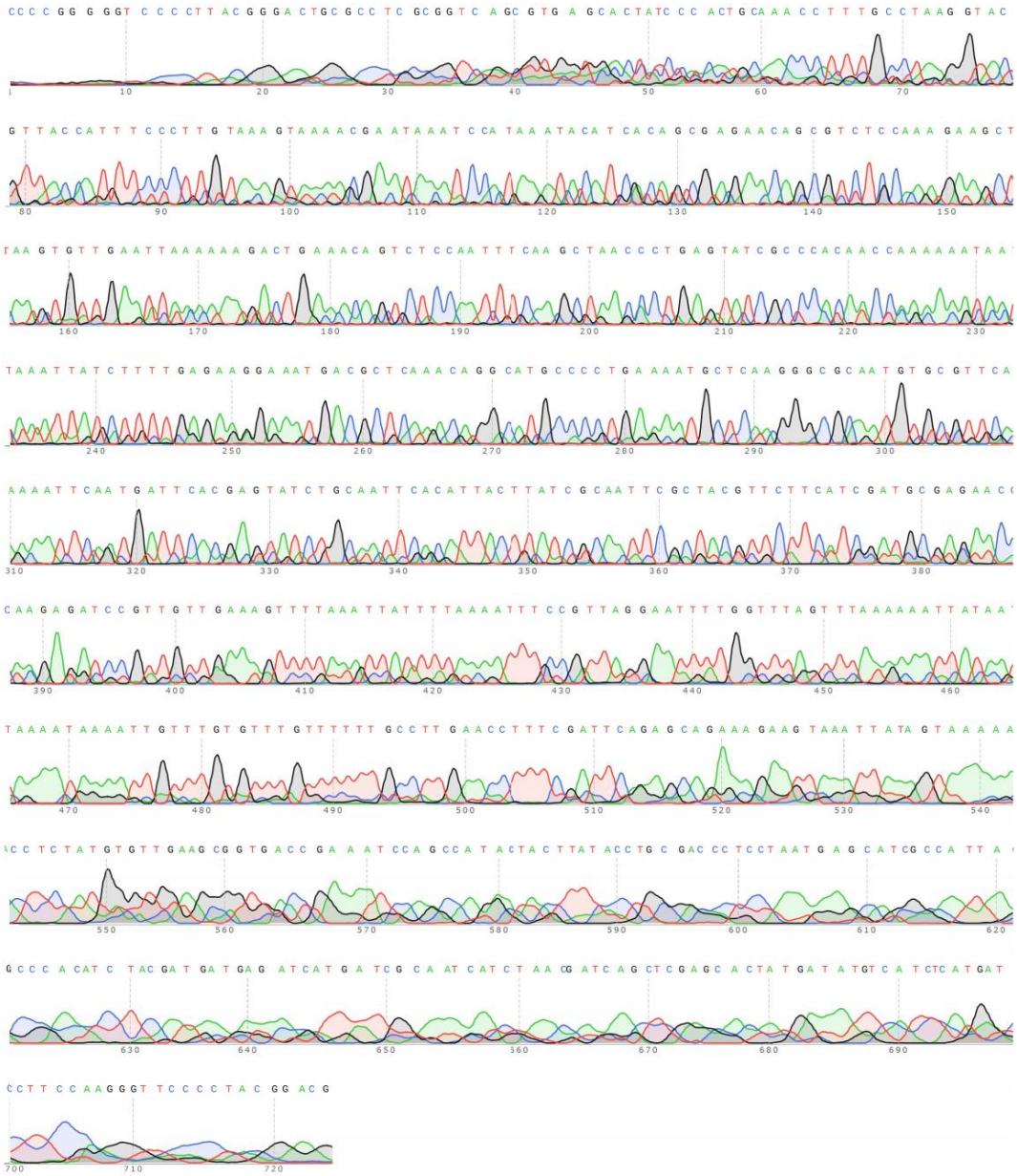

Figure B.3: S3 ITS1-ITS4 Region Sequence

S4 ITS1-ITS4 Region Sequence

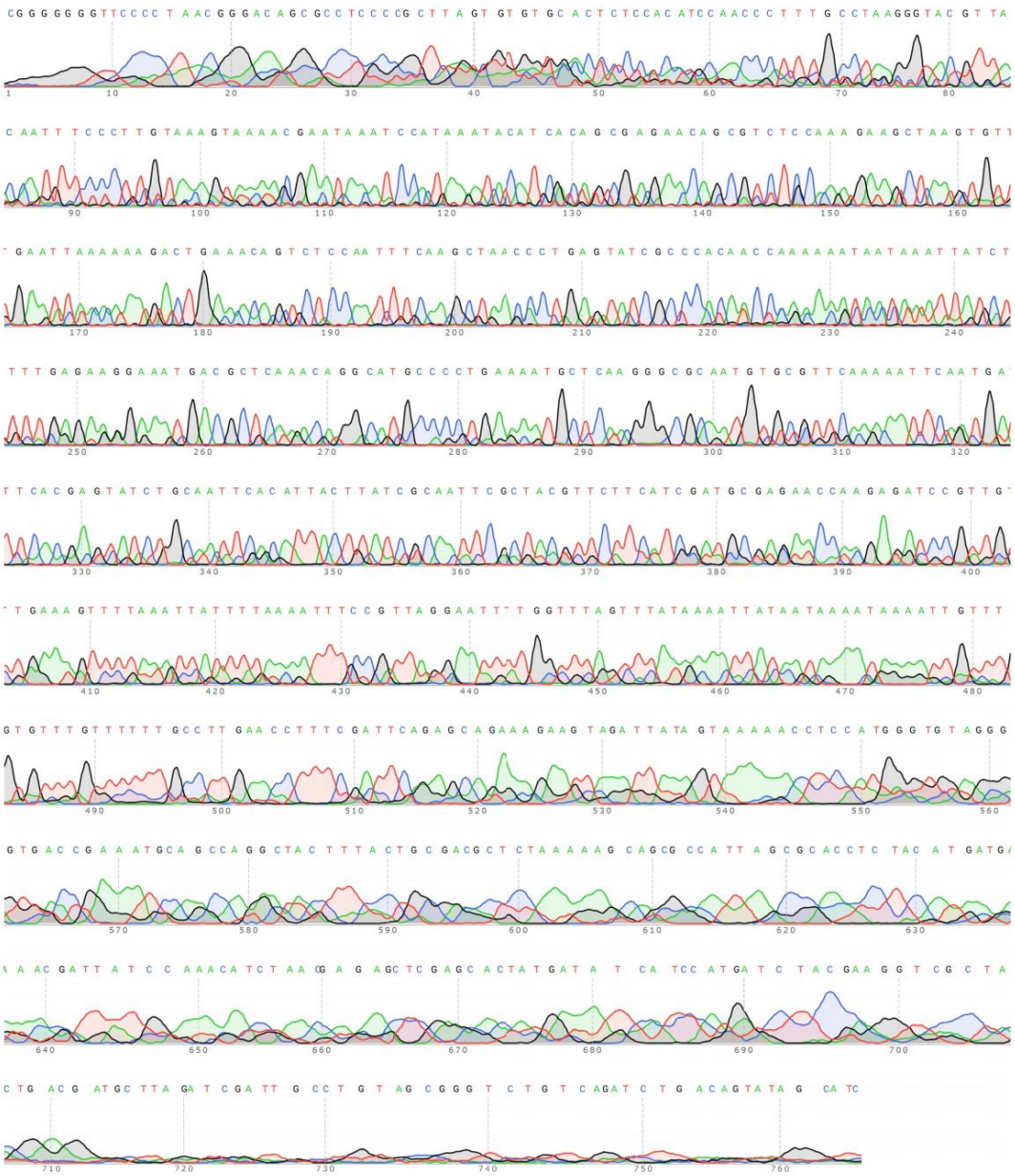

Figure B.4: S4 ITS1-ITS4 Region Sequence

S5 ITS1-ITS4 Region Sequence

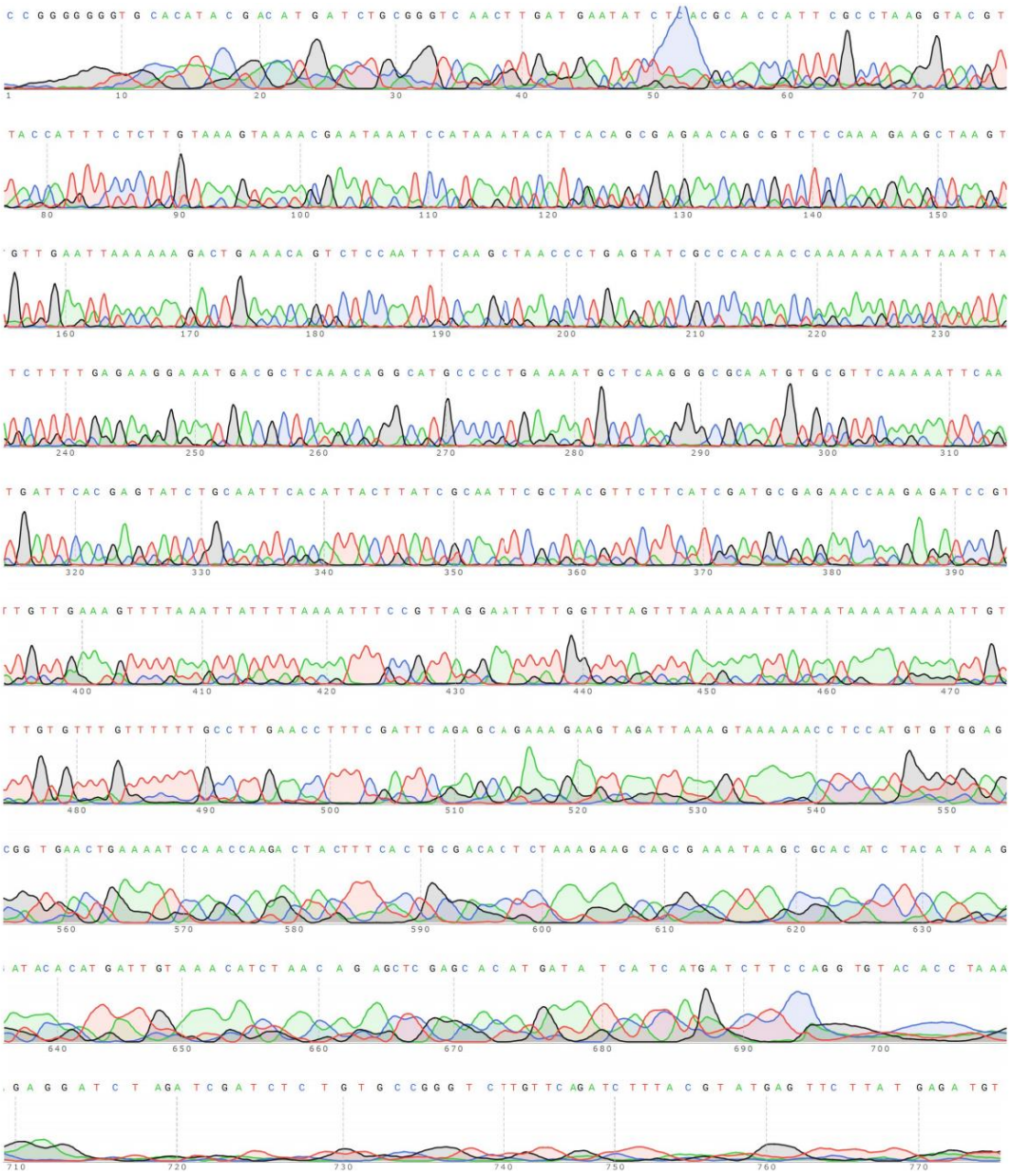

Figure B.5: S5 ITS1-ITS4 Region Sequence

S6 ITS1-ITS4 Region Sequence

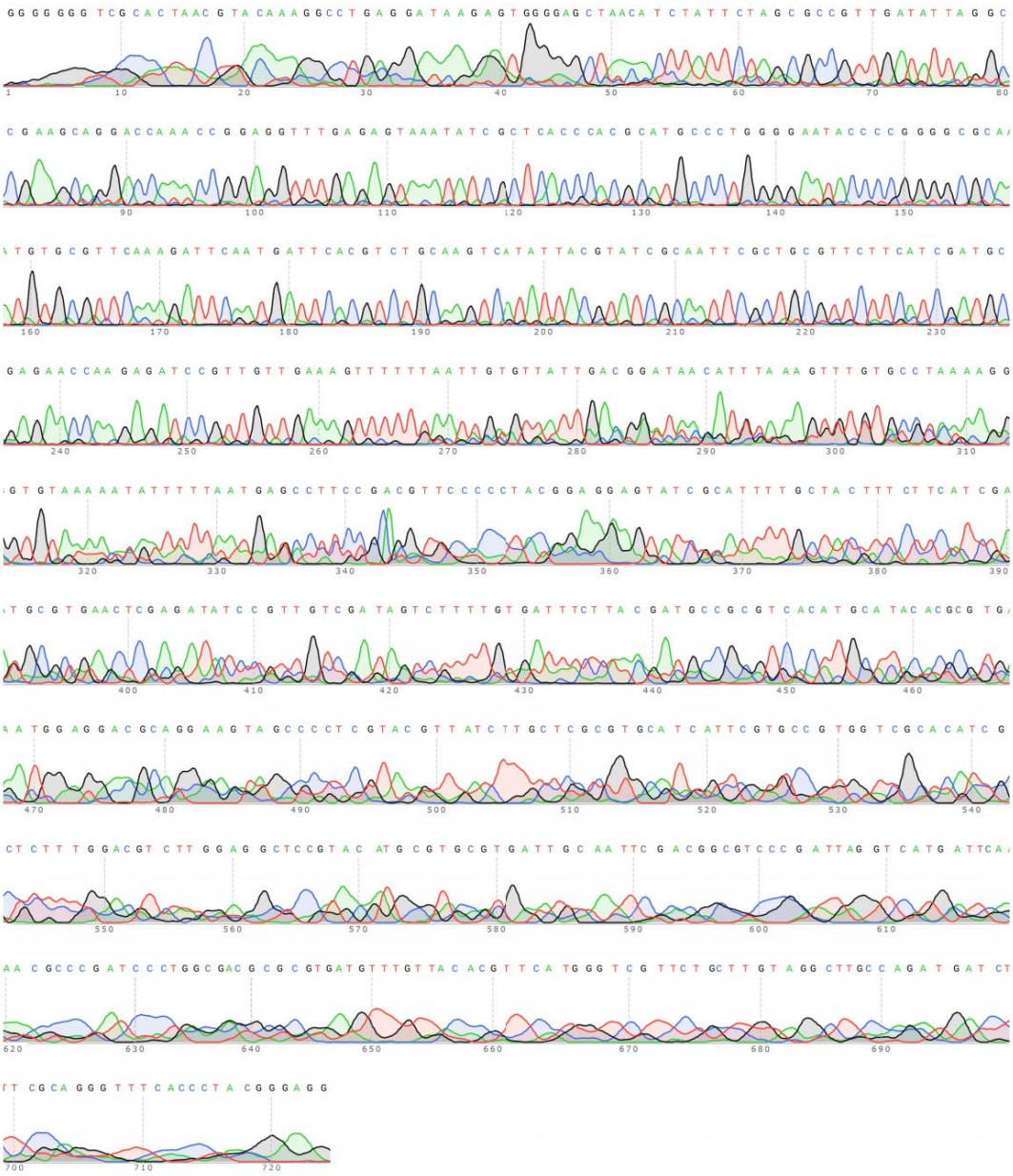

Figure B.6: S6 ITS1-ITS4 Region Sequence

S7 ITS1-ITS4 Region Sequence

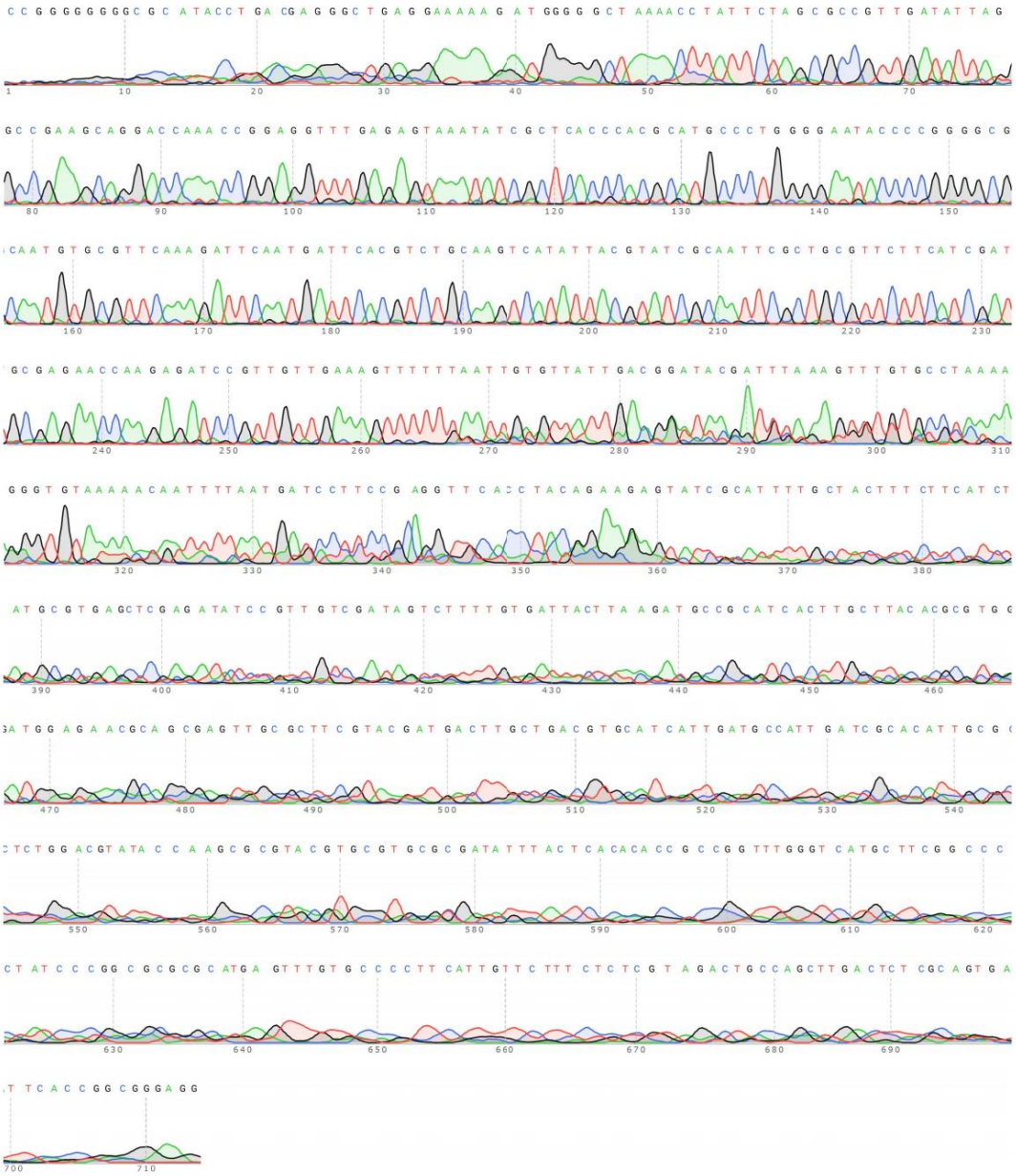

Figure B.7: S7 ITS1-ITS4 Region Sequence

S8 ITS1-ITS4 Region Sequence

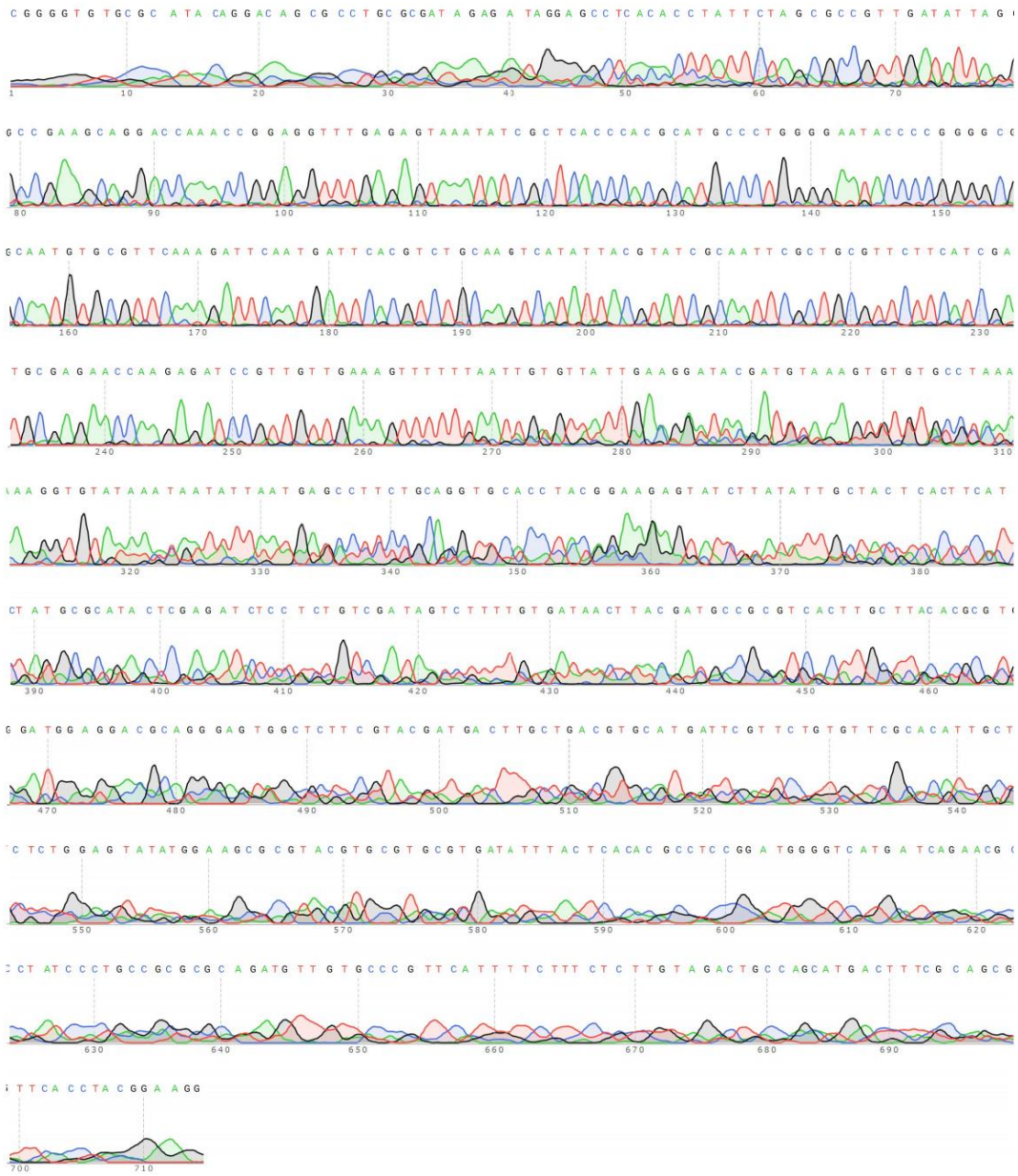

Figure B.8: S8 ITS1-ITS4 Region Sequence

S9 ITS1-ITS4 Region Sequence

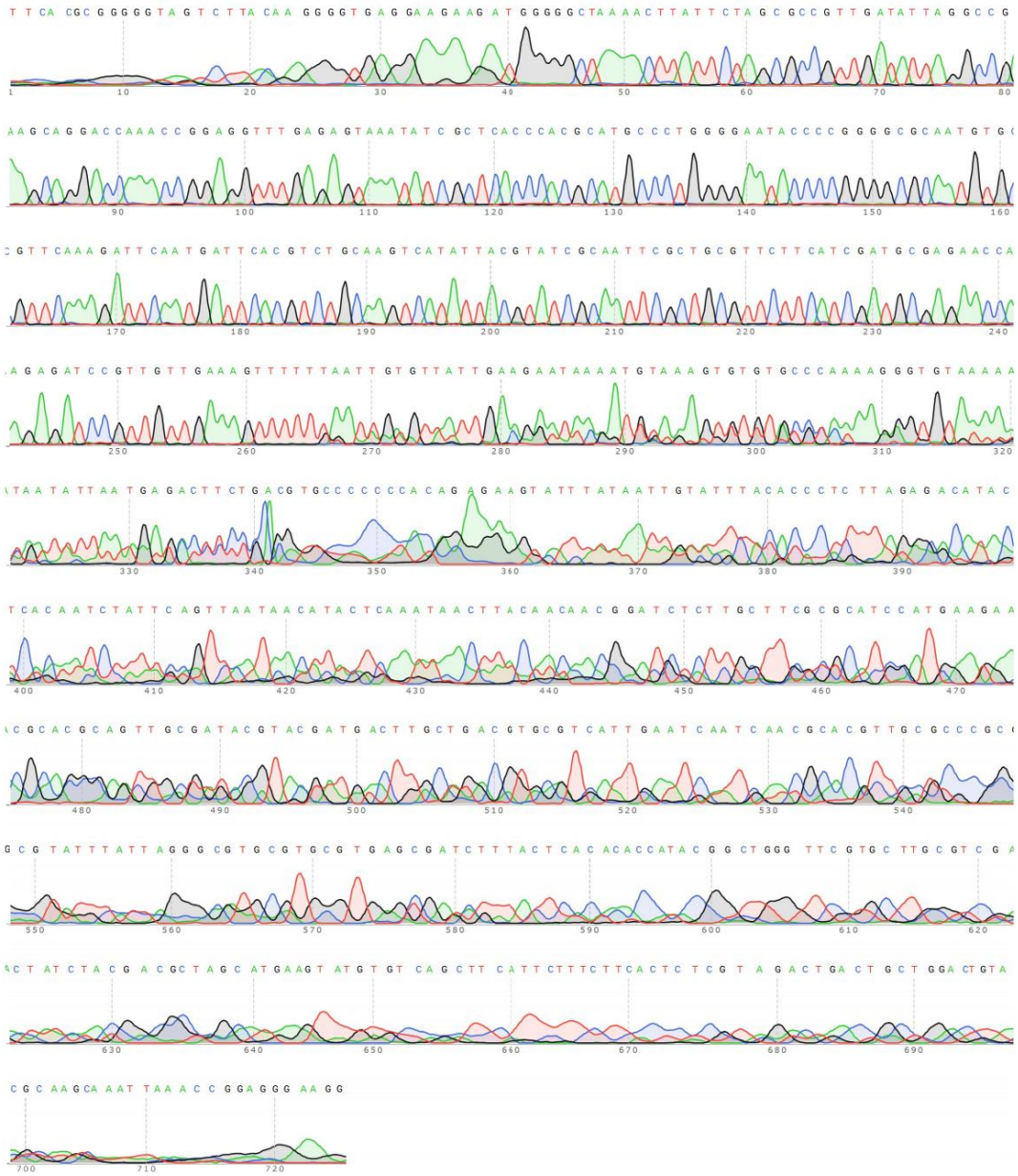

Figure B.9: S9 ITS1-ITS4 Region Sequence

S10 ITS1-ITS4 Region Sequence

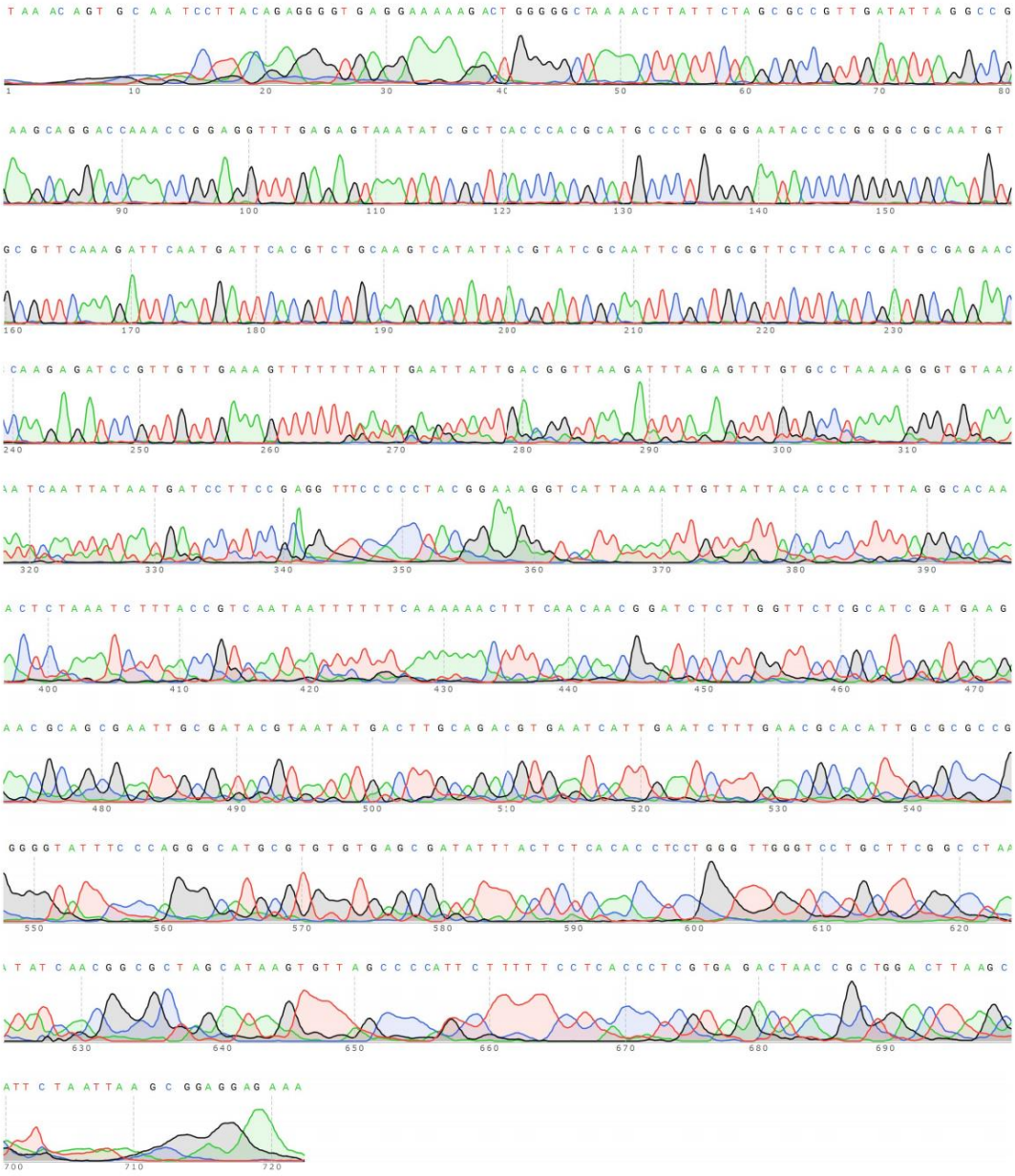

Figure B.10: S10 ITS1-ITS4 Region Sequence

S11 ITS1-ITS4 Region Sequence

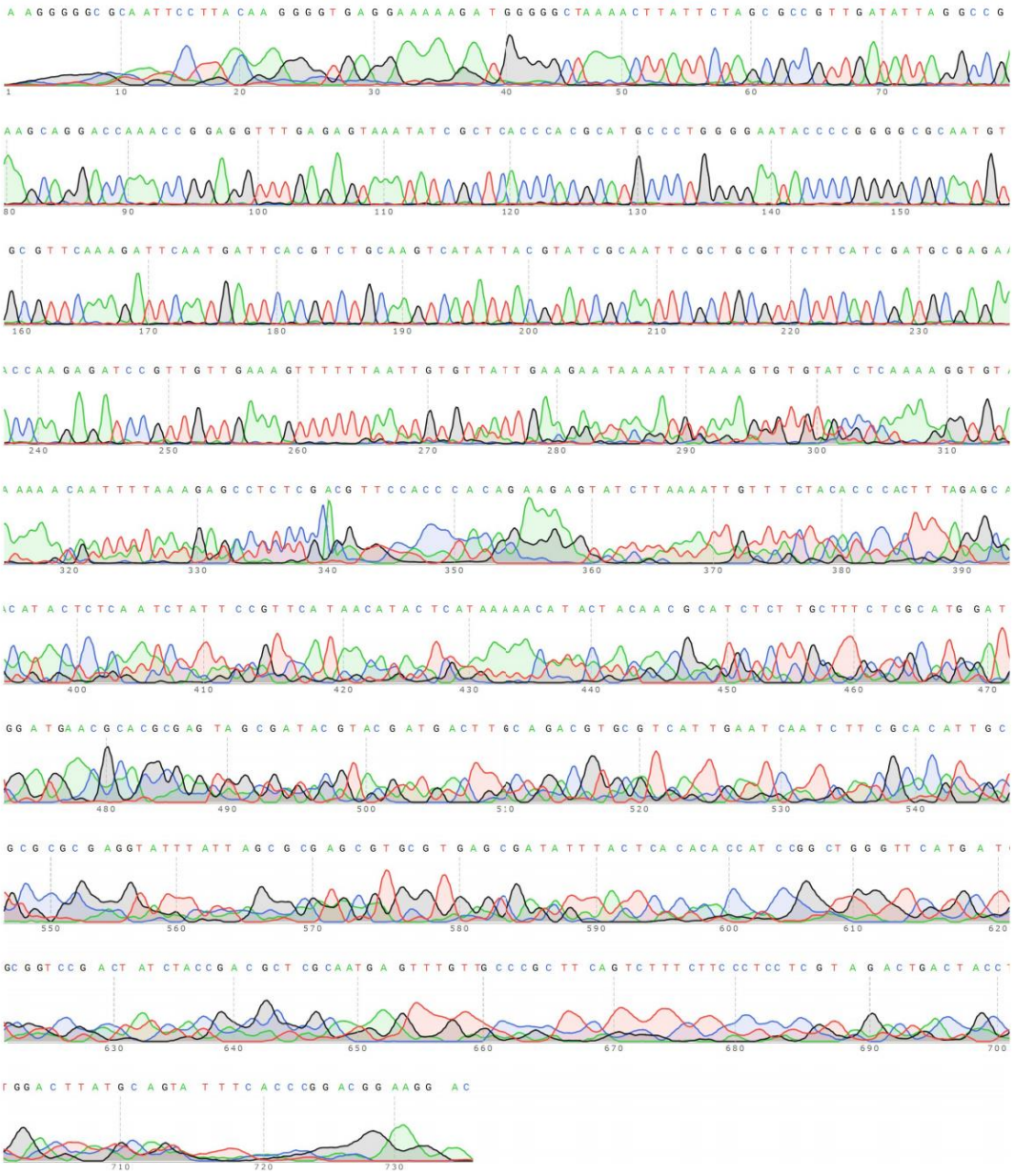

Figure B.116: S11 ITS1-ITS4 Region Sequence

S12 ITS1-ITS4 Region Sequence

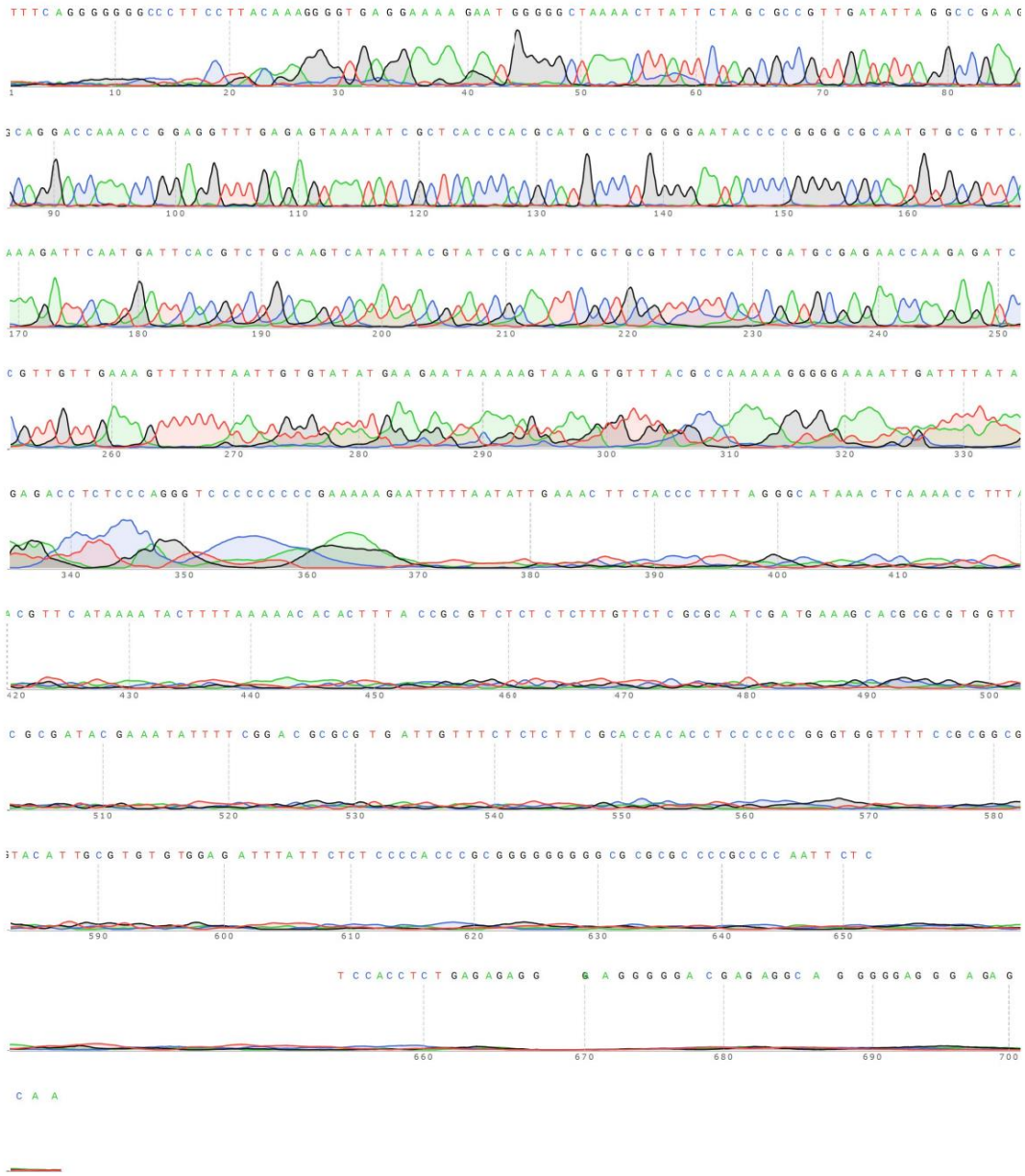

Figure B.12: S12 ITS1-ITS4 Region Sequence

S13 ITS1-ITS4 Region Sequence

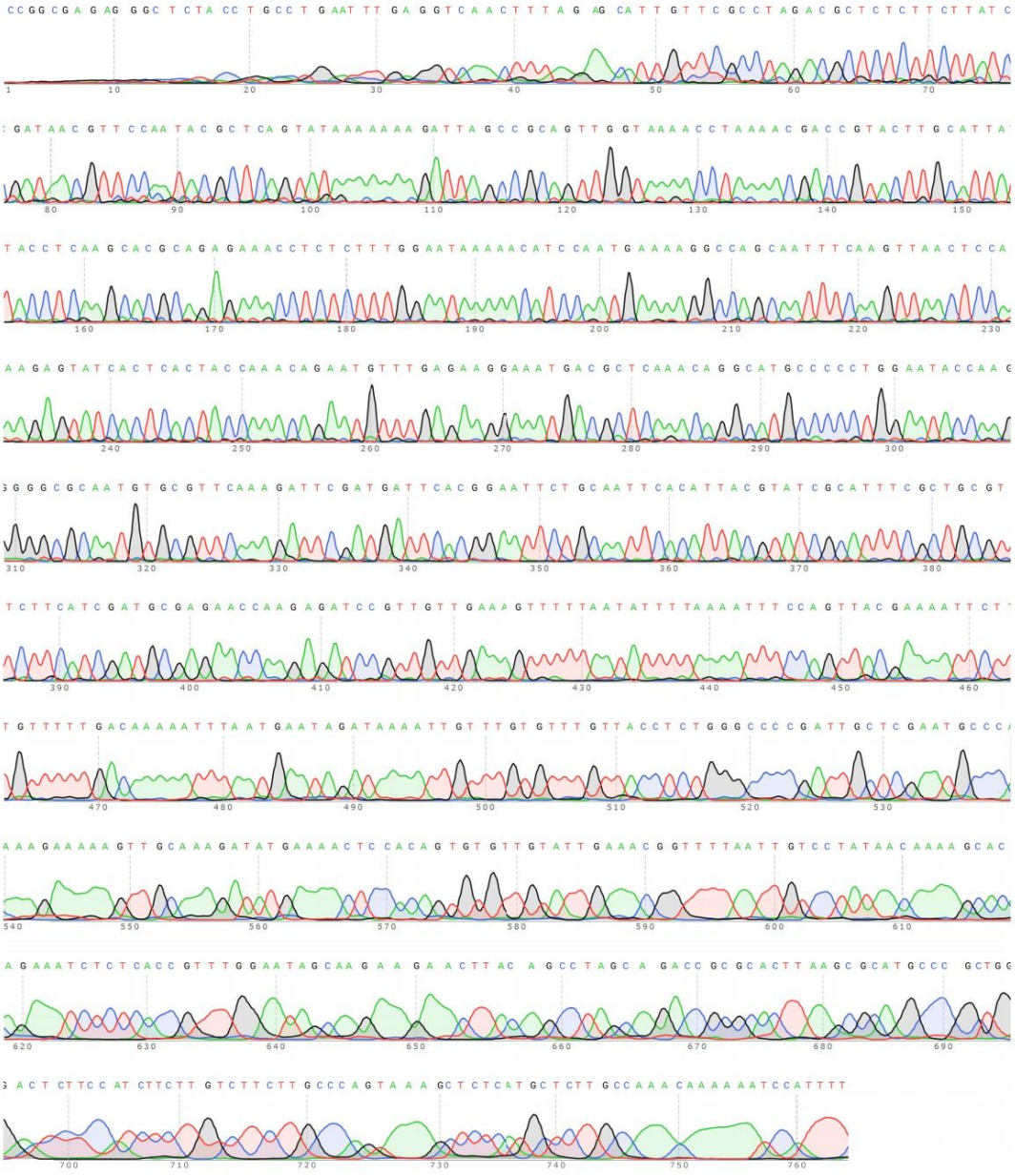

Figure B.13: S13 ITS1-ITS4 Region Sequence

S14 ITS1-ITS4 Region Sequence

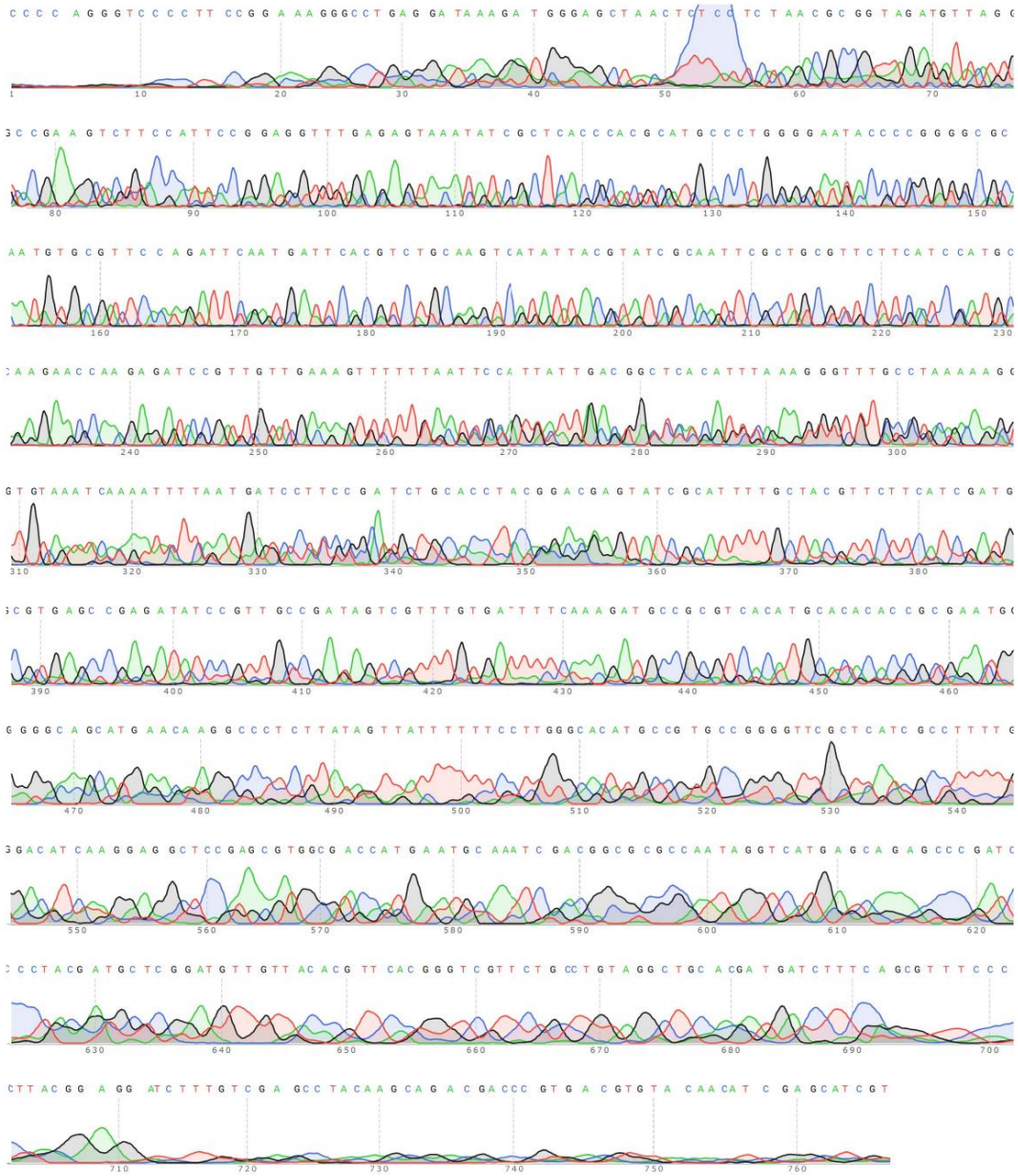

Figure B.14: S14 ITS1-ITS4 Region Sequence

S15 ITS1-ITS4 Region Sequence

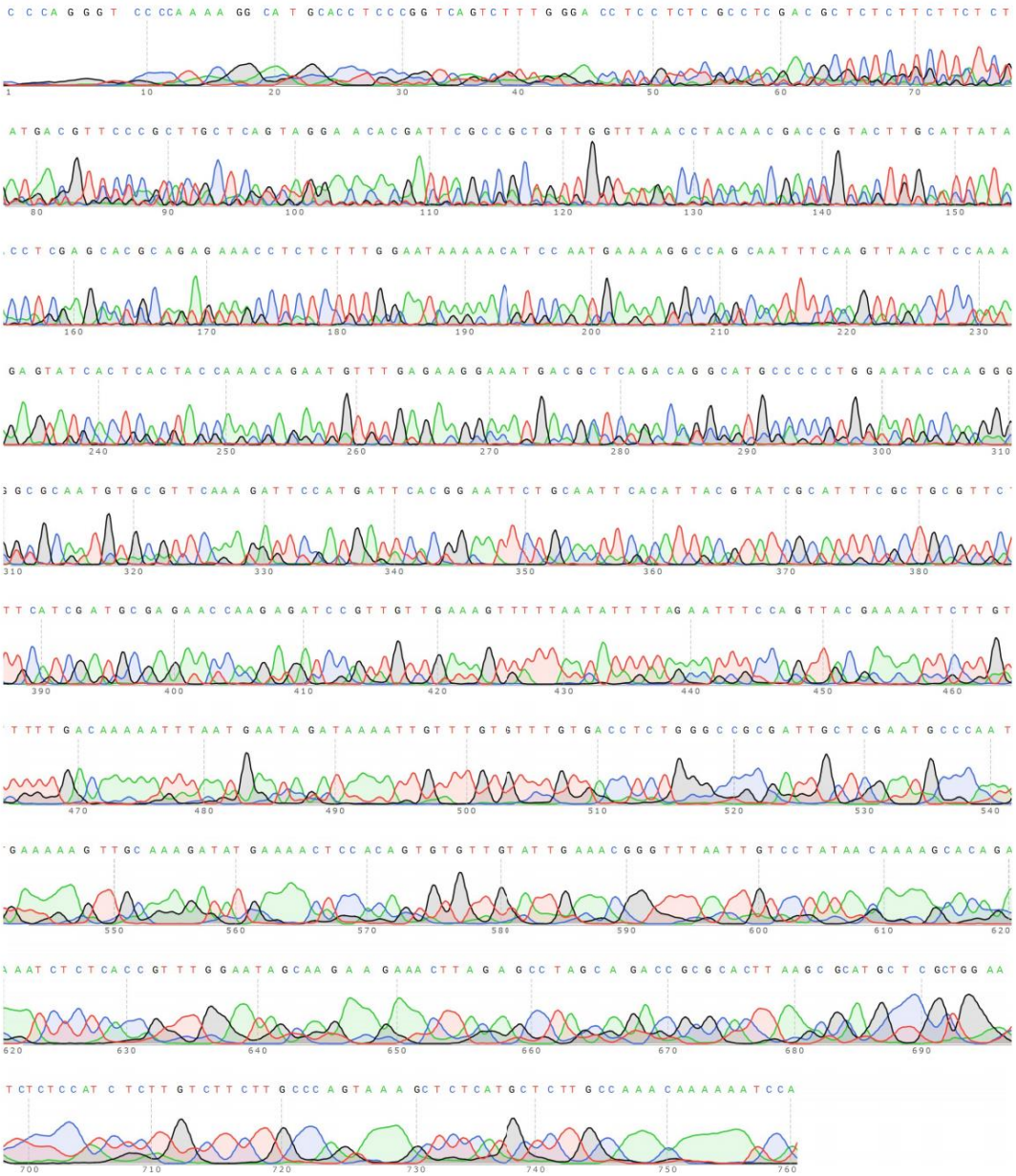

Figure B.157: S15 ITS1-ITS4 Region Sequence

S16 ITS1-ITS4 Region Sequence

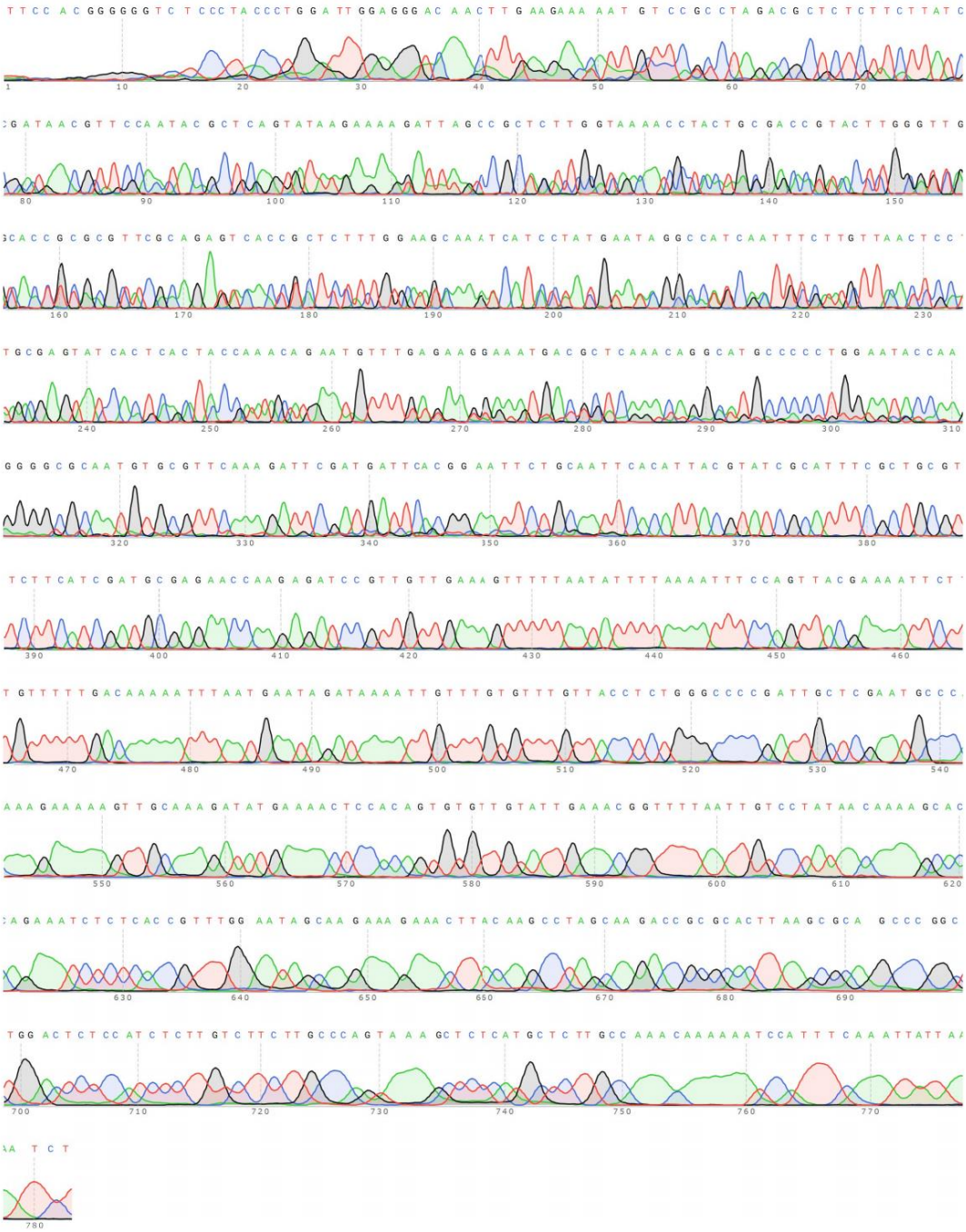

Figure B.16: S16 ITS1-ITS4 Region Sequence

S17 ITS1-ITS4 Region Sequence

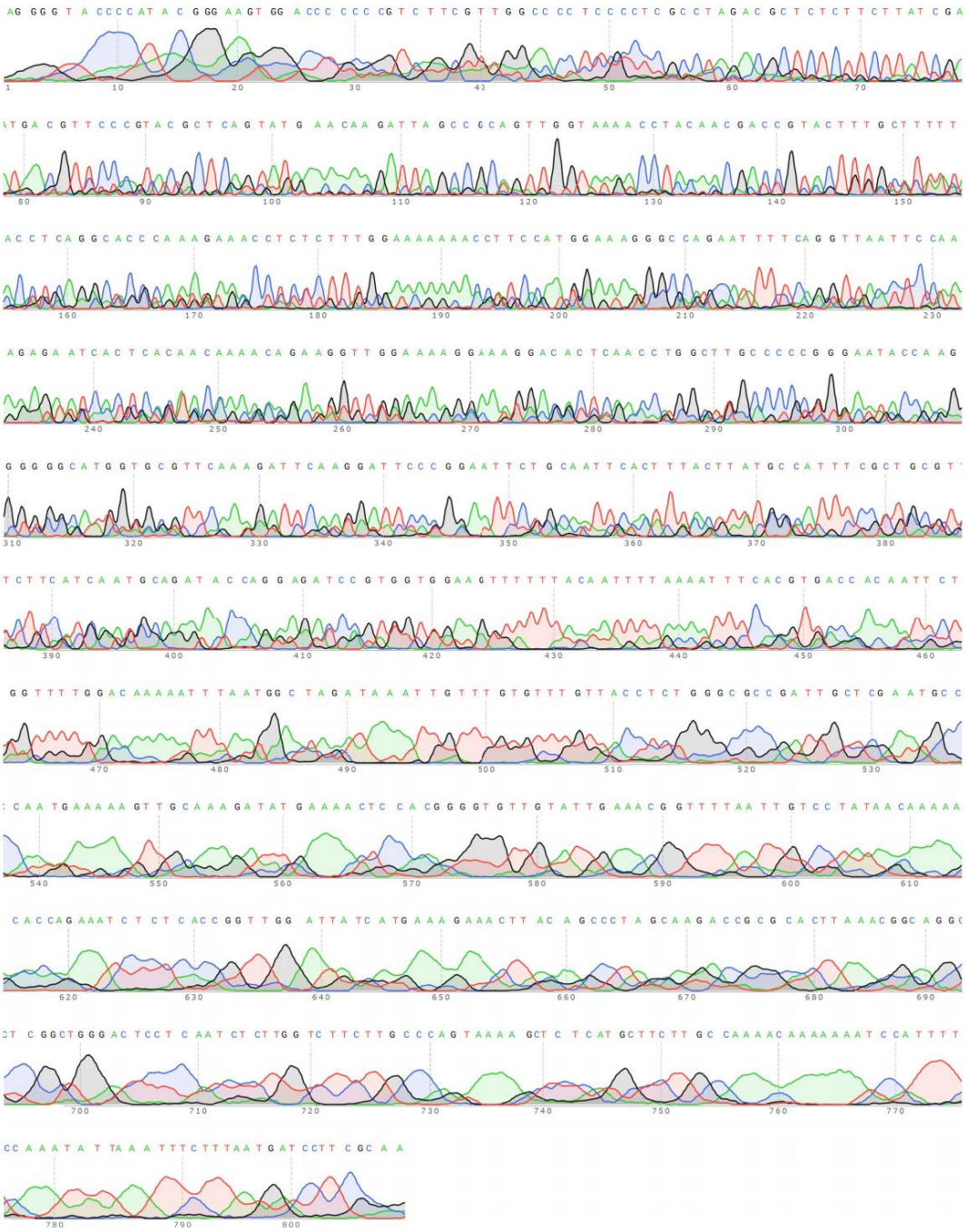

Figure B.17: S17 ITS1-ITS4 Region Sequence