# GENETIC DIVERSITY OF ALMONDS (*Prunus dulcis)* OF DATÇA

by

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B.S., Molecular Biology and Genetics, Boğaziçi University, 2010

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Molecular Biology and Genetics
Boğaziçi University
2012

*To my father*

# ACKNOWLEDGEMENTS

First and foremost I offer my sincerest gratitude to my supervisor Neş'e Bilgin for her endless guidance, encouragement and valuable criticism throughout my research.

I would like to express my thanks to the jury members, Raşit Bilgin and Uğur Sezerman for devoting their time in evaluating this work.

This work was supported by Boğaziçi University Research Fund (BAP-5068), Ali Nihat Gökyiğit Vakfı and SGP-GEF (TUR/SGP/OP4/RAF/07/01/01).

This project would not be possible without the invaluable help of Elisabeth Tüzün for the collection of almond leaves any time it is required. I would like to express my gratitude to her and to the Fruit Heritage group members.

I would like to thank my lab members Uğur Kaplan and Levent Baş for their invaluable assistance in every crisis I had.

I could also not forget to mention my friends İrem Ünlü, Harun Niron, Burçin Duan Şahbaz, Gizem Gül, Güneş Tunçgenç, Sercan Sayın, Sandra Rode, Ümit Bayraktar, Güner Kaçmaz, Cenan Özbakır, Vahap Kapıkıran, Balkan Canher, Alperen Erdoğan, Yıldız Koca, Özden Akay and Erdem Yılmaz. They were always friendly, full of joy and support.

I wish to thank my best friend Gözde Turan. Even though she had to be in far oceans away from me most of the time, she always supported me and never let me walk alone.

Finally I would like to thank to my mom and dad for supporting me throughout all my studies at University and for their neverending love.

# ABSTRACT

## GENETIC DIVERSITY OF ALMONDS (*Prunus dulcis*) OF DATÇA

Anatolia's agricultural biodiversity, which has evolved with the contributions of many different cultures over thousands of years, is today being rapidly eroded as a result of climate change, population growth, globalization and agricultural policies. On the other hand, in every part of Anatolia, there are fruit varieties that are adapted to local soil and climate conditions, resistant to local pests and diseases, and require no irrigation, artificial fertilizer or pesticides. Unfortunately, most of these fruit varieties are today threatened by urbanization, soaring land prices and marketing problems in addition to agricultural policies. These fruit varieties are important genetic resources essential for the food security in the face of climate change and with their diverse flavors and traditional uses they are a significant part of Turkey's cultural heritage. Since 2007, as a part of the "FRUIT LANDRACES OF MUĞLA: CULTURAL HERITAGE, DATABASE AND CONSERVATION PROJECT", 96 almond landraces (*Prunus dulcis*) has been recorded in Datça. Molecular characterization and the genetic diversity of these almonds is the subject of this thesis. Using microsatellite (SSR) markers, molecular data were gathered in the form of DNA fingerprints and phylogenetic relatedness of these varieties was constructed on a dendrogram. From this data 82 unique almond varieties were identified. DNA fingerprints could allow the registration of these unique varieties as Datça's landraces. Moreover, this data can be used for the selection of proper varieties for agricultural hybridization studies for the development of new and better cultivars.

# ÖZET

## DATÇA BADEMLERİNİN (*Prunus dulcis)* GENETİK ÇEŞİTLİLİĞİ

Binlerce yıl boyunca, birçok farklı kültürün katkılarıyla gelişen, Anadolu'nun tarımsal biyoçeşitliliği; iklim değişikliği, nüfus artışı, küreselleşme ve tarım politikalarının bir sonucu olarak kaybolmaktadır. Halbuki, Anadolu'nun çoğu yerinde, hiçbir sulama, suni gübre ve zirai ilaç gerektirmeyen, yerel hastalık ve zararlılara dayanıklı meyve çeşitleri mevcuttur. Ne yazık ki, bu meyve çeşitlerinin çoğu günümüzde tarım politikalarının yanı sıra artan arsa fiyatları, pazarlama sorunları ve hızlı kentleşmenin tehdidi altındadır. Bu yerel meyve çeşitleri, iklim değişikliği karşısında gıda güvencemizi sağlamanın yanı sıra farklı tatları ve geleneksel kullanımları ile Türkiye'nin kültürel mirasının önemli bir parçasını oluşturmaktadır. 2007 yılından beri "Muğla'nın Yerel Meyve Çeşitleri: Kültürel Miras, Veritabanı ve Koruma Projesi"nin bir parçası olarak, Datça'da 96 yerel badem (*Prunus dulcis*) çeşidi kaydedilmiştir. Bu yerel çeşitlerin moleküler karakterizasyonu ve genetik çeşitliliğinin belirlenmesi bu tezin konusudur. DNA Mikrosatellit belirteçleri (SSR) kullanılarak, yerel badem çeşitlerinin DNA parmak izleri belirlenmiş ve bu çeşitlerin filogenetik akrabalığını göstern bir dendrogram oluşturulmuştur. Elde edilen moleküler veriler 82 özgün yerel badem çeşidini ortaya çıkartmıştır. DNA parmak izleri bu çeşitlerin tescili için kullanılabilir. Ayrıca bu moleküler veriler, yeni ve daha iyi çeşitlerin geliştirilmesi için tarımsal hibridizasyon çalışmalarında yararlı olabilir.

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| | |
|---|---|
| AFLP | Amplified Fragment Length Polymorphism |
| ATP | Adenosine 5’-triphosphate |
| A-value | Alleles per locus |
| bp | Base pairs |
| BPB | Bromophenol Blue |
| cM | Centimorgan |
| CTAB | Cetyl Trimethyl Ammonium Bromide |
| dATP | 2’-deoxyadenosine 5’-triphosphate |
| dCTP | 2’-deoxycytosine 5’-triphosphate |
| dGTP | 2’-deoxyguanosine 5’-triphosphate |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotides |
| EDTA | Ethylenediaminetetraacetatic acid |
| EtBr | Ethidium bromide |
| EST | Expresses sequence tag |
| $H_e$ | Expected heterozygosity |
| $H_o$ | Observed heterozygosity |
| $H_2O$ | Water |
| ISSR | Internal simple sequence repeats |
| ITS | Internal Transcribed Spacer |
| Kb | Kilo base |
| µg | Microgram (10-6 g) |
| µl | Microliter (10-6 L) |
| µM | Micromolar (10-6 M) |
| M | Molar |
| Mbp | Mega base pairs |
| $Mg^{+2}$ | Magnesium |
| $MgCl_2$ | Magnesium chloride |
| mL | Milliliter (10-3 L) |
| mM | Millimolar |

| | |
|---|---|
| mRNA | Messenger RNA |
| NaCl | Sodium Chloride |
| NaOAc | Sodium Acetate |
| No | Number |
| OD | Optical density |
| PAGE | Polyacrylamide gel electrophoresis |
| PCO | Principal Component Analysis |
| PCR | Polymerase chain reaction |
| PVP | Polyvinylpyrrolidone |
| RAPD | Random amplified polymorphic DNA |
| RFLP | Restriction Fragment Length Polymorphism |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal RNA |
| rpm | Revolutions per minute |
| RT | Room Temperature |
| SDS | Sodiumdodecylsulphate |
| SI | Self-incompatibility |
| SNP | Single nucleotide polymorphism |
| S-Rnase | S-locus encoding ribonuclease |
| SSR | Simple sequence repeat |
| TAE | Tris-Acetate-EDTA |
| *Taq* | *Thermus aquaticus* |
| TE | Tris-EDTA |
| UPGMA | Unweighted pair group method with arithmetic mean |
| USA | United States of America |
| UV | Ultraviolet |

# 1. INTRODUCTION

## 1.1..The Genus *Prunus*

*Prunus* is *a* genus belonging to Angiosprermae (flowering plants) of the Plantae kingdom (Figure 1.1). It is composed of trees and shrubs known as stone fruits such as peach, plum, apricot, cherry and almond. Genus *Prunus* has nearly four hundred species and most of them are cultivated, so both wild and domesticated forms exist (Das *et al.*, 2011).

- Domain: Eukaryota
  - Kingdom: Plantae (Plants)
    - Phylum: Viridiplantae (Green plants)
      - Subphylum: Streptophyta
        - Subkingdom: Embryophyta (Land plants)
          - Tracheophyta (Vascular plants)
            - Superdivision: Spermatophyta (Seed plants)
              - Division: Magnoliophyta (Flowering plants)
                - Clade: Eudicotyledons
                  - Clade: Core eudicotyledons
                    - Class: Magnoliopsida (Dicotyledons)
                      - Rosids
                        - Fabids
                          - Subclass: Rosidae
                            - Order: Rosales
                              - Family: Rosaceae (Rose family)
                                - Subfamily: Amygdaloideae
                                  - Amygdaleae
                                    - Genus: *Prunus* L.

Figure 1.1. Taxonomy of the Genus *Prunus.*

### *1.2.Prunus dulcis*

Almond [*Prunus dulcis* (Mill.) D.A. Webb; syn. *Prunus amygdalus* Batsch] is a species within the genus *Prunus*, which originated in Central Asia and spread to all around the world from this region (Ladizinsky, 1999). Wild forms of almond have bitter kernel taste due to the presence of high levels of cyanogenic glycoside amygdalin (Conn, 1980; Kester *et al.,* 1991; Das *et al.,* 2011). According to one hypothesis, cultivated almond has emerged from a species known as *Amygdalus communis* L. (syn. *Prunus communis* Archang); after selection from two different natural populations mostly composed of sweet-kernel individuals rather than bitter-kernel ones (Watkins, 1979; Arús *et al.,* 2009). In this perspective, the distinction between cultivated and wild forms of almonds gradually disappeared with human selection (Arús *et al.,* 2009). This man-directed-selection hypothesis is also supported by the natural range of *A. communis* that extends across Iran, the Transcaucausus, Eastern Turkey and the present-day Syria, the known sites of early civilizations and almond cultivations (Denisov, 1988; Kester *et al.,* 1991; Arús *et al.,* 2009). As the agrarian societies emerged in Asia and Mediterranean regions, the evolution of the cultivated almond also occurred in parallel. With respect to archeological studies, in present day Israel, it is claimed that the use of almond is as ancient as 23000 BC (Weiss *et al.,* 2004; Arús *et al.,* 2009). There are common cultural references and legends of almond from the Levant to China (Rosengarten, 1984; Arús *et al.,* 2009).

The evolution and distribution of almonds have been reviewed by Martínez-Gómez *et al.,* (2007). According to one view, semi-wild almonds migrated through man-made selection and cultivation from Central Asia to Mediterranean regions. Almonds have further evolved in the Mediterranean region and then spread to the other parts of the world from Tunisia and Morocco, creating an additional route across Central Africa between 500 to 600 AD (Das *et al.,* 2011). Almond was introduced to California from the Mediterranean region during the Spanish mission and during the Gold Rush periods in the $18^{th}$ and the $19^{th}$ centuries (Wood, 1925) (Figure 1.2.). About the same time, almond was also introduced to Australia, Chile, Argentina and South Africa.

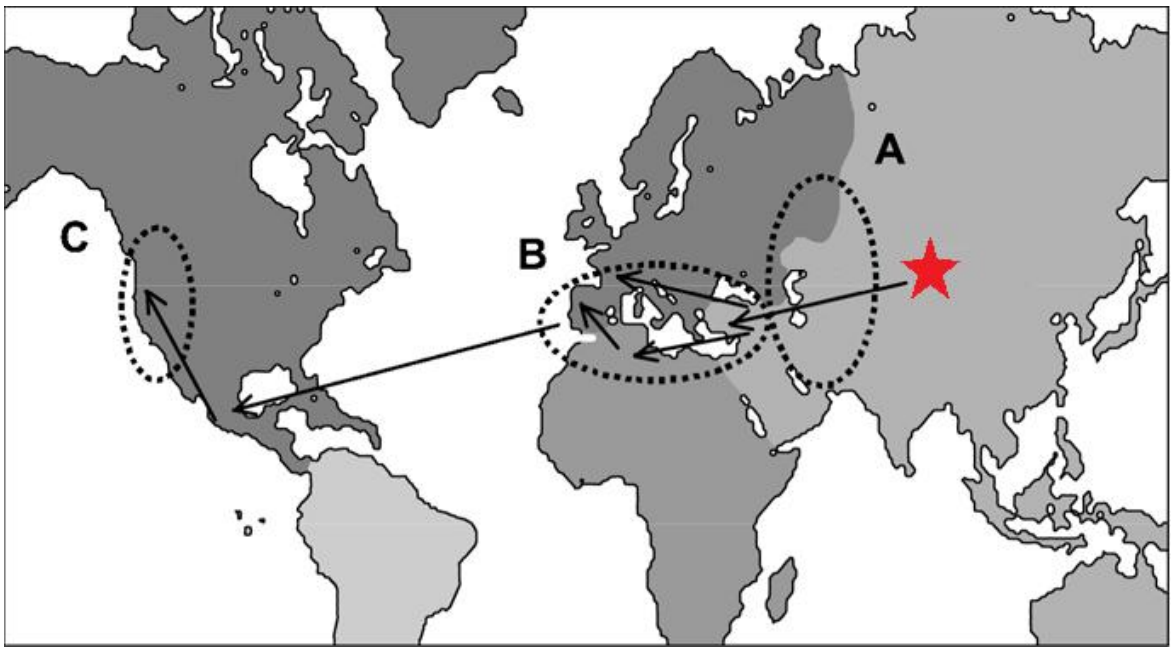


Figure 1.2. Almond evolution and distribution map. [Adapted from: (Martínez-Gómez *et al.*, 2007)].

Almonds prefer to grow in harsh stony soils. They do not require much water therefore are resistant to drought climates. In fact, they do not prefer moisture and cold winds. Almonds usually patch off their leaves earlier than the other nut trees. It blooms in early spring because of its low winter chilling requirements and ability to grow quickly in response to warm temperatures.

Genetically, almond is the most polymorphic stone fruit within the genus *Prunus*. It has a diploid genome with sixteen (2n=16) chromosomes at a size of 300 Mbp. In breeding, almond is predominantly self-incompatible which may explain why it is highly polymorphic. However, self-compatible almond cultivars were also reported in Puglia region of Italy, and since then self-compatibility studies became important for almond breeding in both Europe and the USA (Martínez-Gómez *et al.*, 2007).

Almond is commercially grown worldwide. Most of the almond production is from the central valley of California corresponding to 50% of the world's total. Spain with 12%, Syria 8%, Iran 6%, Italy 5%, Greece 2% and Turkey 2% are the other major almond-growing countries (FAO, 2004).

## 1.3. Identification of Almond Cultivars

### 1.3.1. Morphological Markers

Until recently, characterization and identification of almond cultivars were mostly based on morphological traits. Taxonomic relationships, morphological distinctions (growth habit, leaf shape, petiole length, leaf margins, flower color, fruit shape, etc.), ecological specificities and geographical distribution were studied (Browicz and Zohary, 1996). However, characterization from morphological traits has disadvantages mostly because they are unstable in changing environmental conditions. Furthermore, some morphological characters may only become visible in adult species making their analysis difficult (Martínez-Gómez *et al.*, 2007).

### 1.3.2. Molecular Markers

Today, molecular markers are widely used for identification studies; which allow more accurate, fast and environmentally stable tests (Wünsch and Hormaza, 2002; Martínez-gómez *et al.*, 2003b; Martínez-Gómez *et al.*, 2007). Additionally, these markers are highly polymorphic.

1.3.2.1. Isoenzymes. Isoenzymes (isozymes) are enzymes that catalyze the same chemical reactions but having different amino acid sequences. Different molecular forms of the enzymes are present not only in the same individual but also in the same tissue (Markert and Møller, 1959). Genes at different loci or different alleles at the same loci may be encoding the isoenzymes (Szikriszt *et al.*, 2011). Isozymes can be used for the identification of almonds. As a molecular marker, they exhibit co-dominant expression, reproducibility and environmental stability (Arulsekar *et al.*, 1986; Hauagge *et al.*, 1987a; Hauagge *et al.*, 1987b; Foolad *et al.*, 1995; Vezvaei *et al.*, 1995; Sathe *et al.*, 2001). Different isozymes are generally differentiated by common protein separation techniques. The most common method is polyacrylamide gel electrophoresis (PAGE) which separates proteins with respect to their molecular sizes or isoelectric points. However, the utilization of isozymes is limited due to presence of a small number of loci for the differentiation process. In addition, most loci have low variation (Martínez-Gómez *et al.*, 2007).

1.3.2.2. DNA Markers. DNA sequence of a gene or a part of an intron may differ from species to species and sometimes differ within species. These markers show Mendelian inheritance. DNA-based molecular markers are useful for identification studies because they are discriminative and environmentally stable. In addition to being used for differentiating species, these sequence variations can be used to link genes to morphological traits. There are several methods that use DNA markers. For the identification of almonds restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) analyses have been used (Szikriszt *et al.*, 2011). In Figure 1.3. (Szikriszt *et al.*, 2011) distinctive features, advantages or disadvantages of each molecular marker are listed.

| Features | RFLP | RAPD | AFLP | SSR | ISSR | SNP |
|---|---|---|---|---|---|---|
| Frequency | high | high | high | moderate | moderate | moderate |
| Reproducibility | high | unreliable | high | high | high | high |
| Degree of polymorphism | moderate | moderate | moderate | moderate | moderate | moderate |
| PCR-based | no | yes | yes | yes | yes | yes |
| Specification of locus | yes | no | no | no | no | no |
| Development cost | low | low | moderate | high | low | high |
| DNA required (μg) | 10 | 0.02 | 0.5–1.0 | 0.05 | 0.05 | 0.05 |
| DNA quality | high | high | moderate | moderate | moderate | high |
| Ease to use | not easy | easy | easy | easy | easy | easy |
| Cost per analysis | high | low | moderate | low | low | low |
| Amenable to automation | low | moderate | moderate | high | moderate | high |
| Expression | co-dominant | dominant | dominant | co-dominant | dominant | co-dominant |
| Predominant application | physical mapping | gene tagging | | analysis of genetic diversity | | |
| No. of studies in almond[a] | 1 | 6 | 2 | 8 | 2 | 1 |

[a]Number of studies found in international literature and used in the review

Figure 1.3. Features of molecular markers used in almond characterization [Taken from: (Szikriszt *et al.*, 2011)].

*Restriction Fragment Length Polymorphism (RFLP).* Restriction fragment length polymorphism method is based on using various restriction enzymes to digest the genomic DNA. The resulting DNA restriction fragments are separated with gel electrophoresis and the restriction fragment profile is used to identify the organism. RFLPs are able to detect virtually unlimited number of markers and they are also co-dominant, which gives an advantage in the discovery of linkages between markers and constructing genetic maps

because each marker is related to a single locus at a time. One almond characterization study was conducted with RFLP analysis to detect the self-incompatibility (SI) genotypes in Portugal (RC Ma and Oliveira, 2001).

For the identification of almond cultivars, RFLP method was proven to be useful (MA Viruel *et al.,* 1995). However, RFLP analyses are laborious and time-consuming, and they require using radioisotopes (Martínez-Gómez *et al.,* 2007).

*Random Amplified Polymorphic DNA (RAPD).* In Random amplified polymorphic DNA method, arbitrary primers are used to amplify the random locations of the genome with polymerase chain reaction (PCR). The resulting DNA fragments are separated on electrophoresis. The molecular sizes of these fragments comprise a pattern as the fingerprint of the organism. This is a much simpler method than RFLP with use of a single oligonucleotide for the random amplification of various loci in the genome. Another advantage of this method is being PCR-based and easy to handle when compared with RFLP analysis where the usage of radioisotopes are required. The disadvantage of the RAPD method, when compared with RFLPs and isozymes, is that, these markers are dominant which yields various patterns of results with multiple loci. This property limits the use of RAPD in cultivar identification (Bartolozzi *et al.,* 1998; Martins *et al.,* 2003). At least in one study with Californian almonds, with the use of 37 RAPD markers, three groupings of cultivar origins could be distinguished (Bartolozzi *et al.,* 1998).

*Amplified Fragment Length Polymorphism (AFLP).* Amplified fragment length polymorphism method exploits genetic variations between closely related genomes in the form of restriction fragment length polymorphisms. In this method, the genomic DNA is first digested using two restriction endonucleases, one rare cutter (having 6-bp recognition site), and a frequent cutter (having a 4 bp recognition site). Then, appropriate adaptor molecules are introduced to be ligated to the sticky ends of the digested DNA fragments. Using oligonucleotide primers specific to adaptors, some of these DNA fragments are amplified using PCR. The selection of primers may be based on the adapter sequences, restrictive site sequences or some other nucleotide sites for the arbitrary selection of the digested fragments. The amplified fragments are then visualized on the denaturing polyacrylamide gels by using autoradiography or fluorescence labeling.

AFLP has been used successfully in the discrimination of almonds. In one study, almond cultivars from Iran, Europe and America were analyzed by AFLP. In total, 19 AFLP primer combinations were used in addition to several agronomic traits. From the 813 PCR fragments, 781 were polymorphic. All genotypes could be identified with the resulting fragment patterns (Sorkheh *et al.,* 2007).

*Single Nucleotide Polymorphism (SNP).* Patterns of SNPs are a method of preference for understanding the genetic diversity of organisms without having detailed prior genetic information. SNPs can be detected by high resolution melting (HRM) method which reveals mutations, polymorphisms and epigenetic differences. After being detected, SNPs can further be analyzed by sequencing. HRM method in detection of SNPs is advantageous because it is cost-effective, powerful, simple and fast; therefore suitable for high-throughput, accurate analyses. However HRM requires real-time PCR procedure with new generation fluorescently labeled primers (Szikriszt *et al.,* 2011).

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In one study with 25 almond cultivars, 17 amplicons were observed after scanning the genome with HRM. After sequencing these amplicons, 100 SNPs were detected (Wu *et al.,* 2008; Szikriszt *et al.,* 2011). SNP rare allele frequencies were between 0.02 and 0.5; and the polymorphic information content was between 0.04 to 0.53, at an average of 0.31. These results indicated that SNPs are efficient in genotyping and discovery especially for species like almond, with little genomic information (Wu *et al.,* 2008).

*Simple Sequence Repeats (SSR).* Simple sequence repeats, also known as microsatellites, are DNA regions that contain two to six base pair DNA sequence repeats. The number of repeats at each SSR locus is varying in different organisms. SSRs are currently the most utilized markers in almond due to their high quality (high polymorphism, co-dominance and reproducibility), the simplicity and robustness of the current analytical methods and the existence of large sets of publicly available markers (Arús *et al.,* 2009).

In *Prunus* species, SSR markers (microsatellites) which are abundantly spread in the genome are detected for apricot, peach, Japanese plum and cherry (Cipriani *et al.,* 1999; Downey and Iezzoni, 2000; Sosinski *et al.,* 2000; Aranzana *et al.,* 2002, 2003;

Dirlewanger *et al.,* 2002; Georgi *et al.,* 2002; Yamamoto *et al.,* 2002; Clarke and Tobutt, 2003; Decroocq *et al.,* 2003; Schueler *et al.,* 2003; Hagen *et al.,* 2004; Messina *et al.,* 2004; Mnejja *et al.,* 2004). Later, SSR markers for the identification of almonds have also been defined (Testolin *et al.,* 2004) and used successfully (Martínez-Gómez *et al.,* 2003a; Testolin *et al.,* 2004) not only for almonds but also for the characterization of other *Prunus* species (Martínez-Gómez *et al.,* 2003b). SSR markers that are used in almonds mostly derived from SSR marker collections of other *Prunus* species, especially from peach (Arús *et al.,* 2005). A large set of SSR markers for various *Prunus* species are available publicly (Arús *et al.,* 2009; Testolin *et al.,* 2004). SSR markers have been obtained either from genomic libraries (Testolin *et al.,* 2004; Mnejja *et al.,* 2005) or EST collections (Xu *et al.,* 2004).

In order to separate and analyze the PCR products of the amplified SSR markers, polyacrylamide gels are used. Fragments are detected either by radioactive labeling or by silver staining. Instead of polyacrylamide, Metaphor® agarose gels can also be used as an alternative (Martínez-Gómez *et al.,* 2007). However, Metaphor® agarose is not a suitable matrix for the separation of fragments with less than 5bp differences. When the fragments being separated are longer than 5bp, Metaphor® agarose may be more convenient because of its lower cost and easier application (Sanchezperez *et al.,* 2006). More recently, the use of automated capillary sequencers for separating and detecting the microsatellite fragments became popular.

In order to understand the allelic variation of SSR's, 38 almond cultivars (23 Chinese and 15 international) were compared in one study using eight EST-SSR and eight genomic-SSR markers (Xie *et al.,* 2006). Six of the SSRs from a total of 16 markers yielded 117 alleles. These SSR alleles were further analyzed by DNA sequencing. The sequences of the 98 alleles of these 117 alleles showed that there were no insertions or deletions in the flanking sequences. Furthermore, some alleles had interrupted repeat sequences with the insertion of different repeat motifs, whereas some were uninterrupted. So there may be different mutational patterns in different alleles of the same loci. In some cases, sequence variation without a change in the size of the marker region (allelic homoplasy) was also observed resulting from base substitutions, interruptions or complex

repeat motifs. So, in addition to the size of the SSR loci fragments, sequence data can also be informative (Xie *et al.,* 2006; Szikriszt *et al.,* 2011).

### 1.4. Linkage Map Analysis of Almonds

Alleles that are inherited together are genetically linked which means they are segregated together during the crossovers of meiotic division. Using this information genetic linkage maps can be constructed. Genetic maps show the locations of genes and genetic markers on the chromosome which are inherited together. Therefore, with the help of genetic maps, one can relate the genetic markers to genes that are responsible for some morphological phenotypes or diseases.

In almond, linkage analysis was first undertaken by using isozyme markers (Arús *et al.,* 1994b; Vezvaei *et al.,* 1995; Szikriszt *et al.,* 2011). However the limited number of isozyme markers that can be analyzed with conventional enzyme staining impeded the use of these markers for the construction of almond linkage maps.

RFLPs were also used for the construction of linkage maps in plants and animals and they gave rise to virtually unlimited source of high quality markers. With 120 RFLPs and seven isozymes, first linkage map of almonds was constructed with using $F_1$ progeny of “Ferragnés” and “Tuono” species (FxT map). In this map, eight linkage groups were detected with a size of 400cM (Viruel *et al.,* 1995; Martínez-Gómez *et al.,* 2007).

Later, the F2 population of an interspecific cross between a peach selection (54P455) and an almond cultivar ‘Padre’ (P$\times$5) was used to construct an almond linkage map. In this map, 101 RFLPs and six isozymes spanning a larger map than the first one of almond by Viruel *et al.,* (1995) was used (Foolad *et al.,* 1995; Martínez-Gómez *et al.,* 2007).

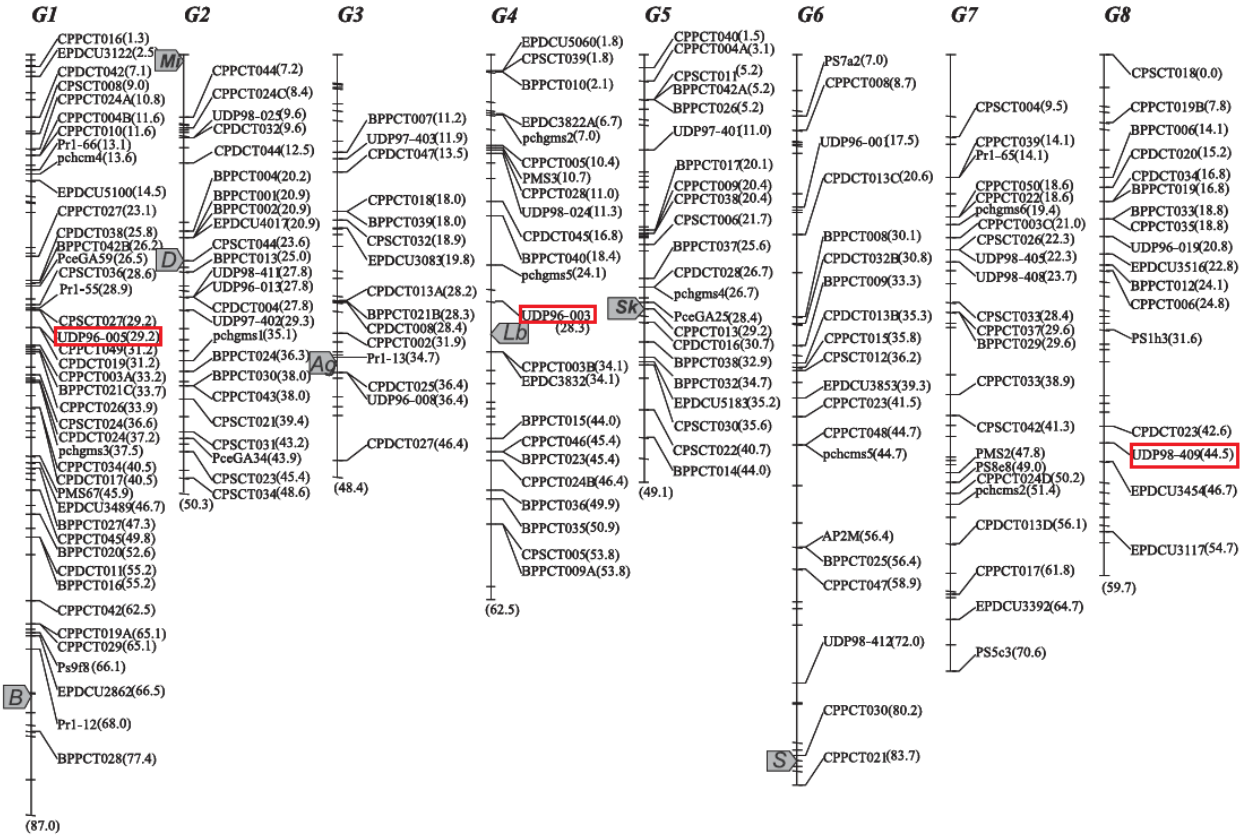



Figure 1.4. Map of the 'Texas' (almond) × 'Earlygold' (peach) F2 population. This map contains the SSR markers of the map of Dirlewanger *et al.*, (2004) and with the approximate location of genes.

In the European *Prunus* project (Arús *et al.*, 1994), six European research groups collaborated to develop genetic markers and linkage maps for the use of *Prunus* breeding programs (Arús *et al.*, 1994a). One of the hybrids prepared in this project was between an almond (cv. "Texas, syn. "Mission") and a peach (cv. "Earlygold") to obtain the $F_2$ progeny (TxE). Using this hybrid, a linkage map was constructed with 246 markers containing 235 RFLPs and 11 isozymes. Eight linkage groups were observed and markers were mapped into them with a total distance of 491cM (Joobeur *et al.*, 1998; Martínez-Gómez *et al.*, 2007). After comparing TxE map and FxT map constructed in the first study, it was observed that they have many common markers with the same distribution in their genomes. In addition, these markers in the same linkage groups were also collinear in both maps. TxE map was considered as the *Prunus* reference map. Because of the

similarities in both maps; FxE almond map was adapted for the TxE map. With the addition of more markers as RFLPs and SSRs, the reference TxE map improved progressively (Aranzana *et al.,* 2003). One of the most recent versions of the reference map contains 562 markers with 361 RFLPs, 185 SSRs, 11 isozymes and 5 Sequence-Tagged Sites (STSs) covering a distance of 519 cM (Dirlewanger *et al.,* 2004) Genes on the map responsible for the morphological traits are flower color (*B*), nematode resistance (*Mi*), shell hardness (*D*), anther color (*Ag*), blooming time (*Lb*), kernel taste (*Sk*), and self-incompatibility (*S*) (adapted from:Martínez-Gómez *et al.,* 2007). Bracketed markers were used in the current study (Figure 1.4).

### 1.5. Polymorphisms in *Prunus* Species

One of the first molecular studies on the polymorphism levels of *Prunus* species was conducted by Bryne (1990). With the use of isozymes, it was observed that almond and Japanese plum had the highest levels of polymorphism; whereas apricot and peach had the least. Almond and Japanese plum are the most polymorphic species within *Prunus*, probably due to their self-incompatibility (SI) (Arús *et al.,* 2009). The self-incompatibility of almond results from the presence of the S-locus which is highly polymorphic and multiallelic (López *et al.,* 2006). The S-locus encodes for an S-ribonuclease (S-RNase) protein which is located in the pistils. This protein degrades the RNA in the self-pollen tubes therefore prevents fruit formation (Szikriszt *et al.,* 2011). On the other hand, apricot and peach are self-compatible, therefore selection for inbred individuals is usually preferred, which is most probably the main reason for them being less polymorphic (Arús *et al.,* 2009).

For *Prunus* species, SSR data are consistent with the isozyme data. In a comparative analysis of variability among *Prunus* species with 125 SSR markers, it was verified that almond was the most polymorphic species, followed by Japanese plum, apricot, cherry and peach (Mnejja *et al.,* 2010). In an earlier study by Xie *et al.,* (2006), 38 almonds were analyzed using 16 SSRs and average number of alleles per locus (A-value) was found as 7.7 with average observed heterozygosity ($H_o$) of 0.65. In another study with a much larger sample size, 212 peaches were analyzed, and A-value was found as 7.3, which is similar to that of almonds. However $H_o$ was found as 0.35, nearly half of the value of the

almonds indicating the huge difference in variability levels of these two *Prunus* species (Aranzana *et al.,* 2003). In another study, 18 SSR markers were used in parallel for almond and peach individuals, and it was found that almond had an A-value of 6 and $H_o$ value of 0.59, whereas peach had an A-value of 3.6 and $H_o$ value of 0.19 (Martínez-Gómez *et al.,* 2003b). As a result, higher marker efficacy for cultivar identification in almond is consistent with high variability (Arús *et al.,* 2009).

In order to establish the phylogenetic relations among *Prunus* species, isozymes have also been used in one study (Mowrey and Werner, 1990). The results were consistent with the previous morphology-based relationship studies. All these studies confirmed that almond was more closely related to peach among other *Prunus* species. DNA sequence analysis confirmed this status (Potter, 2003). SSR markers can be used to clearly distinguish almond and peach species (Martínez-Gómez *et al.,* 2003b; Martínez-Gómez *et al.,* 2003a). Additionally, using SSR markers, Chinese and Mediterranean cultivars were compared and it was found that Chinese cultivars exhibited higher level of variation, suggesting a possible founder role in the evolution of cultivated almond (Xu *et al.,* 2004; Xie *et al.,* 2006; Ma *et al.,* 2004). Further evaluation of the differences between Chinese and Mediterranean species via using molecular markers may help us to understand the environmental forces those determine the cultivar evolution from different origins (Arús *et al.,* 2009).

### 1.6. Plant Diversity in Turkey

In terms of plant diversity, Turkey is one of the richest lands in the middle latitudes; as a result of its climate varieties, soil and geomorphological diversity and being located in an area at the intersection of Euro-Siberian, Mediterranean and Irano-Turanian regions (Avci, 2005). The diverse climate of Turkey ranges from subtropical to cold-temperate. This leads to ecological diversity with successful introduction and cultivation of great number of fruit taxa with high genetic diversity (Ercisli, 2004). The rich flora of Turkey covers about 12000 species (Avci, 2005). This includes many wild, transitional and cultivated forms of annual and perennial, herbaceous and woody plants (Agaoglu *et al.,* 1997). Among these plants, fruits have an important role both in agricultural economy and cultural diversity.

Fruit culture has played an important role throughout Anatolia's history. There are subtropical and tropical fruits growing in the south with mild winters and hot summers; and nearly all deciduous fruits can be grown all over the country (Ercisli, 2004). These include fruits such as apricot, almond, walnut, chestnut, cornelian cherry, mulberry, plum and pomegranate, species which propagate to a large extend from seeds with high variability (Ozbek, 1978).

### 1.6.1. Almond Studies in Turkey

Most of the almond studies in Turkey were on morphological clustering of varieties, adaptation, selection and discovery for agricultural purposes (Şimşek *et al.*, 2010; Şimşek and Osmanoğlu, 2010; Polat *et al.*, 2001; Küden, 2001; Karagoz, 2001; Küden *et al.*, 1994; Küden, 1997; Aslanta, 2001).

Until now, almonds in different regions of Turkey such as Datça, Acıpayam, Tavas (Dokuzoğuz and Gülcan, 1973), Akdamar island (Bostan *et al.*, 1995), Erzincan-Kemaliye (Aslantaş and Güleryüz, 1995), Siirt (Karadeniz and Erman, 1996), Adır island (Karadeniz *et al.*, 1996), Aegean region (Dokuzoğuz and Gülcan, 1973), Southeast region (Kaşka *et al.*, 1993; Ak *et al.*, 1999), Mediterranean region (Önal *et al.*, 1994) were studied based on morphological observations.

There is also one study that was carried out to find the most adaptable almond types to Mediterranean ecological conditions, which used phenological observations and pomological analysis (Küden *et al.*, 1994).

There have been only a few almond studies in Turkey, which used molecular markers: In one study, microsatellite markers were used for fingerprinting and segregation analyses (Kaçar *et al.*, 2003). In this study, eight almond varieties and 21 hybrid individuals were analyzed with 12 microsatellite primers designed for peach (Kaçar *et al.*, 2003; Bayazit, 2007). Apart from two identical varieties, all other almond cultivars were successfully identified. Among the 12 microsatellite markers, 10 of them were polymorphic, while two of them could not be amplified (Bayazit, 2007).

In another molecular study on Turkish almonds, RAPD markers were used in order to assess the genetic identities of both early and late flowering type almonds ( Küden *et al.*, 2004). In this work, almonds were collected from İzmir, Urla, Mardin, Muğla, Çanakkale (Umurbey), Çanakkale (Lapseki), Tekirdağ (Şarköy), Birecik and compared with Californian almonds. Using 11 primer pairs, 107 alleles were obtained from 15 almond varieties, 94 of which were polymorphic. The UPGMA dendrogram indicated that the 15 almond cultivars of this study clustered in two main groups containing both Turkish and Californian types.

In another molecular study using RAPD markers, 69 almond varieties from Konya, Ayrancı Barajı, Nevşehir, Göreme-Kayseri, Niğde, Gaziantep (Nizip), Küllü Yolu, İbrahim Şehri, Şanlıurfa and Cennet Bahçesi regions were compared with cultivated almonds from abroad (Nonpareil and Cristomorto types) and with Turkish commercial selections (Hacı Alibey, Gülcan-1 and 101-13). In this study, molecular analysis was also supported with phenological, pomological and morphological observations for three years. It was concluded that almonds from Konya, Nevşehir and Niğde were found to be similar both genetically and morphologically. This group was the most similar to cultivated almonds. Likewise, Gaziantep and Şanlıurfa samples grouped into two different clusters. These two clusters were also divergent from the Konya, Nevşehir and Niğde samples (Bayazit, 2007).

Despite the fact that, the highest almond production in Turkey is from Datça/Muğla region, and these almonds have the highest commercial value, no adequate molecular identification study of almonds in this region has been carried out until now.

## 1.7. Muğla's Local Fruit Varieties: Cultural Heritage, Database and Preservation Project

In every part of Turkey there are many fruit varieties that are adapted to local soil and climate conditions, resistant to local pests and diseases, and require no irrigation, artificial fertilizer or pesticides. However, most of these fruit varieties are threatened today by urbanization, soaring land prices, agricultural policies and marketing problems. Not

only are these fruit varieties an important genetic resource essential for food security in the face of climate change, but with their diverse flavors and traditional uses they are a significant part of Turkey's cultural heritage (Fruit Heritage, 2008).

The province of Muğla includes three of Turkey's most important wild plant habitats (Ozhatay *et al*., 2005) as well as 15 of Turkey's important natural resources (Eken *et al*., 2006). In addition to wild plants, the local fruit varieties of Datça are about to disappear due to the land usage for hotels and summer villas as a consequence of tourism expansion. Furthermore, farmers are nearly forced to plant commercial varieties which are mostly introduced from abroad, due to agricultural loans offered to farmers being only valid for certified varieties. Unfortunately none of the certified varieties are local, even though growing local varieties are far more efficient, low-cost and easy to produce.

For all the reasons above, Mugla's Local Fruit Varieties: Cultural Heritage, Database and Preservation Project has been started by volunteers in 2007 (Fruit Heritage, 2008). The project aims to identify the local fruit varieties, constructing databases, molecular identification of these varieties and in-situ/ex-situ conservation studies. So far, more than 550 local fruit varieties (heirloom varieties) have been identified in the course of the project. Among these local fruits, 97 local almond varieties have been identified in Datça (http://www.fruitheritage.org). Molecular characterization and the genetic diversity of these almonds is the subject of this thesis.

# 2. PURPOSE

Datça is the center of production of almonds in Turkey. As a part of the Fruit Heritage Project, supported by United Nations Development Program (SGP-GEF, project number TUR/SGP/OP4/RAF/07/01/01) 97 almond (*P. dulcis*) varieties have been listed since December 2007. None of these local varieties have been studied at the molecular level or yet registered as Datça's landraces.

The purpose of this thesis is to gather molecular data, in the form of DNA sequencing and DNA fingerprints in order to study the molecular diversity of Datça's almond landraces. These data will then be used for their identification and registration of Datça's landraces.

# 3. MATERIALS

## 3.1. Plant Tissue

Plant tissue was obtained from fresh leaves of 97 cultivated almonds and five wild species (Şeytan payamı) collected early in summer 2008. The geographical coordinates of the trees where the leaves were taken, the name of the village, and the owner of the garden were recorded.

## 3.2. Chemicals

Table 3.1. Chemicals used in this study.

| | |
|---|---|
| Absolute Ethanol | Merck, Germany |
| Absolute Isopropanol | Merck, Germany |
| Acetic acid, glacial | Merck, Germany |
| Agarose | Bioron, Germany |
| Bromophenol blue | Merck, Germany |
| Chloroform | Merck, Germany |
| CTAB (cetyl trimethylammonium bromide | Sigma, USA |
| DNA Molecular Size Standard (100bp) | Fermentas, Canada |
| DNA Molecular Size Standard (1 kb) | Fermentas, Canada |
| dNTP (dATP, dCTP, dTTP, dGTP) | Fermentas, Canada |
| Ethanol | Merck Germany |
| Ethidium Bromide | Sigma, USA |
| Glycerol | Riedel, Germany |
| Isopropanol | Merck, Germany |
| Isoamyl alcohol | Merck, Germany |
| 2-mercaptoethanol | Merck, Germany |
| Sodium EDTA | Merck, Germany |
| Sodium acetate | Merck, Germany |
| Sodium bisulfate ($NaHSO_3$) | Merck, Germany |
| Phenol | Merck, Germany |
| Sarcosyl | Sigma Aldrich, USA |
| Sorbitol | Merck, Germany |
| Xylene cyanol | Merck, Germany |
| TRIZMA(Tris(hydroxymethyl)aminomethane) | Sigma, USA |

### 3.3. Buffers and Solutions

Table 3.2. Stock Solutions for Genomic DNA Isolation.

| | |
|---|---|
| CTAB Extraction Buffer | 0.8 M NaCl<br>1% Sarkosyl<br>0.14 M Sorbitol<br>22 mM EDTA<br>220 mM Tris-HCl (pH 8.0)<br>0.8% CTAB |
| Na-Acetate (pH 4.5) | 3 M Na-acetate adjusted to pH 4.5 with Acetic Acid (Glacial) |
| Phenol:chloroform:isoamylalcohol | 25% (v/v) Phenol<br>24% (v/v) Chloroform<br>1% (v/v) Isoamylalcohol |
| TE Buffer | 10 mM Tris-HCl (pH 8.0)<br>1 mM Na-EDTA |

Table 3.3. Stock Solutions for Polymerase Chain Reaction.

| | |
|---|---|
| 5X PCR Buffer | Proprietary formulation of Promega at pH 8.5 |
| $MgCl_2$ | 25 mM $MgCl_2$ (Promega) |
| dNTP mix | 10 mM of each dATP, dGTP, dTTP in $H_2O$ |

Table 3.4. Stock Solutions for Agarose Gel Electrophoresis.

| | |
|---|---|
| 25 X TAE Buffer | 121 g/l Tris (Base)<br>28.55 ml/l Glacial acetic acid<br>50 mM EDTA (pH 8.0) |
| 1% Agarose Gel | 1% (w/v) Agarose (Bioron) in 1 X TAE Buffer, containing 0.5 µg/ml Ethidium Bromide |
| 2% Agarose Gel | 2% (w/v) Agarose (Bioron) in 1 X TAE Buffer containing 0.5 µg/ml Ethidium Bromide |
| 6 X Loading Dye | 10 mM Tris-HCl (pH 7.6)<br>0.03% bromophenol blue<br>0.03% xylene cyanol FF<br>60% glycerol<br>60 mM EDTA |

## 3.4. Enzymes

Table 3.5. Enzymes used in this study.

| | |
|---|---|
| *Taq* DNA polymerase (Go *Taq* Flexi) | Promega, USA |
| Proteinase K | Promega, USA |
| RNase-A | Promega, USA or Sigma, USA |

## 3.5. Oligonucleotide Primers

### 3.5.1. Primers for the amplification of the chloroplast DNA region

Table 3.6. List of the oligonucleotide primers for the amplification of the intergenic spacer region between chloroplast *trn*L(UAA) 3’ exon and the *trn*F(GAA) gene.

| | |
|---|---|
| E | 5'-GGTTCAAGTCCCTCTATCCC |
| F | 5'-ATTTGAACTGGTGACACGAG |

### 3.5.2. Primers for the amplification of the nuclear rRNA regions

Table 3.7. List of the oligonucleotide primers used for the amplification of nuclear rRNA ITS region.

| | |
|---|---|
| ITS4 | 5'-TCCTCCGCTTATTGATATGC |
| ITS5 | 5'-GGAAGTAAAAGTCGTAACAAGG |

Table 3.8. List of the oligonucleotide primers for the amplification of 16S RNA region.

| | |
|---|---|
| B27F | 5’-AGA GTT TGA TCC TGG CTC AG |
| 533F | 5’-GTG CCA GCM GCC GCG GTA A |
| U1492R | 5’-GGT TAC CTT GTT ACG ACT T |

### 3.5.3. Primers for the amplification SSR Marker Loci

Table 3.9. Primers used for amplifying the nuclear SSR marker region.

| | |
|---|---|
| UDP96003F | 5’-TTGCTCAAAAGTGTCGTTGC |
| UDP96003R | 5’-ACACGTAGTGCAACACTGGC |
| UDP96005F | 5’-GTAACGCTCGCTACCACAAA |
| UDP96005R | 5’-CCTGCATATCACCACCCAG |
| UDP98409F | 5’-GCTGATGGGTTTTATGGTTTTC |
| UDP98409R | 5’-CGGACTCTTATCCTCTATCAACA |

## 3.6. Equipment

Table 3.10. Equipments used in this study.

| | |
|---|---|
| Autoclaves | Astell Scientific, UK |
| Beads (2.3mm zirconium) | Biospec Products, USA |
| Centrifuge Tubes | BD, USA<br>TPP, Switzerland |
| Cryo Tubes | VWR, USA |
| Electronic Balances | Mettler Toledo, Switzerland<br>Sartorius, Germany |
| Electrophoresis tanks | Thermo Fisher Scientific, USA |
| Freezers | -80°C Thermo Scientific, USA<br>-20°C Arçelik, Turkey |
| Gel Documentation | Bio-Rad Labs, USA (GelDoc XR) |
| Hot Block | Grant, UK |
| Micro Centrifuge | Eppendorf, Germany (5417R) |
| Microcentrifuge Tubes | Treff, Germany |
| Micropipetters | Gilson, USA (Pipetman) |
| Microwave owen | Arçelik, Turkey |
| Nanodrop Spectrophotometer | Thermo Scientific, USA |
| Refrigerator | Arçelik, Turkey |
| Tissue Homogenizator | Roche, Switzerland (Magna Lyser) |
| Nanodrop Spectrophotometer | Thermo Scientific, USA |
| Vortex | Heidolph, Germany |
| Water Baths | Grant, UK<br>Bibby, UK |

## 3.7. Software

Table 3.11. Software used in this study.

| | |
|---|---|
| Peak Scanner | v.1.0 |
| Popgene | v.1.32 |
| NTSYSpc | v.2.02i |
| Micro Checker | v.2.2.3 |
| Populations | v.1.2.32 |
| Genepop | v.4.1.4 |
| MVSP | v.3.21 |

# 4. METHODS

## 4.1. Preperation of Almond Leaves as Genetic Material

Fresh almond leaves were collected during early summer when the leaves were still soft and transported immediately to the laboratory within a day. Fresh leaves were washed first with tap water to remove dirt, rinsed with distilled water, dried with a tissue paper and then placed into a porcelain mortar. Leaves were grinded under liquid nitrogen (-196 °C) to a fine powder and then immediately transferred into sterile cryotubes and stored at -80 °C until further analysis.

## 4.2. Extraction of the Genomic DNA

Genomic DNA was isolated using the procedure of Doyle and Doyle (Doyle, 1987). Approximately 100 mg of frozen pulvarized almond leaves were transferred into 1.5 ml microcentrifuge tubes. 750 µl of CTAB extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% mercaptoethanol, 0.1% $NaHSO_3$) was added on each sample powder with 5-6 pieces of 2.3 mm zirconium beads. Samples were homogenized using MagNaLyser two times for 60 seconds at 7000 rpm to rupture the cell wall and the cell membrane. Samples were then incubated at 65 °C for 1 hour and 500 µl of chloroform was added. Samples were vortexed for 5 minutes and then centrifuged at 14000 rpm for 5 minutes to precipitate cell debris. Upper phases were transferred into new 2 ml microcentrifuge tubes. An equal volume of isopropanol was added to each tube and the samples were shaken gently until the presence of cloudy DNA was visually observed. Samples were left on ice for 10 minutes to precipitate the DNA and the precipitate was collected by centrifugation for 10 minutes. Supernatants were discarded. DNA pellets were dried at 50 °C for a few minutes and then 650 µl TE Buffer and 3 µl RNAse A (17 mg/ml) were added. Samples were incubated at room temperature for 20 minutes. An equal volume of phenol was added, vortexed for 2 minutes and then centrifuged at maximum speed for 10 minutes to separate the two phases. Upper phases were transferred into new 2 ml microcentrifuge tubes. The extraction step was repeated, once with using an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and then with using an

equal volume of chloroform. To the upper phase of the chloroform extract 0.1 volume of 3 M Na-acetate was added, samples were briefly vortexed and DNA was precipitated by adding two volumes of ethanol. The presence of DNA was observed visually after gently shaking the tubes. Samples at this step were either stored at -20 °C or they were incubated on ice for 20 minutes to continue with extraction. Precipitated DNA was collected by centrifugation at maximum speed for 5 minutes and supernatants were discarded. Pellets were washed with 1 ml of ice-cold 70% ethanol. Pellets containing DNAs were dried at 50 °C for 5 minutes to get rid of the remaining alcohol and 150 µl of TE buffer were added onto the pellets to dissolve DNA. Samples were left at 4 °C for at least overnight for complete dissolution of DNAs and then stored at -20 °C until further analysis.

The concentrations of the genomic DNAs were determined spectrophotometrically using a nanodrop spectrophotometer. Quality of the genomic DNAs were also observed after loading 10 µl of each DNA mixed with 2 µl of 6X loading dye (Fermentas) on 1% Agarose gel. Electrophoresis was at 150V for 20 minutes. Purified DNA samples were diluted to 18 ng/µl with 1X TE Buffer and these aliquots were stored at -20 °C until further use.

### 4.3. PCR Amplification of the Chloroplast Markers

Using purified almond DNA, the intergenic spacer between the *trnL*(UAA) 3' and *trnF*(GAA) 5' genes was amplified using the universal primers designed from conserved chloroplast tRNA gene sequences (Taberlet *et al.,* 1991).

In a total volume of 25 µl, PCR mix contained 1X GoTaq Flexi Reaction Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dGTP, dCTP and dTTP), 1 µM of each primer (E and F), 90 ng of DNA and 1 unit of Taq Polymerase (Go Taq Flexi DNA Polymerase, Promega). PCR cycles were as follows: 94 $^{o}$C for 150 seconds for initial denaturing, then 30 cycles of 95 $^{o}$C for 30 seconds, 52 $^{o}$C for 90 seconds, 72 $^{o}$C for 180 seconds, followed by a final extension at 72 $^{o}$C for 7 minutes. If sequencing of the PCR products were planned, PCR reactions were prepared in 100 µl.

After PCR amplification, reaction products were analyzed electrophoretically on 1% agarose gels. For this purpose, 5 µl of each product was mixed with 1 µl of 6X Loading Dye and ran on a 1% Agarose gel containing 0.5 µg/ml ethidium bromide in TAE buffer. Electrophoresis was for 20 minutes at 150V. DNA bands were visualized under UV and photographed.

### 4.4. Purification of the PCR Products

PCR products, were purified using High Pure PCR Clean up Micro Kit (Roche) for further sequencing analysis. For each 100 µl of PCR mixture, 400 µl of Binding Buffer was added. Each sample was vortexed for 4 seconds and centrifuged briefly. This mixture is transferred to a high pure column which was inserted into a collection tube. Samples were centrifuged for 1 minute at 11000 rpm at RT. The flowthrough was discarded and 400 µl wash buffer was added to the upper reservoir of the column and centrifuged at 8000 g for 1 minute and the flowthrough was discarded. Washing of the column was repeated once more with 300 µl of wash buffer for optimal purity. After washing the columns, samples were centrifuged at 11000 rpm for 1 minute to ensure the complete removal of the wash buffer. Finally, columns were placed onto a clean, sterile 1.5 ml microcentrifuge tubes. For each sample, 20 µl of elution buffer was added and the samples were centrifuged at 11000 rpm for 1 minute to recover the DNA in the flowthrough. Recovery of the PCR products were further analyzed by electrophoresis on 1% agarose gels for 15 minutes at 150V.

### 4.5. PCR Amplification of the SSR Loci

In order to genetically compare almond cultivars, selected SSR loci were amplified by PCR using oligonucleotide primer pairs which were complementary to the flanking regions of the SSR marker loci. These primers were fluorescently labeled which allows direct visual analysis of the PCR products. Primer pairs were either labeled with WellRed dye or with 6-FAM dye.

In a total volume of 25 µl, the PCR mix contained 1X GoTaq Flexi Reaction Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dGTP, dCTP and dTTP), 0.2 µM of each primer, 90 ng of DNA and 1 unit of Taq Polymerase (Go Taq Flexi DNA

Polymerase, Promega). PCR cycles were as follows: 95 $^{o}$C for 3 minutes for initial denaturing, followed by 35 cycles of denaturation at 94 $^{o}$C for 1 minute, annealing at 57 $^{o}$C for 1 minute, extension at 72 $^{o}$C for 1 minute, and a final extension at 72 $^{o}$C for 10 minutes.

After PCR amplification, 5 µl of each product was mixed with 1 µl of 6X Loading Dye and electrophoretically analyzed on 2% Agarose gel for 20 minutes at 150V.

### 4.6. Fragment Analysis

Amplified PCR products of the SSR loci were analyzed either on Beckman GenomeLab™ GeXP Genetic Analysis System using automated sequencer capillary electrophoresis in the core facility of the department or sent for custom analysis to Macrogen Inc. (Korea).

PCR products labeled with 6-FAM fluorescent dye were sent to the Macrogen Inc. (Korea) for fragment analysis. Peak Scanner v1.0 (Applied Biosystems) software was used for the analysis of the alleles and band scoring.

PCR products labeled with WellRed dye were analyzed in the core facility of the department using GEXP Genetic Analysis System. As size standard, GenomeLab™ DNA Size Standard Kit – 400 labeled with WellRed D1 dye was added to the PCR products so that the fragments and size marker ran together during each run. After data collection, the data was processed with GenomeLab GeXP Analysis Software for allele identification and band scoring.

Sometimes ghost peaks in the fragment analysis were observed, representing insignificant alleles. For these samples, the whole procedure was repeated after a gradient PCR. For each sample, two new PCR products having annealing temperatures of 58 °C and 59 °C were obtained and re-analyzed in fragment analysis with previously obtained 57 °C products as controls.

### 4.7. Multiplex PCR

To perform multiplex PCR with two set of primers for the UDP96003 and the UDP96005 loci, to be analyzed in Beckman Genome Lab System; both of the loci were PCR amplified in a single reaction. In a total volume of 25 µl, PCR mix contained 1X GoTaq Flexi Reaction Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dGTP, dCTP and dTTP), 0.2 µM of each primer, 90 ng of DNA and 1 unit of Taq Polymerase (Go Taq Flexi DNA Polymerase, Promega). PCR cycles were as follows: 95 $^oC$ for 3 minutes for initial denaturing, followed by 35 cycles of denaturation at 94 $^oC$ for 1 minute, annealing at 57 $^oC$ for 1 minute, extension at 72 $^oC$ for 1 minute, and a final extension at 72 $^oC$ for 10 minutes.

After PCR cycle, PCR products were diluted ten times and each sample was mixed with 0.4 µl of size marker-400 and 37.1 µl of Sample Loading Solution (SLS). Genescan analysis was performed and allele results were visualized with Beckman GenomeLab software.

### 4.8. Amplification of the Nuclear rRNA ITS Region

For the PCR amplification of nuclear ribosomal RNA ITS region oligonucleotide primers ITS4 and ITS5 were used. In a total volume of 25 µl, PCR mix contained 1X GoTaq Flexi Reaction Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dGTP, dCTP and dTTP), 1 µM of each primer, 90 ng of DNA and 1.25 unit of Taq Polymerase (Go Taq Flexi DNA Polymerase, Promega). PCR cycles were as follows: 94 $^oC$ for 150 seconds for initial denaturing, followed by 30 cycles of denaturation at 95 $^oC$ for 30 seconds, annealing at 54 $^oC$ for 90 seconds, extension at 72 $^oC$ for 3 minutes, and a final extension at 72 $^oC$ for 7 minutes.

After PCR amplification, 5 µl of each product was mixed with 1 µl of 6X Loading Dye and electrophoretically analyzed on 1% Agarose gel for 20 minutes at 150V.

### 4.9. Data Analyses

For each almond variety, the allele data from all SSR marker regions were combined in a single matrix where the presence of an allele was scored as "1" and its absence as "0". This matrix was used for further analysis of the data. The number of alleles, the observed and the expected heterozygosities ($H_o$ and $H_e$) were calculated for each locus using Popgene (Yeh and Boyle, 1997) software. $H_o$ is defined as the heterozygous genotypes of a given locus divided by total genotypes. Expected genetic heterozygosity (He) was calculated as $1 - \sum_{i=1}^{k} p_i^2$ where $p_i$ is the frequency of the $i^{th}$ allele (Nei, 1973; Sánchez-Pérez *et al.,* 2005).

Hardy-Weinberg tests were performed for each locus independently and for all alleles using Genepop program (Raymond and Rousset, 1995) with Markov chain method. Linkage disequilibrium between each locus was also determined with Genepop. For both analyses, 0.05 value was set as the significance border.

The consistencies of the allele data was determined using Micro Checker software (Shipley, 2003). In addition, homozygote frequencies, which is the ratio of homozygote individuals to the total number of individuals, were also measured.

Microsatellite marker 1/0 data was analyzed using NTSYS-pc program (Rohlf, 2008). Using the SIMQUAL module of the program based on the Jaccard's similarity coefficient, 1/0 data were used to calculate the genetic distance. Jaccard's similarity coefficient is measured by the intersection divided by the union of the sample sets. With the similarity matrix, unweighted pair group method with arithmetic mean (UPGMA) analysis was performed using SAHN module in the NTSYS-pc package. In UPGMA analysis, a dendrogram is created using a constant-rate evolution assumed clustering method. An additional UPGMA tree and Neighbor-Joining (NJ) tree were generated based on Nei's genetic distance (Nei *et al.,* 1983) with Populations software with 1000 bootsraps. Trees were visualized using Treeview version 1.6.6. (Page, 2001)

Moreover, microsatellite data was analyzed using MVSP program (Kovach, 1998) to perform principal coordinate analysis (PCO). In PCO, similarities or dissimilarities of a

raw data or distance matrix are explored and visualized with two or three dimensional graphs (Anderson, 2003).

For each almond landrace a barcode was created. For this purpose, the 44 digit 1/0 data was used in a free web page (http://www.roubaixinteractive.com/) ASCII text generator, with the addition of four more zero digits to complete the 1/0 code to be dividable by 8. The ASCII code generated is then used in another website to convert the text into various types of barcodes (Reinardt, 2012).

# 5. RESULTS

## 5.1. Collection of Almond Leaves

In this study, 97 local varieties of cultivated almonds (*Prunus dulcis)* and five wild species (Şeytan payamı) were collected from several locations at Datça peninsula (Table 5.1.). For the selection of the local varieties and for their locations, almond growers were used as reference. Almost all of the cultivated local varieties (92 samples) have well-known characteristics and recognizable trees by the local people. Few of the most known varieties were collected from more than one location. Almonds were grown from seeds (called Sıra Payamı, in the region) therefore genetic mixtures were also collected for comparisons (five samples). Morphological differences between the varieties were significant, as demonstrated for the 34 almonds from Sındı village (Figure 5.1).

Table 5.1. Almond cultivars of Datça Peninsula used in this study.

| Sample No | Pres no | Local Name | Village collected |
|---|---|---|---|
| 1 | 377 | Çinko payamı | Palamutbükü |
| 2 | 378 | Diş payamı | Palamutbükü |
| 3 | 379 | Haşmet payamı | Yazıköy |
| 4 | 380 | Çete payamı | Yazıköy |
| 5 | 381 | Sıra payamı (1) | Yazıköy |
| 6 | 382 | Sıra payamı (2) | Yazıköy |
| 7 | 383 | Yaşar payamı | Palamutbükü |
| 8 | 384 | Karıncalı payam | Palamutbükü |
| 9 | 385 | Toker payamı | Palamutbükü |
| 10 | 386 | Cevizpayamı | Palamutbükü |
| 11 | 387 | Yazılıpayam | Palamutbükü |
| 12 | 388 | Joe payamı | Palamutbükü |
| 13 | 389 | Uğur payamı | Palamutbükü |
| 14 | 390 | Puf payamı | Palamutbükü |
| 15 | 391 | İhsan payamı | Cumalı Köyü |
| 16 | 392 | Hafızali payamı-1 | Cumalı Köyü |
| 17 | 392 | Baha payamı-1 | Cumalı Köyü |

Table 5.1. Almond cultivars of Datça Peninsula used in this study (cont.).

| Sample No | Pres no | Local Name | Village collected |
|---|---|---|---|
| 18 | 393 | Acıpayam | Cumalı Köyü |
| 19 | 394 | Tumbanali payamı | Cumalı Köyü |
| 20 | 395 | Dedebağ payamı | Cumalı Köyü |
| 21 | 396 | Nurlu payamı | Palamutbükü |
| 22 | 397 | İkigöynek payamı | Palamutbükü |
| 23 | 398 | Akpayam | Palamutbükü |
| 24 | 399 | Kababağ payamı | Palamutbükü |
| 25 | 400 | Haceli payamı | Palamutbükü |
| 26 | 401 | Kocamehmet payamı | Palamutbükü |
| 27 | 402 | Ayvamandal payamı | Mesudiye Köyü |
| 28 | 403 | Şeytam payamı | Mesudiye Köyü |
| 29 | 404 | Keram payamı | Eski Datça |
| 30 | 405 | Osman payamı | Yaka Köyü |
| 31 | 406 | Acımsıpayam | Yaka Köyü |
| 32 | 407 | Tatlıçağlapayam | Yaka Köyü |
| 33 | 408 | Kızılkis(t)le payamı | Yaka Köyü |
| 34 | 409 | Aviniye payamı | Yaka Köyü |
| 35 | 410 | Tekerlek (yazılı )payam | Yaka Köyü |
| 36 | 411 | Sıra payamı (3) | Yaka Köyü |
| 37 | 412 | Karataşak payamı | Yaka Köyü |
| 38 | 413 | Sıra payamı (4) | Yaka Köyü |
| 39 | 414 | Sıra payamı (5) | Yaka Köyü |
| 40 | 415 | Karaali payamı | Yaka Köyü |
| 41 | 416 | Acıpayam | Hızırşah Köyü |
| 42 | 417 | Bekir payamı | Hızırşah Köyü |
| 43 | 418 | Gülbekir payamı | Hızırşah Köyü |
| 44 | 419 | Rüştü payamı | Hızırşah Köyü |
| 45 | 420 | Ömer payamı (Benzinlik payamı) | Hızırşah Köyü |
| 46 | 421 | İsmet payamı | Hızırşah Köyü |
| 47 | 422 | Çakalkuyu payamı | Kızlan Köyü |
| 48 | 423 | Tatlıpayam | Kızlan Köyü |
| 49 | 424 | Kaymak payamı/Kemal payamı | Kızlan Köyü |
| 50 | 425 | Yanışlıpayam (Nakışlıpayam) | Kızlan Köyü |
| 51 | 426 | Zenginpayam | Kızlan Köyü |
| 52 | 427 | Kırmızıpayam | Kızlan Köyü |
| 53 | 428 | Sivriburun payamı | Kızlan Köyü |
| 54 | 429 | Hampayam | Kızlan Köyü |
| 55 | 430 | Fındık payamı (Çentikli payam) | Kızlan Köyü |
| 56 | 431 | Dikikpayam | Kızlan Köyü |

Table 5.1. Almond cultivars of Datça Peninsula used in this study (cont.).

| Sample No | Pres no | Local Name | Village collected |
|---|---|---|---|
| 57 | 432 | Tekir payamı (sandıkp/dişp) | Kızlan Köyü |
| 58 | 433 | Baha Payamı-2 | Cumalı Köyü |
| 59 | 434 | Hafızali Payamı-2 | Cumalı Köyü |
| 60 | 538 | Semihahanım/ Hanımdiş payamı | Sındı Köyü |
| 61 | 539 | Kemalamca payamı | Sındı Köyü |
| 62 | 540 | Çıtır payamı | Sındı Köyü |
| 63 | 541 | Fehmiçavuş / Acımsıpayam | Sındı Köyü |
| 64 | 542 | Ayşenine payamı | Sındı Köyü |
| 65 | 537 | Paşa payamı | Sındı Köyü |
| 66 | 535 | Telat payamı | Sındı Köyü |
| 67 | 536 | Amad payamı | Sındı Köyü |
| 68 | 543 | Çavuş payamı | Sındı Köyü |
| 69 | 544 | Ergun payamı | Sındı Köyü |
| 70 | 545 | Osman payamı (pres no 405) * | Sındı Köyü |
| 71 | 546 | Kızılkistle payamı (pres no 408) * | Sındı Köyü |
| 72 | 547 | Kadiroğlu payamı | Sındı Köyü |
| 73 | 548 | Goz/Kozpayam | Sındı Köyü |
| 74 | 521 | Yazılıpayam | Sındı Köyü |
| 75 | 522 | Akpayam (pres no 398) * | Sındı Köyü |
| 76 | 523 | Hacelipayam (pres no 400) * | Sındı Köyü |
| 77 | 524 | Nurlupayam (pres no 396) * | Sındı Köyü |
| 78 | 525 | Kababağ payamı (pres no 399) * | Sındı Köyü |
| 79 | 526 | Halil payamı | Sındı Köyü |
| 80 | 527 | Osmanok payamı | Sındı Köyü |
| 81 | 528 | Nine payamı | Sındı Köyü |
| 82 | 529 | Semihahanım payamı II | Sındı Köyü |
| 83 | 530 | Diş payamı (Hasanohan) | Sındı Köyü |
| 84 | 531 | Arif payamı | Sındı Köyü |
| 85 | 532 | Şemşe payamı | Sındı Köyü |
| 86 | 533 | Acıpayam | Sındı Köyü |
| 87 | 549 | Hilmi payamı | Sındı Köyü |
| 88 | 534 | Barbalıpayam | Sındı Köyü |
| 89 | 550 | Domapayam | Sındı Köyü |
| 90 | 551 | Gocapaşa payamı | Sındı Köyü |
| 91 | 552 | Fındık payamı | Sındı Köyü |
| 92 | 553 | Dedebağ payamı | Sındı Köyü |
| 93 | 554 | Acıpayam II (Dişpayamı) | Sındı Köyü |

Table 5.1. Almond cultivars of Datça Peninsula used in this study (cont.).

| Sample No | Pres no | Local Name | Village collected |
|---|---|---|---|
| 94 | 555 | Asırlık I | Sındı Köyü |
| 95 | 556 | Asırlık II | Sındı Köyü |
| 96 | 557 | Asırlık III | Sındı Köyü |
| 97 | 558 | Asırlık IV: semihahanımpayamı III | Sındı Köyü |
| 98 | 403A | Şeytan payam (identical to 28) | Mesudiye Köyü |
| 99 | 559 | Şeytan payamı II | Mesudiye Köyü |
| 100 | 560 | Şeytan payamı III | Mesudiye Köyü |
| 101 | 561 | Şeytan payamı IV | İskele mahallesi |
| 102 | 562 | Şeytan payamı V | İskele mahallesi |
| 103 | 563 | Şeytan payamı VI | İskele mahallesi |

Figure 5.1. Almond landraces collected from Sındı village.

## 5.2. DNA Extraction of Almond Leaves

DNA extraction from almond leaves worked best when CTAB method was used with some modifications (Doyle, 1987). The use of commercial DNA extraction kits were also investigated, but they were not successful to obtain good quality of DNA. The qualities of the purified DNAs were routinely analyzed spectrophotometrically by measuring the ratio of $OD_{260/280}$ and also by agarose gel electrophoresis. Figure 5.2. shows some of the purified almond genomic DNA samples after agarose gel electrophoresis.

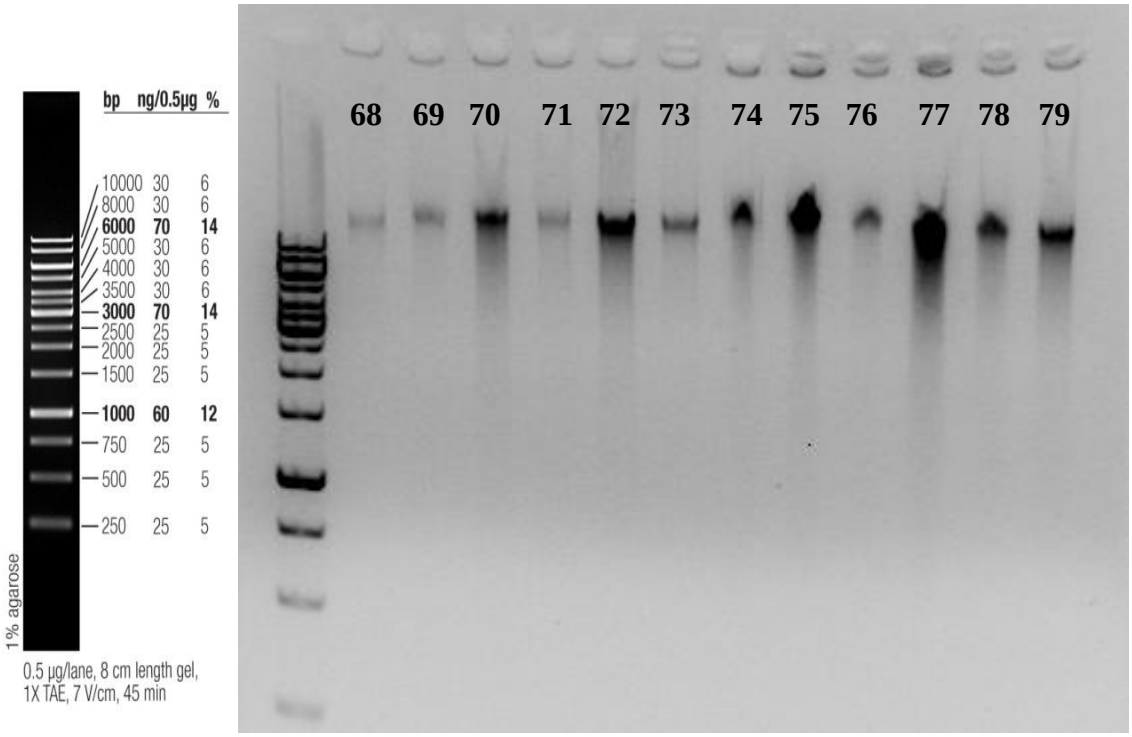



Figure 5.2. Electrophoretic analysis of the purified almond DNA's. Samples were analyzed on 1% agarose gel: from left to right: DNA size marker (SM0311, Fermentas), samples 68-79.

## 5.3 PCR Amplification and Sequencing of the Chloroplast Markers

The non-coding intergenic spacer between the *trn*L(UAA) 3' exon and the *trn*F(GAA) genes in the chloroplast DNA is highly conserved in almonds (Bortiri *et al*., 2001). Therefore the PCR amplification of the *trn*L-F intergenic spacer was used as a

control in order to confirm the quality of the DNA samples for PCR amplifications, before moving on to the amplification of the SSR markers. Except sample 28, which was un-amplifiable with the primer pair E and F, all other almond samples gave a single PCR product which was about 450bp long (Figure 5.3.).

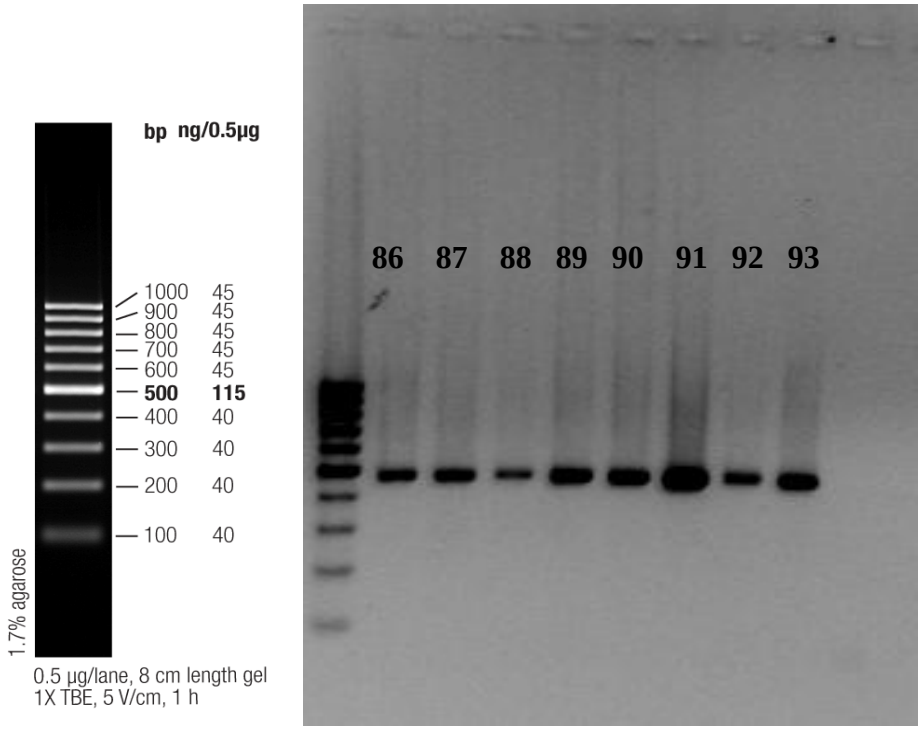



Figure 5.3. Electrophoretic analysis of the PCR amplification of the chloroplast intergenic spacer between the *trn*L(UAA) 3' exon and the *trn*F(GAA) gene. PCR products for almond samples 86-93 were analyzed on 1% agarose gel. The far left lane is the DNA size marker SM0242 (Fermentas).

Sequencing of the intergenic spacer between the *trn*L(UAA) 3' exon and the *trn*F(GAA) genes for ten almond samples (samples 1 to 10) showed that these sequences are absolutely identical. The alignment of the sequences is given in the Appendix. BLAST analysis of this sequence confirmed that this region is fully conserved among *P. dulcis* (Figure 5.4.)

Sequence analysis of the intergenic spacer between the *trn*L(UAA) 3' exon and the *trn*F(GAA) genes showed a slight difference between the wild almond samples (102 and 103) and cultivated ones. There were one base transversion in one locus and one base

deletion in other locus as seen in the Figure 5.5. The sequence data of wild almonds was identical to several different *Prunus* species as seen in the BLAST analysis in Figure 5.6.

**Sequences producing significant alignments:**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| AF318707.1 | Prunus dulcis trnL-trnF spacer region, chloroplast sequence | 776 | 776 | 95% | 0.0 | 100% |
| GQ179668.1 | Prunus dulcis voucher GIL-047 tRNA-Leu (trnL) gene and trnL-trnF | 414 | 414 | 51% | 3e-117 | 99% |

Figure 5.4. BLAST analysis of the 378 bp sequence of the *trn*L-*trn*F intergenic spacer region of the chloroplast DNA of cultivated *P. dulcis*.

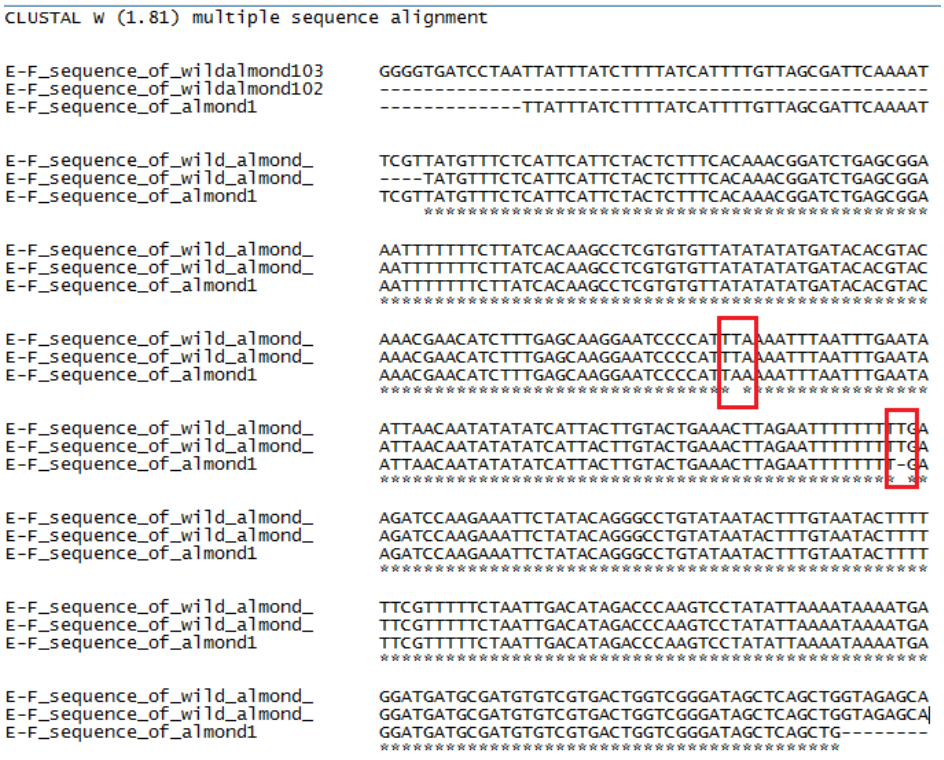
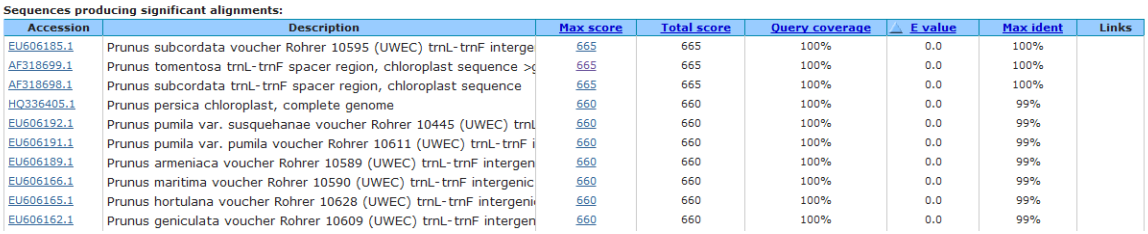



Figure 5.5. Alignment of chloroplast *trn*L-*trn*F intergenic spacer region sequence of wild and cultivated almonds. Rectangles show the base differences.



Sequences producing significant alignments:

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident | Links |
|---|---|---|---|---|---|---|---|
| EU606185.1 | Prunus subcordata voucher Rohrer 10595 (UWEC) trnL-trnF interge | 665 | 665 | 100% | 0.0 | 100% | |
| AF318699.1 | Prunus tomentosa trnL-trnF spacer region, chloroplast sequence >s | 665 | 665 | 100% | 0.0 | 100% | |
| AF318698.1 | Prunus subcordata trnL-trnF spacer region, chloroplast sequence | 665 | 665 | 100% | 0.0 | 100% | |
| HQ336405.1 | Prunus persica chloroplast, complete genome | 660 | 660 | 100% | 0.0 | 99% | |
| EU606192.1 | Prunus pumila var. susquehanae voucher Rohrer 10445 (UWEC) trnL | 660 | 660 | 100% | 0.0 | 99% | |
| EU606191.1 | Prunus pumila var. pumila voucher Rohrer 10611 (UWEC) trnL-trnF i | 660 | 660 | 100% | 0.0 | 99% | |
| EU606189.1 | Prunus armeniaca voucher Rohrer 10589 (UWEC) trnL-trnF intergen | 660 | 660 | 100% | 0.0 | 99% | |
| EU606166.1 | Prunus maritima voucher Rohrer 10590 (UWEC) trnL-trnF intergenic | 660 | 660 | 100% | 0.0 | 99% | |
| EU606165.1 | Prunus hortulana voucher Rohrer 10628 (UWEC) trnL-trnF intergeni | 660 | 660 | 100% | 0.0 | 99% | |
| EU606162.1 | Prunus geniculata voucher Rohrer 10609 (UWEC) trnL-trnF intergen | 660 | 660 | 100% | 0.0 | 99% | |

Figure 5.6. BLAST analysis of the 360bp sequence of the chloroplast *trn*L-*trn*F intergenic spacer region of wild almond.

## 5.4. Control Experiments for PCR Amplification of SSR Loci

For each SSR primer pair, a gradient PCR was applied with annealing temperatures, which varied between 57 °C to 60 °C, in order to determine the optimum annealing temperature for PCR amplifications of each SSR locus. The PCR products were analyzed electrophoretically. The results with highest quality of products were obtained at the annealing temperature of 57 °C for all the three loci. Several amplification results were analyzed by agarose gel electrophoresis as can be seen in Figure 5.7.

In order to optimize the PCR conditions, $MgCl_2$ concentration was varied as 1.5 mM or 2 mM. Optimum primer concentrations were also determined by varying the primers between 0.2 µM to 1 µM. Best results were obtained with 1.5 mM $MgCl_2$ and 0.2 mM of each primer.

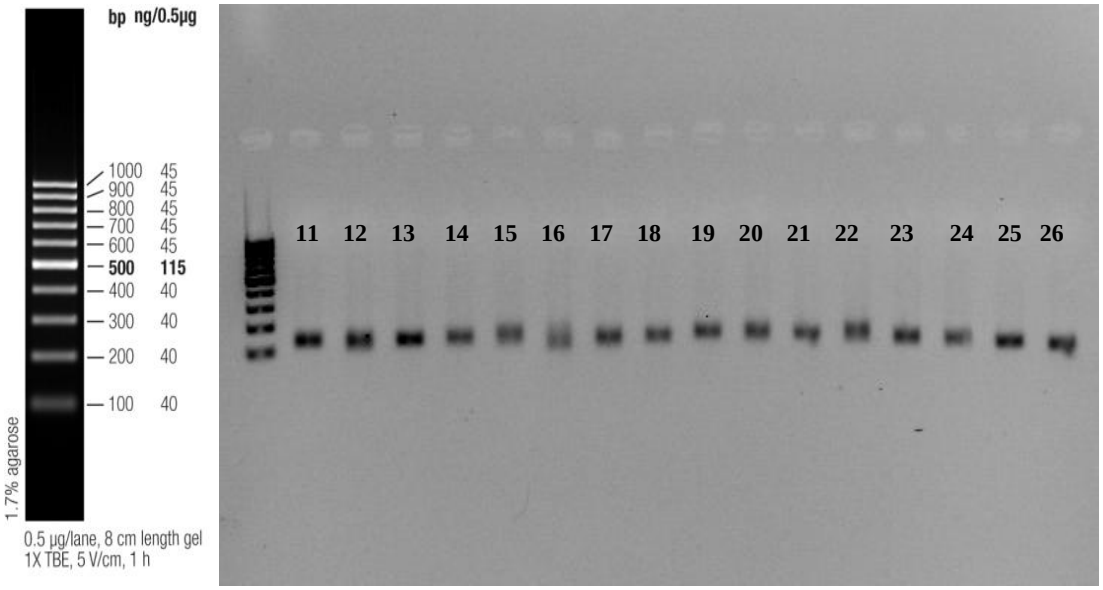



Figure 5.7. Electrophoretic analysis of the PCR products of almond DNA's at the UDP96003 SSR Loci. Samples were analyzed on 2% Agarose Gel. From left to right: DNA size marker (SM0242, Fermentas), almond samples 11-26.

### 5.4.1. UDP96005 PCR and Control Experiments for Fragment Analysis

The samples which yield no fragment or yield aberrant fragments during the fragment analysis with UDP96005 marker locus were re-analyzed in the genescan system. Amplifications were repeated for a second time. Repeat experiments resulted in proper allele peaks.

When fragment analysis showed several ghost peaks, PCR amplifications were repeated using gradient PCR. In the gradient system, 58 °C and 59 °C temperatures were also used as annealing temperatures in addition to standard 57 °C. When the PCR products of the three different annealing temperatures for the same sample were compared, ghost peaks did not disappear completely. The change in the ghost peaks were not consistent; in some cases ghost peak signals decreased with the increase of annealing temperature, but in others vice versa (Figure 5.8. and Figure 5.9.)

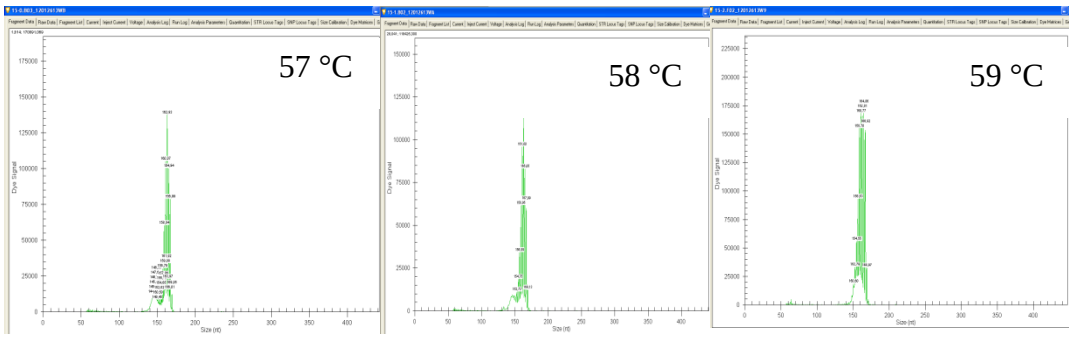



Figure 5.8. Fragment analysis of sample 15 at the UDP96005 locus. As the annealing temperature increased, ghost peaks' signals also increased.

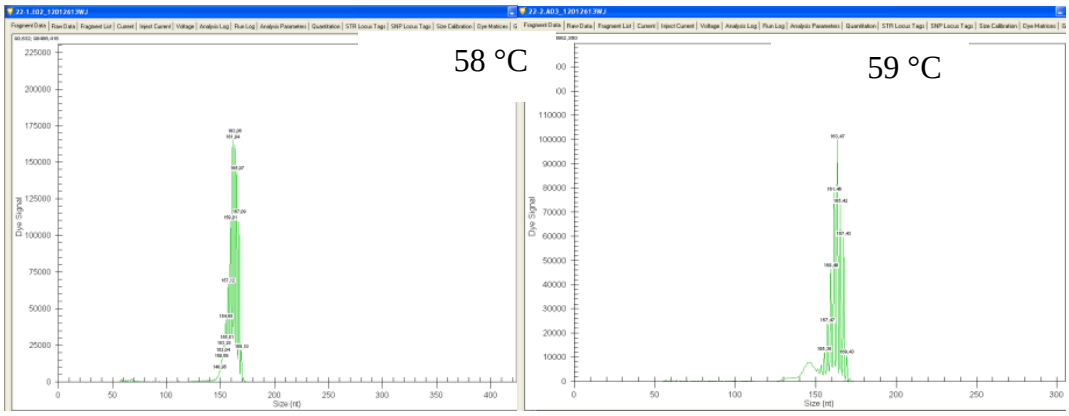



Figure 5.9. Fragment analysis of sample 22 at the UDP96005 locus. Ghost peaks decreased with the increase of the annealing temperature.

### 5.4.2. Multiplex PCR

A multiplex PCR was performed using UDP96003 and UDP96005 primers. Labeled with different wellred dyes. Fragment analysis of the PCR products showed that the fragments were consistent with the separate analysis of each primer pair as seen in Figure 5.10.

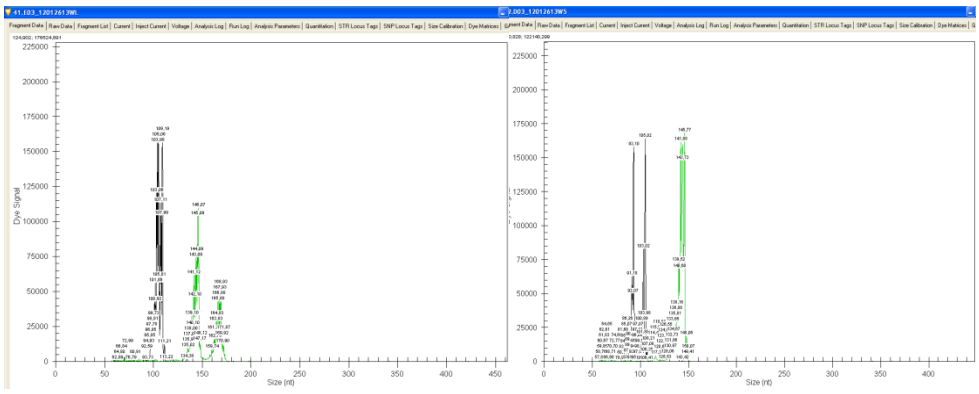


Figure 5.10. Multiplex PCR results of almond 12 and almond 41 for both UDP96003 and UDP96005 loci. Results were consistent with single locus analyses.

## 5.5. Wild (Şeytan Payamı) Samples

It was not possible to PCR amplify some of the wild almond samples collected from the same location with sample 28 but from other wild trees (99,100 and 101), most probably due to the DNA quality. PCR amplification failed for chloroplast E-F primers, nuclear ribosomal ITS4 and ITS5 primers and with SSR primers. In order to improve DNA quality, several times fresh leaves were collected and DNA extractions were repeated with various revisions. In all cases the results were negative. However, wild almonds collected from a different location (samples 102 and 103) could be amplified (Figure 5.11). Furthermore PCR amplifications for samples 99, 100 and 101 were also conducted using universal 16S rRNA primers in order to decide whether the amplification problem is due to the sequence of the region to be amplified or due to the DNA-quality. The absence of PCR products indicated that the DNA quality is not sufficient. Even though all the wild type samples were treated in parallel, an unknown parameter from the location where samples 99-101 were collected interferes with the DNA. The presence of a quarry very near to this location may be related to the problem.

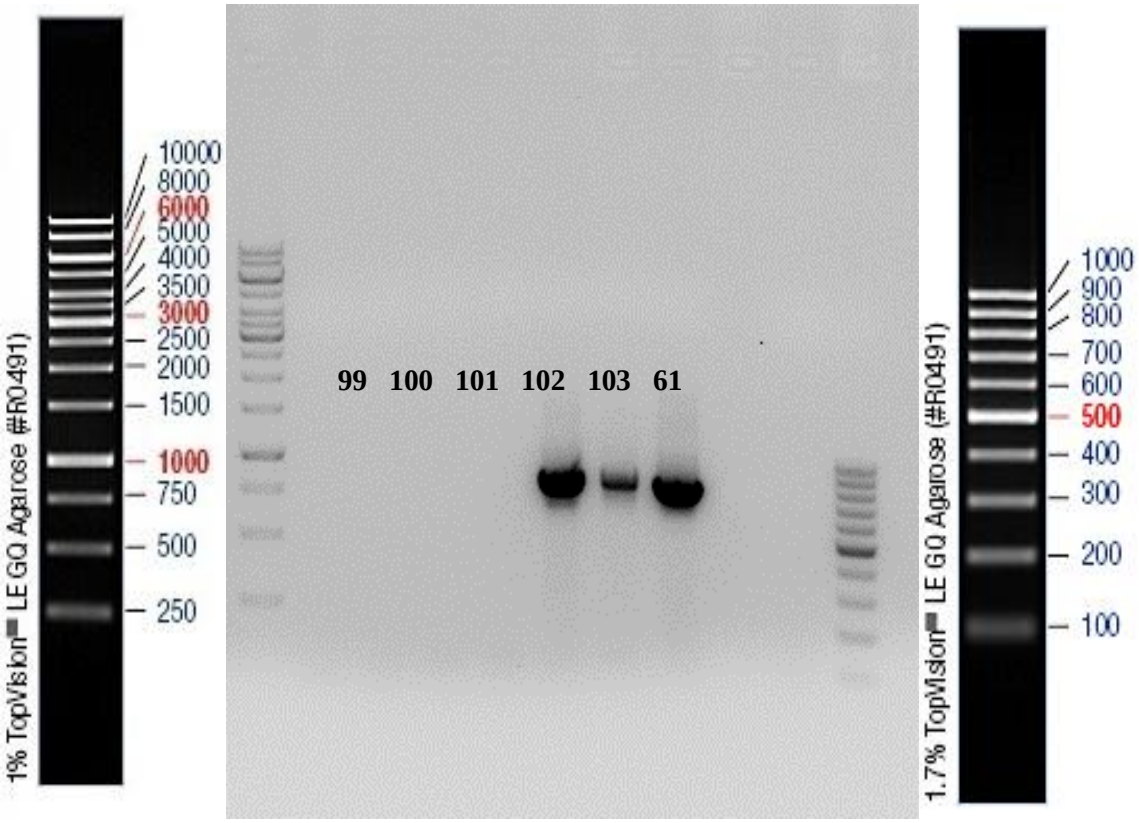



Figure 5.11. Electrophoretic analysis of the PCR products of wild almond DNAs at the nuclear ribosomal RNA ITS region. Samples (98-103) shown on 1% agarose gel with sample 61 as positive control. SM0311 and SM0242 (Fermentas) size markers were used.

Samples 28 (98), 102 and 103, which give sufficiently high quality of data to be amplified with ITS primers were processed further. The result of the samples 102 and 103 multiplex PCR using UDP96003 and UDP96005 primers was shown in Figure 5.12.

For the samples that could not be amplified neither with ITS primers nor with SSR primers, ribosomal 16S and the chloroplast region amplification trials were also unsuccessful.

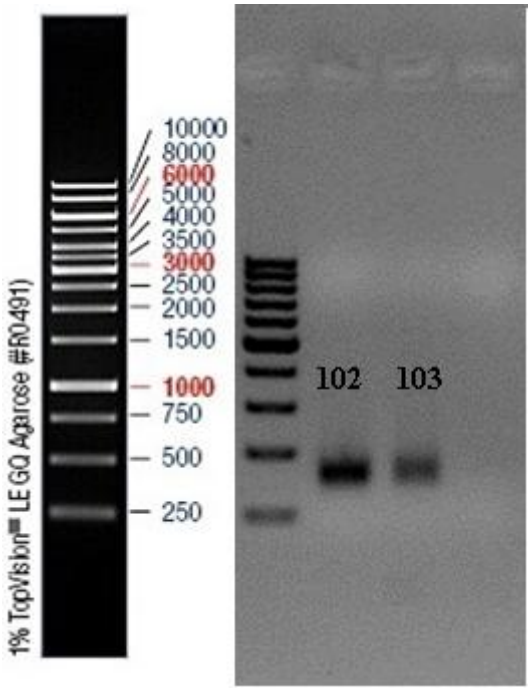



Figure 5.12. Electrophoretic analysis of the multiplex PCR products of the wild almond samples 102 and 103 at the SSR loci UDP96003 and UDP96005. Samples were analyzed on 1% agarose gel. SM0311 (Fermentas) size marker was used.

### 5.6. Fragment (Genescan) Analysis of the SSR Markers

Fluorescently labeled primers were used for the SSR loci amplifications which enabled the direct visualization of the PCR products during capillary electrophoresis where the PCR products were separated according to their size. The size of each fragment was determined with respect to a fluorescently-labeled DNA size marker (labeled with a different fluorescent dye) during the same run. Two such fragment analysis results, one for a homozygous sample (Figure 5.13) and the other for a heterozygous sample (Figure 5.14) are shown for the same SSR locus.

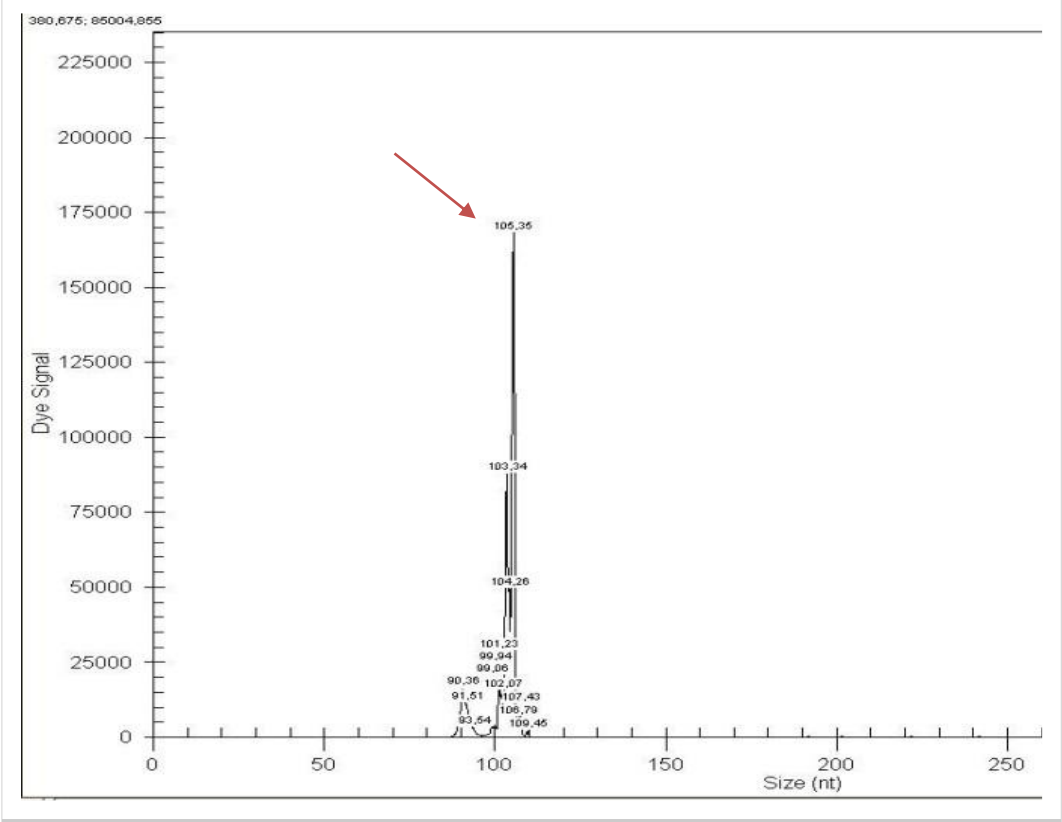



Figure 5.13. Fragment Analysis of UDP96003 SSR region for almond sample 6.

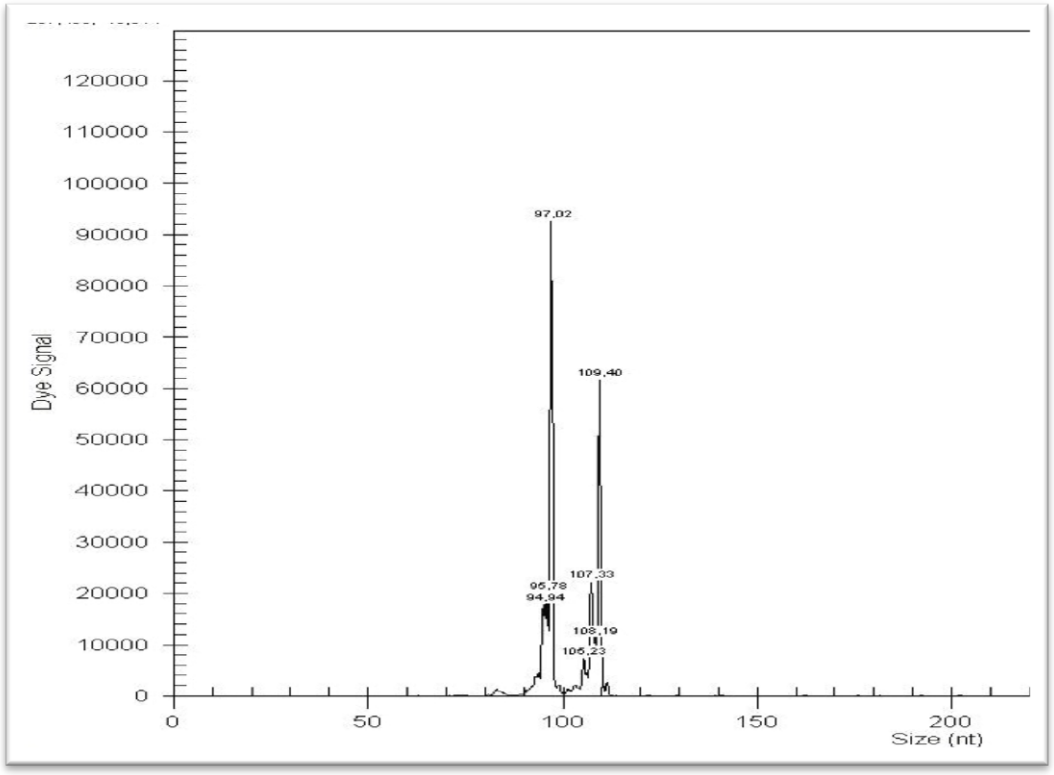



Figure 5.14. Fragment Analysis of UDP96003 SSR region for almond sample 10.

Once the allele information for each almond sample was converted into 1/0 presence/absence data, similarity matrix and principal coordinate analysis was performed using NTSYSpc 2.2 (Rohlf, 2008) and MVSP (Kovach, 1998) programs. The result of the principal coordinate analysis of all the almond samples studied is given in Figure 5.15 as a scatter plot. Using the data from all three SSR loci the principal coordinate analysis indicated that 87 of the almond samples were unique whereas some of the samples had duplicates or triplicates (Table 5.2.)

Table 5.2. Almonds samples which are identical with respect to the three SSR loci.

| |
|---|
| Sıra payamı (no: 6) = Haceli payamı (no: 25) = Haceli payamı (no: 76) |
| Akpayam (no: 75, pres no: 398) Kadiroğlu payamı (no: 72) |
| Amad payamı (no: 67) = İkigöynek payamı (no: 22) İhsan payamı (no: 15) |
| Sıra payamı (no: 5) = Ceviz payamı (no: 10) |
| Kababağ payamı (no: 24) = Yanışlı/nakışlı payam (no: 50) ~ Baha payamı (no: 16) |
| Hilmi payamı (no: 87) = Doma payamı (no: 89) |

## 5.7. Biostatistical Analyses

PCO analysis was first performed with single marker data separately as seen in Figure 5.15, Figure 5.16 and Figure 5.17. These single analyses resulted in several groupings of 97 samples.

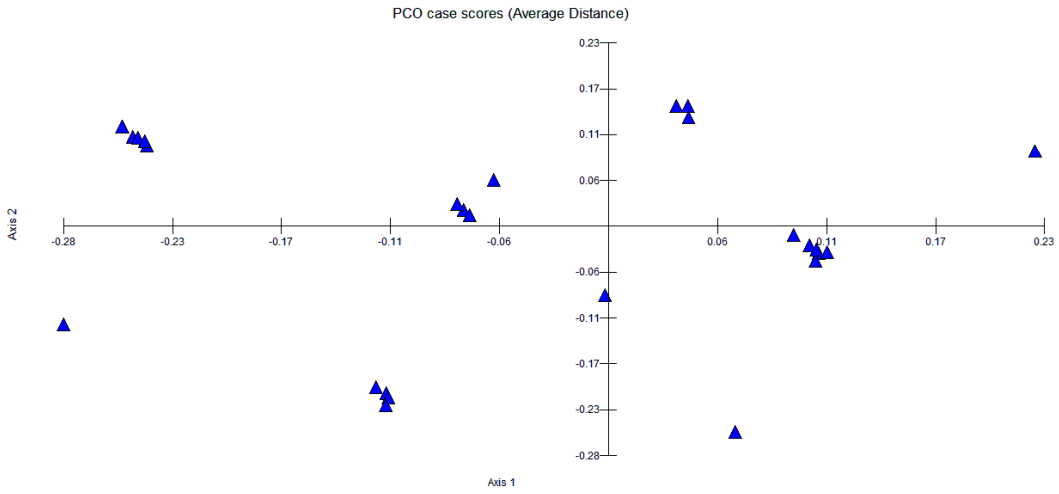



Figure 5.15. Principal Coordinate Analysis of the 96 almond samples for the UDP98409 SSR marker locus. Each almond sample is represented by a triangle. Samples apart from each other in the plot are genetically distant; samples near to each other are closely related.

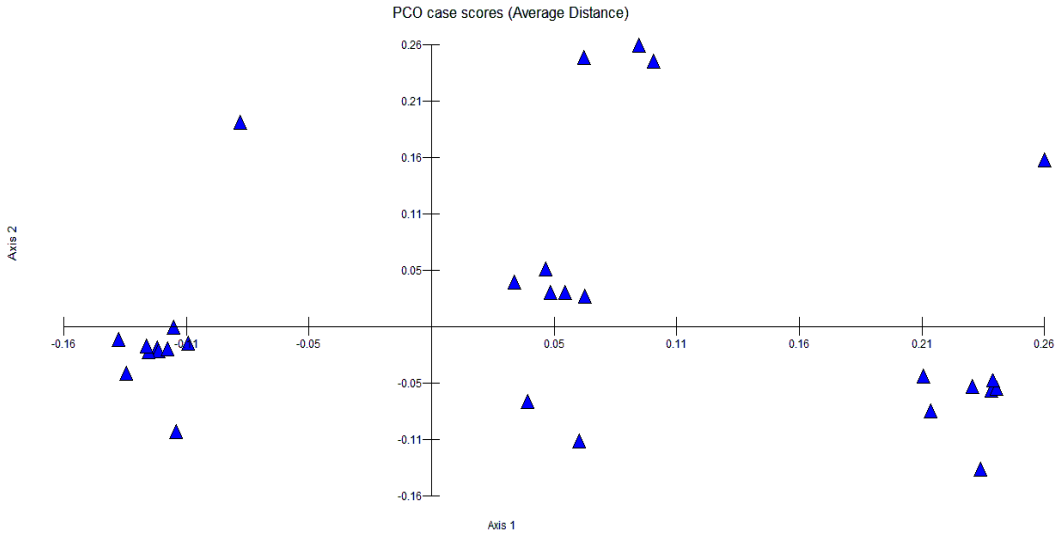



Figure 5.16. Principal Coordinate Analysis for the 96 almond samples with UDP96003 SSR markerlocus.

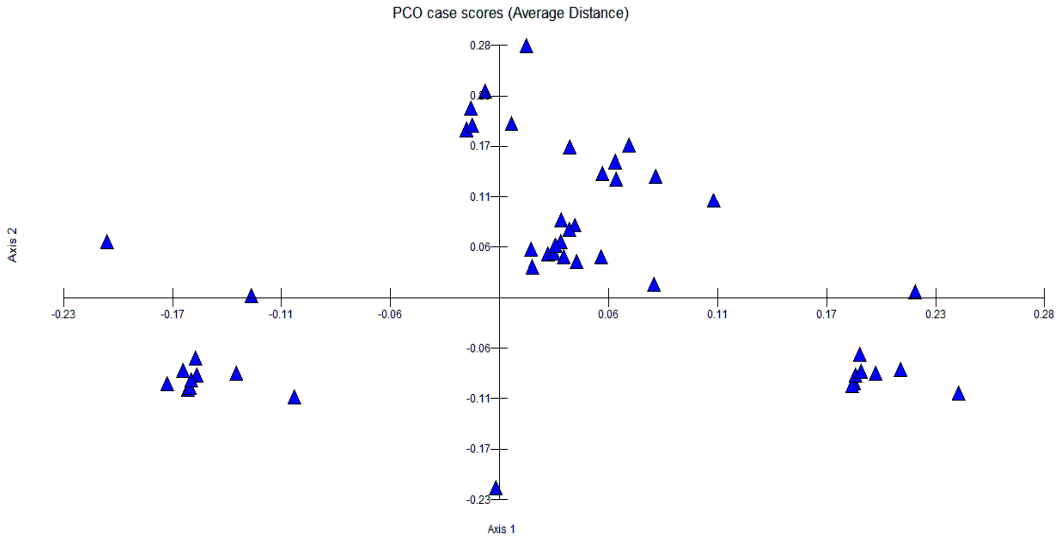



Figure 5.17. Principal Coordinate Analysis of the 96 almond samples for the UDP96005 SSR marker locus.

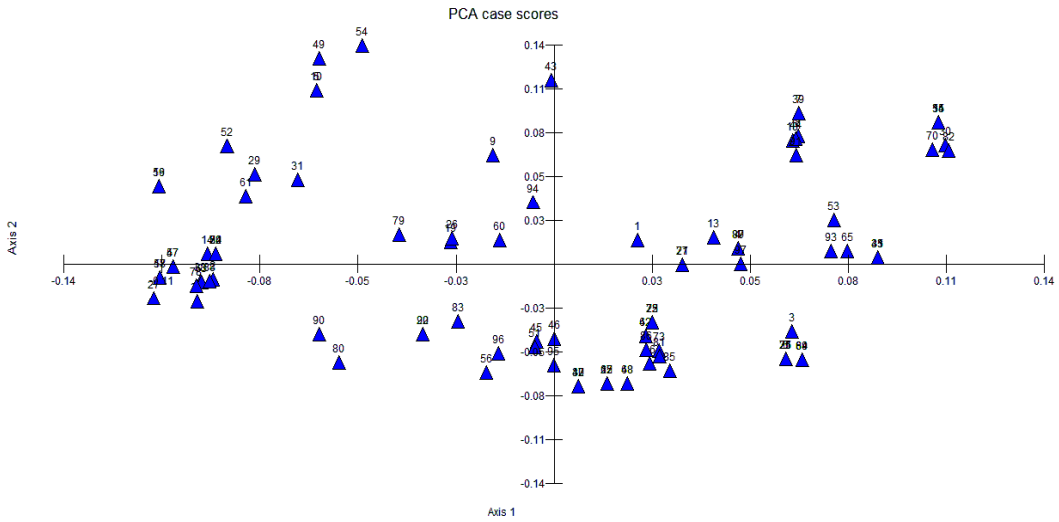



Figure 5.18. Principal Coordinate Analysis of the 96 almond samples for both UDP98409 and UDP96003 SSR marker loci.

PCO with two marker loci (UDP98409 and UDP96003) resulted in a scatter plot with better isolation of different samples (Figure 5.18)

PCO with all three marker loci gave the best separation of samples as seen in Figure 5.19. This scatter plot graph allowed the identification of unique and identical samples (Table 5.2.).

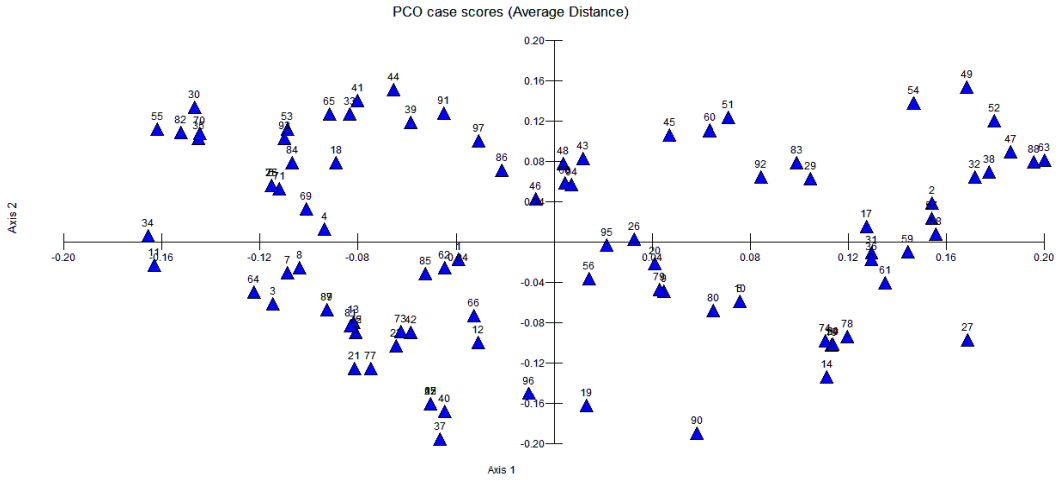



Figure 5.19. Principal Coordinate Analysis of the 96 almond samples for the UDP98409, UDP96003 and UDP96005 SSR marker loci.

From the UPGMA analysis of similarity matrix, UPGMA dendrogram was obtained (Figure 5.20.). The result of the UPGMA analysis with 1000 bootstraps is given in Figure 5.21. NJ tree with 1000 bootstraps, for comparison, is shown in Figure 5.22.

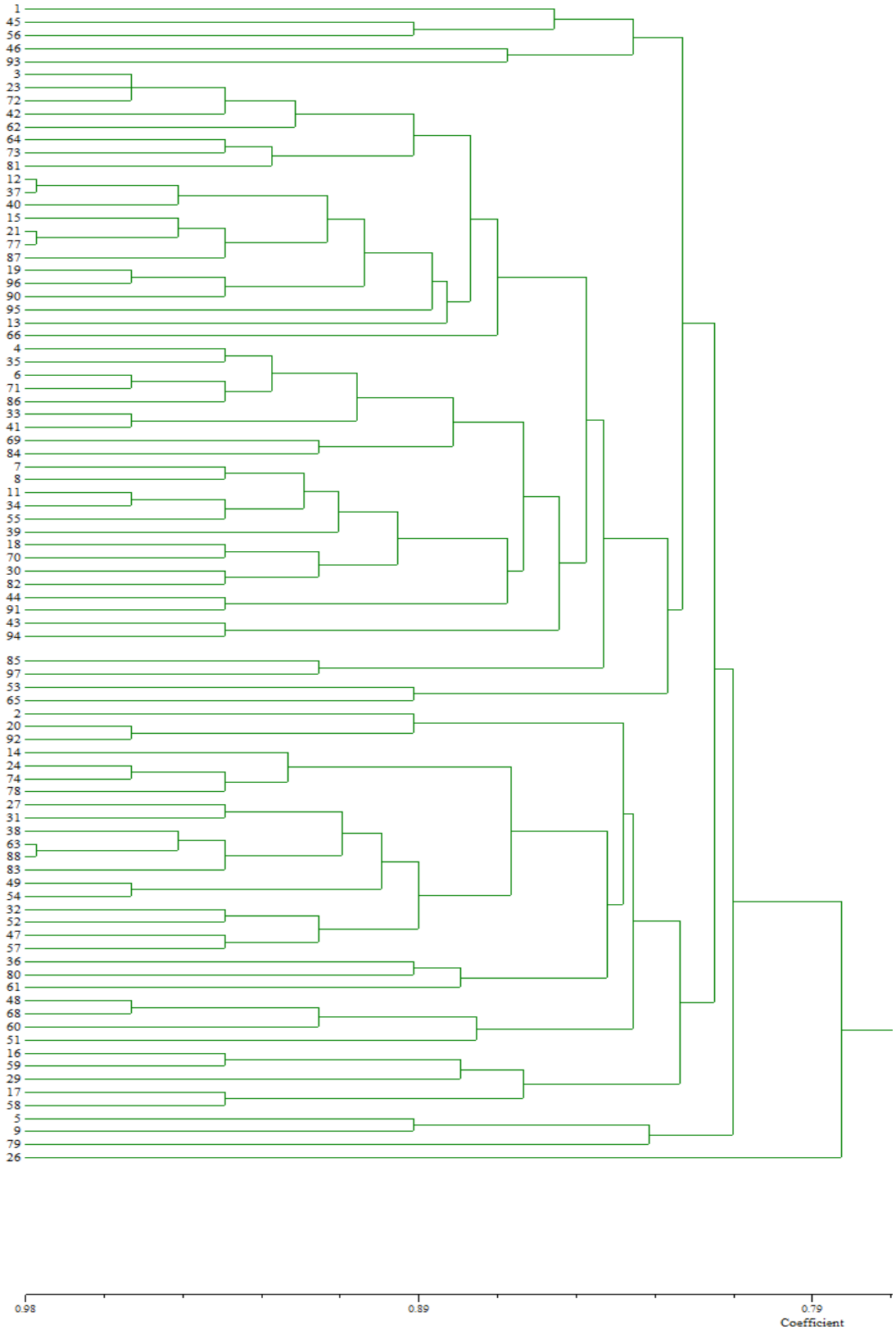



Figure 5.20. UPGMA dendrogram of distinct almond varieties (constructed with NTSYSpc).

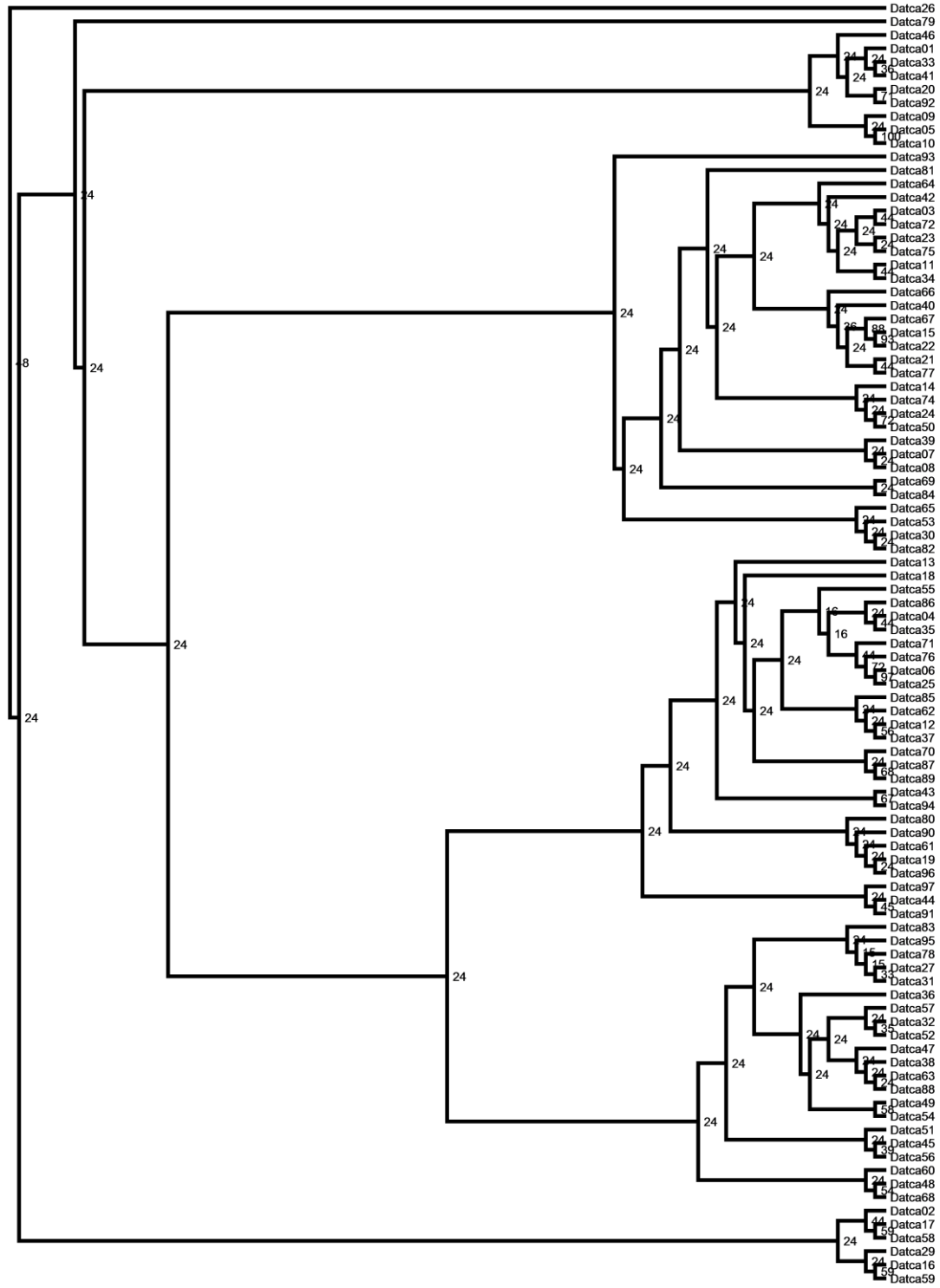



Figure 5.21. UPGMA tree of 97 samples constructed using Populations software with 1000 bootstraps.

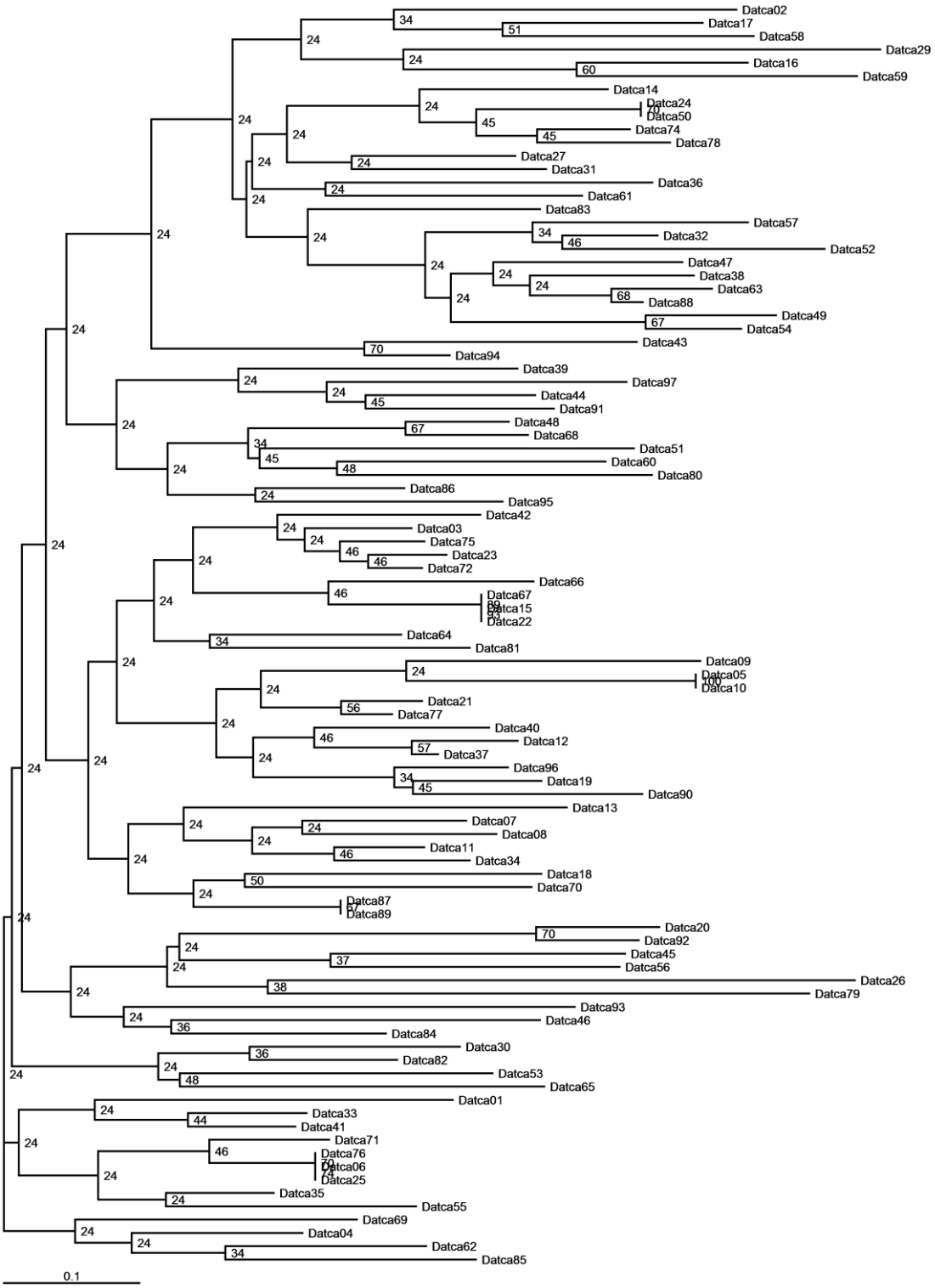



Figure 5.22. NJ tree constructed with Populations software with 1000 bootstraps.

Observed and expected heterozygosities were obtained for each SSR locus (Table 5.3.) Additional tests such as Hardy-Weinberg test and genotypic linkage disequilibrium (LD) tests were performed (Table 5.4. and Table 5.5.). Observed heterozygosity values were slightly different than the expected ones. Hardy-Weinberg tests indicated that, population was not in HW equilibrium and LD test suggested that the UDP98409 locus is linked to the other two loci.

Table 5.3. Observed and expected heterozygosity values of almond samples at each SSR marker region.

| SSR Locus | Number of Alleles | Observed. Heterozygosity | Expected Heterozygosity |
|---|---|---|---|
| **UDP98409** | 13 | 0.6667 | 0.7932 |
| **UDP96003** | 16 | 0.8438 | 0.7926 |
| **UDP96005** | 15 | 0.7396 | 0.8852 |

Table 5.4. Hardy-Weinberg Test for each locus.

| Locus | P-value | Standard Error |
|---|---|---|
| UDP98409 | 0.0000 | 0.0000 |
| UDP96003 | 0.0711 | 0.0255 |
| UDP96005 | 0.0000 | 0.0000 |

Table 5.5. Genotypic Linkage Disequilibrium.

| Locus 1 | Locus 2 | P-Value | Standard Error | Switches |
|---|---|---|---|---|
| UDP98409 | UDP96003 | 0 | 0 | 4112 |
| UDP98409 | UDP96005 | 0 | 0 | 2086 |
| UDP96003 | UDP96005 | 0.112914 | 0.02927 | 1888 |

Homozygote frequency tests were performed with Micro Checker software (Van Oosterhout *et al*., 2004) and results were visualized by graphs as seen in Figures 5.23, 5.24 and 5.25.

Using the 1/0 data, a barcode was created for each almond landrace (Figure 5.26).

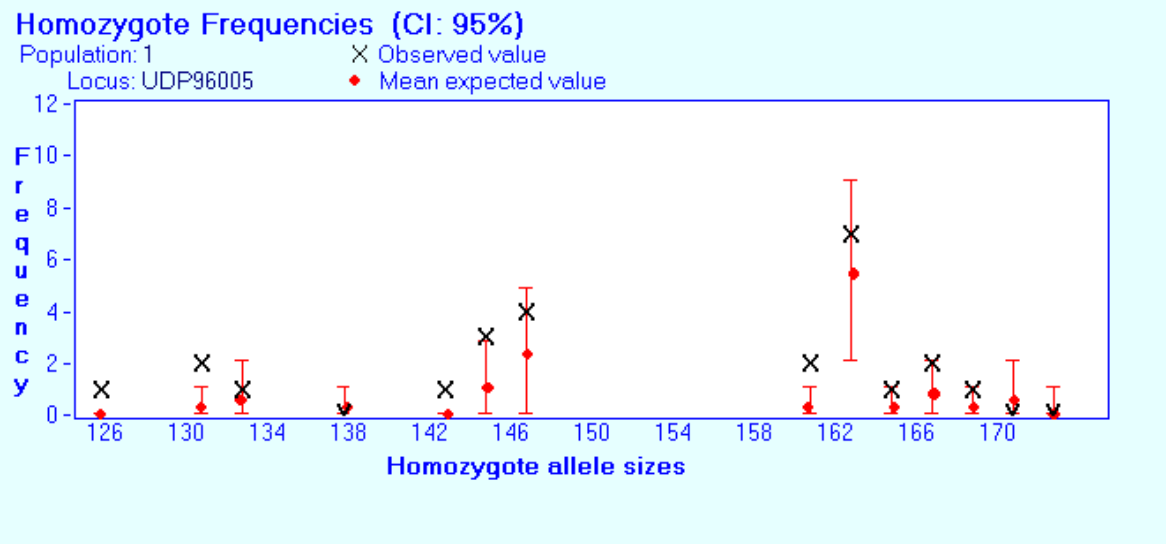



Figure 5.23 Homozygote frequency graph of the UDP96005 locus.

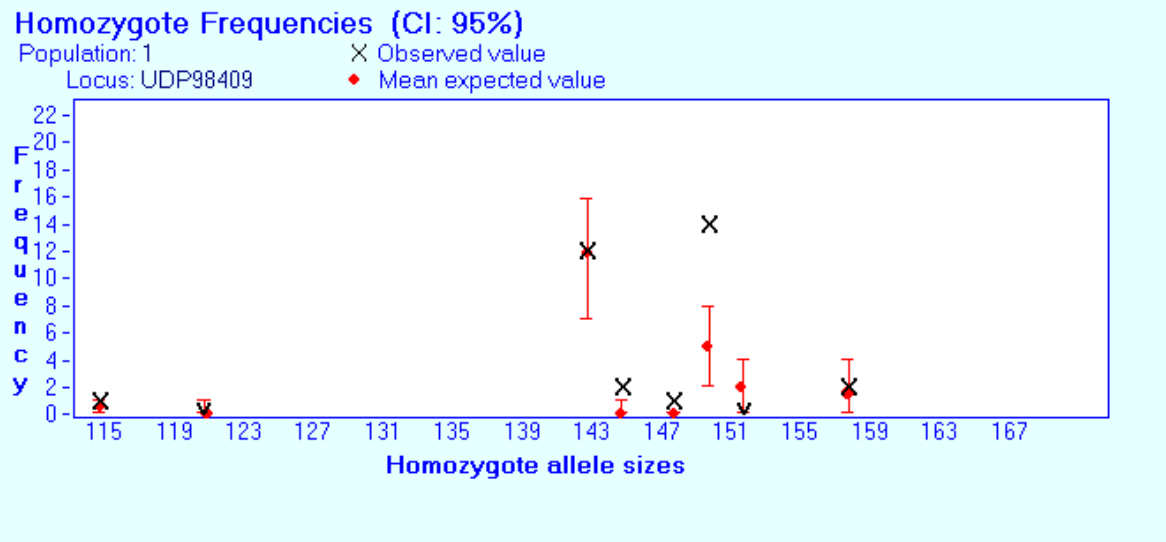



Figure 5.24 Homozygote frequency graph of the UDP98409 locus.

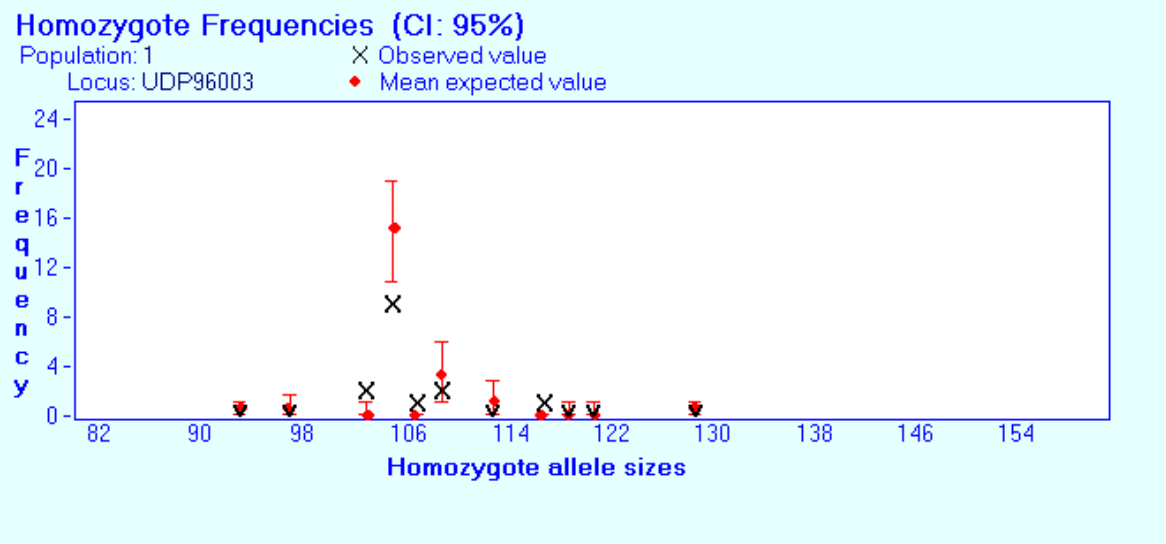



Figure 5.25. Homozygote frequency graph of the UDP96003 locus.

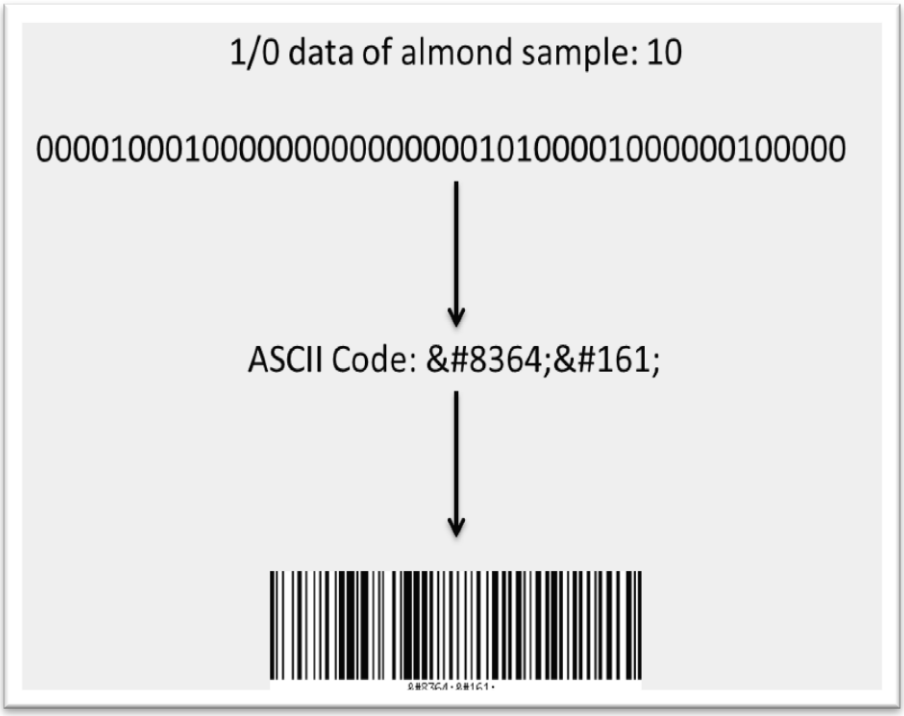



Figure 5.26. Barcode for almond sample 10.

# 6. DISCUSSION

Among the several options for molecular differentiation of Datça's local almond varieties, the use of SSR markers was preferred in this study. There were several reasons for choosing SSR markers: with respect to other markers, especially RFLP, SSR markers are easier to investigate using a PCR-based method, therefore require less genomic DNA for analysis. In addition, during the PCR amplification of the SSR markers, the use of fluorescently labeled primers makes the automated genescan analysis possible, which is easy to handle. With low cost, SSR markers yield high reproducibility. They are co-dominant, that is, each marker is related to a single locus on each homologous chromosomes, therefore, it is possible to further analyze the heterozygosity of the individuals. SSR markers are predominantly used in analyses of genetic diversity. A large set of SSR markers for *Prunus* species were already known and had been used to study the genetic diversity of wild or cultivated almond varieties. For all these reasons the use of SSR markers were chosen for the study of Datça's almond diversity and molecular identification of the local almond varieties.

After three SSR region amplifications, fragment analyses procedures were conducted in two different places.

First group of fragments belonging to the UDP98409 locus, were analyzed by Macrogen Inc. (Korea). These results were not all clear with one or two alleles with respect to the variety being homozygote or heterozygote in that locus; but instead there were many other fluorescent peaks, signs of many insignificant fragments coming from the PCR reaction of that locus. Some of these fragment peaks were so weak in signal when compared to the main high-fluorescent-signal peaks so all insignificant peaks were ignored. But in some cases there were more than two peaks with high signal strength; so it was hard to detect the peaks representing the alleles. In all complicated situations, fragments with highest signal strengths among the others were chosen as alleles. In some cases there were several fragment signals around a base pair spot and far from them there were other fragment signals in another base pair spot. In those cases these two fragment-signal spots were considered as two distinct alleles and the several signal peaks in each

spot were regarded as an error of PCR reaction producing more than one fragment in each repeat region because of the fidelity problem of amplifying repetitive DNA regions.

Second primer pair belonging to the SSR locus called UDP96003, was labeled with WellRed dye for processing in the Beckman GenomeLab™ GeXP Genetic Analysis System in our department. After the PCR procedure with fluorescently labeled primers, PCR products were run in capillary electrophoresis system of Beckman Coulter instrument. Results of the analysis were reviewed with Beckman GenomeLab software and for each almond variety, alleles were determined and recorded. In this locus, allele peaks were very clean, most of the time without noisy signals behind the main peaks. So it was easy to determine the fluorescent signal peaks representing the alleles.

Third primer pair belonging to the SSR locus named UDP96005 was also labeled with WellRed dye for analysis in the Beckman GenomeLab™ GeXP Genetic Analysis System. Results were reviewed with the Beckman GenomeLab software. However this locus was similar to the SSR locus UDP98409 with respect to the presence of noisy peaks which make it difficult to detect alleles. For this reason, results were analyzed several times to prevent mistakes in allele determination and noisy signals were ignored carefully. For the samples which gave several peaks, PCR amplification was repeated as gradient PCR with changing the annealing temperatures. Higher temperatures like 58°C and 59°C instead of 57°C were used in order to decrease the noisy signals in the PCR products, which may be due to the improper primer binding. However there were no improvement. On the other hand, these peaks may be the result of DNA polymerization errors due to the repetitive nature of the sequence at the SSR loci. For this reason, other PCR enzymes, *Taq* polymerases from other sources were investigated. Go *Taq* Polymerase (Promega) was found as to be the best suitable enzyme for SSR amplifications.

After analyzing all three SSR loci, it was concluded that, the quality of the data (clear allele peaks) depend strongly on the nature of the SSR loci. Changing the annealing temperature of the PCR amplifications or changing the PCR chemicals to eliminate the ghost peaks in some of the samples had no noticeable improvements. Multiplex PCR trials were successful and can be applied to new almond samples to save time instead of separate analyses.

In PCO analysis obtained with the UDP98409 locus alone resulted in some grouping of samples along the x-y axis. The basis of this grouping, which morphological features relate to this grouping, should be investigated further. The PCO analysis using all three marker loci resulted in a fine separation of samples on the x-y axis as seen in Figure 5.17 and verified the identical samples which appeared on the same coordinates as expected. The PCO analysis also shows the high polymorphic genetic structure of the almonds. In the future, PCO analysis of each SSR marker that is linked to a morphological property can be used to understand the phenotypic distribution of almond landraces.

Heterozygosity is a parameter of genetic variation in a population and expected heterozygosity is calculated with respect to Hardy-Weinberg equation. It was found that, only the UDP96003 marker has higher degree of observed heterozygosity compared to the expected heterozygosity. When observed heterozygosity is higher than the expected; this may be an indicator of isolate-breaking effect which may be the case for the almond population studied in this work. Last but not least, all heterozygosity values were relatively high, which means markers selected were statistically successful for the discrimination of almond landraces.

Hardy-Weinberg tests indicated that, the almond population was not in HW equilibrium. Taking the man-made selections of these almond landraces into consideration, this result is reasonable; after all, the evolution and distribution of these almonds are not natural.

Linkage disequilibrium test between marker loci pointed out that, UDP98409 locus is linked to the other two loci. However, it is known that, all these three markers are located on different chromosomes, so these loci cannot be linked. However, linkage disequilibrium is not solely related with genetic linkage. It is also affected by selection, non-random mating and population structure. So, the population dynamic of man-made selections on these almonds may be effective on linkage disequilibrium results.

Homozygous allele frequencies for the SSR locus UDP96005 indicated that, there were more homozygous individuals in this region compared to the expected value. There is no evidence for stuttering or large allele dropout. However, null alleles may be present.

Homozygous allele frequency test for the SSR locus UDP98409 showed that, there are excess homozygous individuals in this region. After the consistency tests with Micro Checker software, it was concluded that, excess homozygosity may be due to stuttering. Stuttering occurs due to the errors in PCR and stutter peaks are seen as small peaks near the real peaks. This is usually due to the shortage of highly heterozygous genotypes with alleles of one repeat unit difference. Null alleles may also be present due to observations of excess homozygotes. But no evidence was seen for large allele dropout.

In the SSR locus UDP96003, there were less homozygous individuals than expected. No evidence for stuttering, allele dropout and null alleles was present.

Sequence and BLAST analyses of chloroplast *trn*L-*trn*F intergenic region confirmed that except Şeytan Payamı samples (28, 102 and 103), other landraces were identical to *P. dulcis* sequences in the GenBank. However, Şeytan payamı samples were more related with other *Prunus* species such as *P. subcordata* (Klamath plum) and *P. tomentosa* (Nanking cherry) than the cultivated almonds of Datça. The similarity of wild and cultivated almonds of Datça was 99% with a transversion and a deletion mutation in two different loci which should be further investigated to understand the phylogenetic clustering of wild almond species in Datça.

UPGMA dendrogram was used as the clustering method with the assumption of equal rate of evolution in between almond landraces. Additionally, UPGMA is the simplest tree-clustering method to observe the genetic relations among varieties. In the UPGMA dendrogram, almond sample 26 which is Kocamehmet payamı from Palamutbükü village appeared as the most distant cultivar. Its morphological properties should be further examined in order to understand the reason of its separate clustering. The dendrogram indicates the presence of four clades; however, this genetic grouping is not related to their geographical locations. Since all the samples were collected from Datça peninsula, a limited area, geographical clustering was not expected. On which common property the almond were clustered is not clear and should be further investigated.

The comparison of two UPGMA dendrograms showed that, both of them were consistent in clustering the samples. In the UPGMA tree with bootstrapping, identical samples were also denoted and found to be clustered as closely related with high bootstrap values. However, the main large branches had lower bootstrap values which show the unreliability of clustering with only three marker regions.

The neighbor-joining tree had a different clustering with respect to UPGMA dendrograms. This situation may be a result of evolutionary forces indicated on NJ tree. For instance, sample 26 which behaves like an outer group in UPGMA dendrograms is not located as an outgroup with the highest evolution rate among the other almond landraces. As a result, evolutionary forces, which are actually human-made selections between almonds, may be different among lineages. The difference between UPGMA and NJ clustering may be affected by these evolutionary forces.

# 7. CONCLUSION

With this study, the genetic diversity of the 96 of Datça's almond landraces were established based on molecular data and DNA fingerprints were created to identify each cultivar. DNA fingerprint data will be very useful and sufficient enough for their registry into formal data bases and help for their recognition as Datça's almond landraces in the future. This registration would help to protect this highly economical and culturally important value of Datça. Genetic relationships between the cultivars, inferred from the molecular data presented in this thesis can also be used for the selection of proper cultivars among Datça's landraces for agricultural hybridization studies in the future for the development of new and better cultivars. The development of such cultivars would ensure our food safety in a changing planet.

The SSR data for each sample can be used in a specific format, to create a unique barcode to define each variety and this may be useful for the registration of these almonds in the ministry of agriculture as the landraces of Datça. However, large number of samples from each landrace has to be tested at each SSR loci for consistency, before finalizing the barcoding. It is also required to analyze almonds from different regions of Turkey as well as commercial almond samples, at these three SSR loci in order to understand whether these three SSR markers are sufficient enough for barcoding studies. Otherwise additional SSR loci should also be investigated.

In the future, to follow up the molecular data for Datça’s landraces should be compared with other almond cultivars in Turkey in order to understand the almond diversification all over the country. To compare the cultivated forms with wild forms using microsatellite markers may also help us to observe the evolutionary pathway of almonds.

In addition, SSR marker analyses can be further used to understand the genetic linkage between the SSR markers and the morphologic features in order to select and breed almonds with specific phenotypes.

# APPENDIX A: NUCLEOTIDE SEQUENCE ALIGNMENT OF INTERGENIC SPACER REGION BETWEEN CHLOROPLAST *trn*L(UAA) 3' EXON AND THE *trn*F(GAA) GENE IN TEN ALMOND SAMPLES

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CLUSTAL 2.0.12 multiple sequence alignment.


1
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2                -
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3
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4
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5
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9
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10
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3
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4
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5
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7
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9
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10
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2
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3
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4
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2
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10
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**********

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2
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3
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10
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2
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3
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4
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5
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6
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9
TGACATAGACCCAAGTCCTATATTAAAATAAAATGAGGATGATGCGATGTGTCGTGACTG

10
TGACATAGACCCAAGTCCTATATTAAAATAAAATGAGGATGATGCGATGTGTCGTGACTG

**********

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2 GTCGGGATAGCTCAGCTG

3 GTCGGGATAGCTCAGCTG

4 GTCGGGATAGCTCAGC--

5 GTCGGGATAGCTCAGCTG

6 GTCGGGATAGCTCAGCTG

7 GTCGGGATA---------

8 GTCGGGATAGCTCAGCTG

9 GTCGGGATAGCTCAGCTG

10 GTCGGGATAGCTCAGCTG

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# APPENDIX B: FRAGMENT ANALYSIS CHROMATOGRAMS OF THE UDP96003 SSR MARKER LOCUS

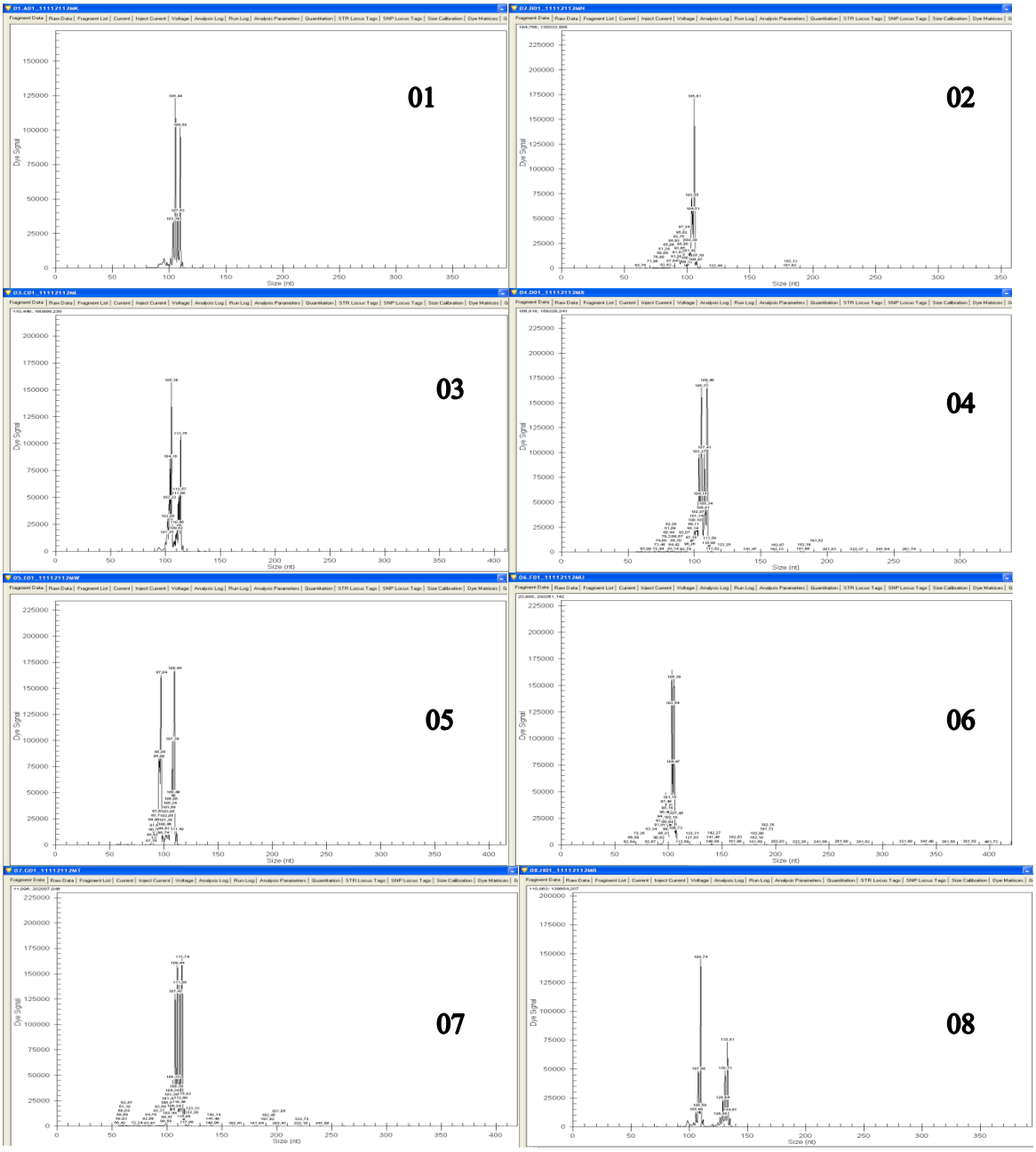


Figure B.1. Fragment analysis graphs of UDP96003 locus for samples 1-8.

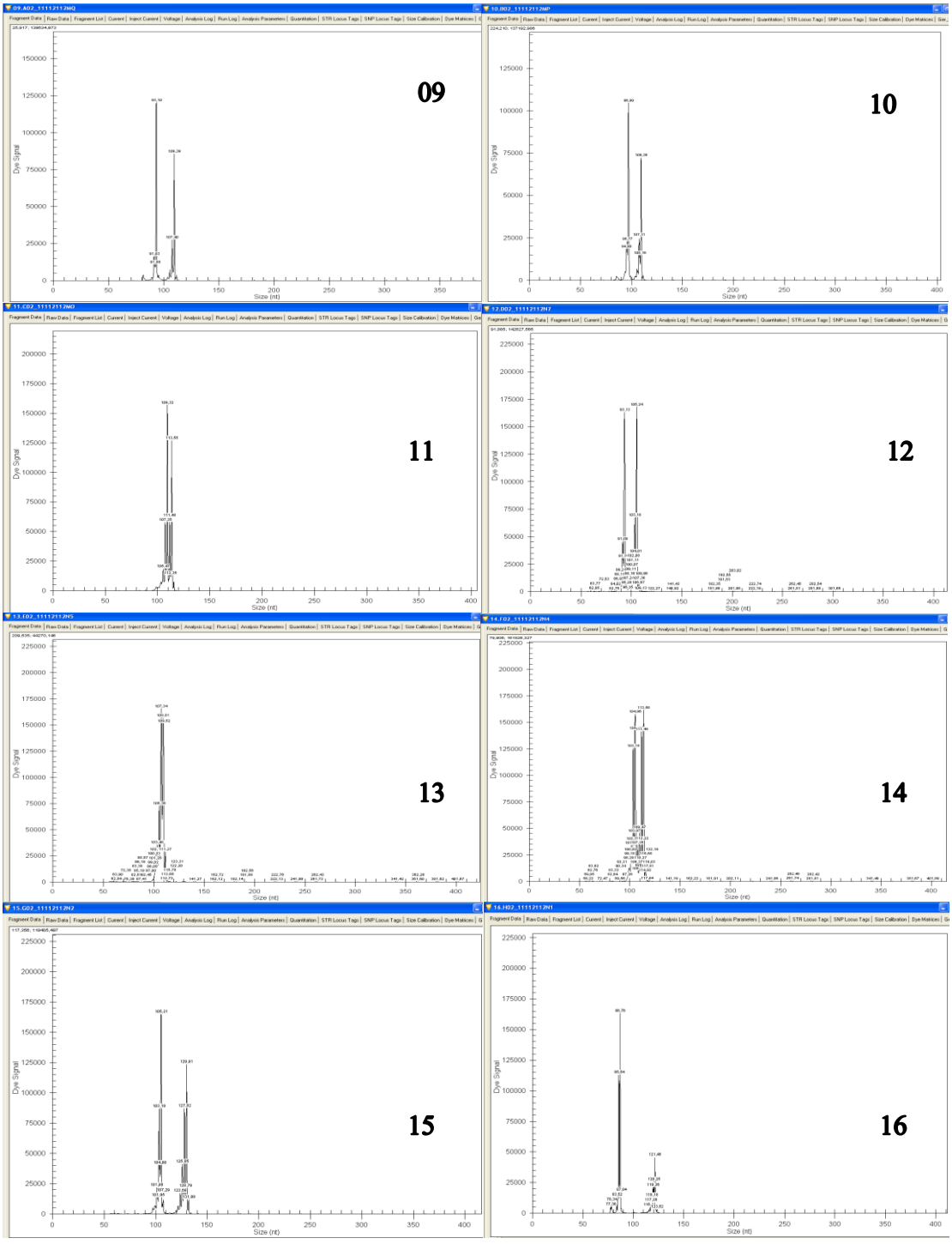


Figure B.2. Fragment analysis graphs of UDP96003 locus for samples 9-16.

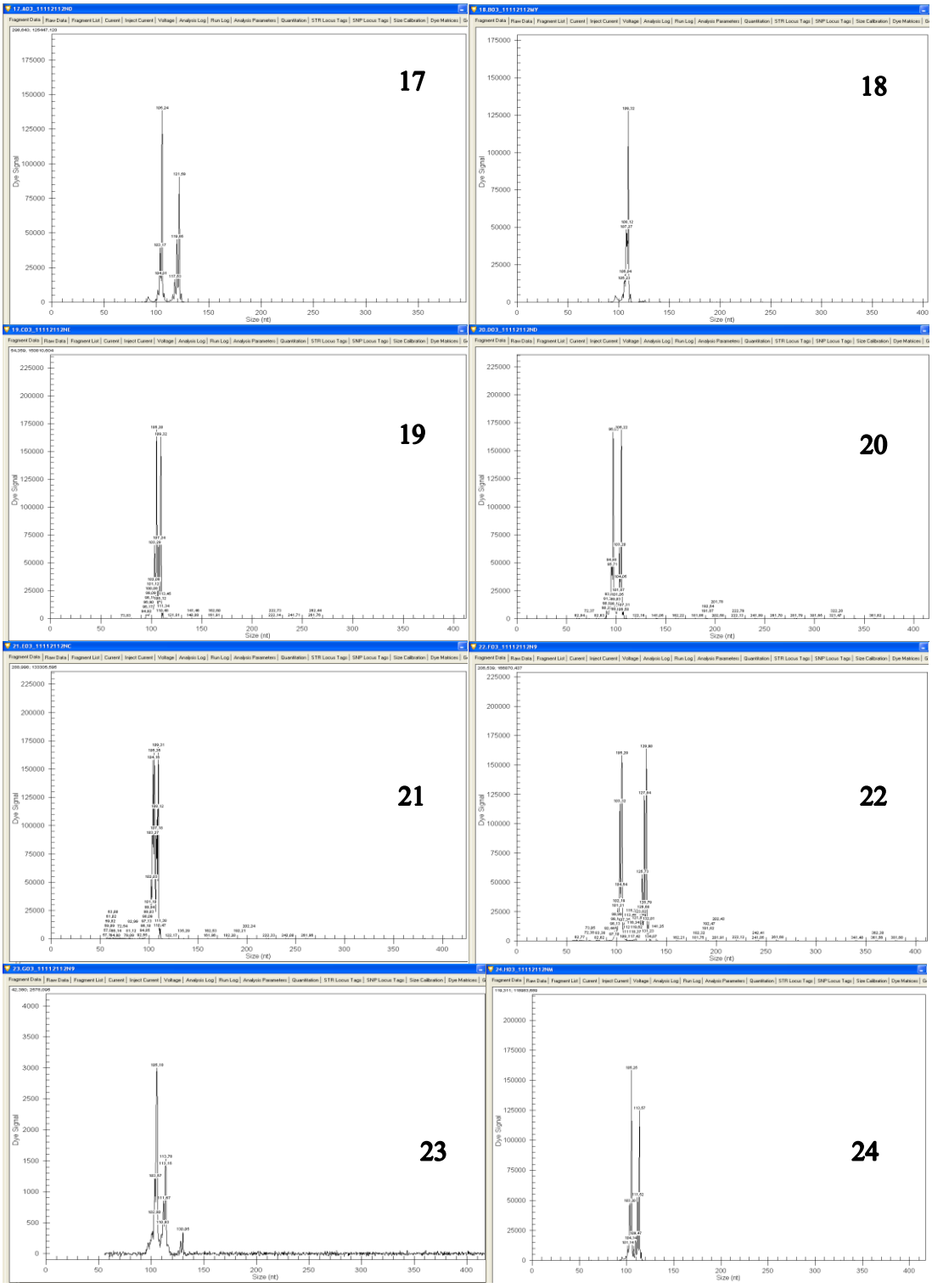


Figure B.3. Fragment analysis graphs of UDP96003 locus for samples 17-24.

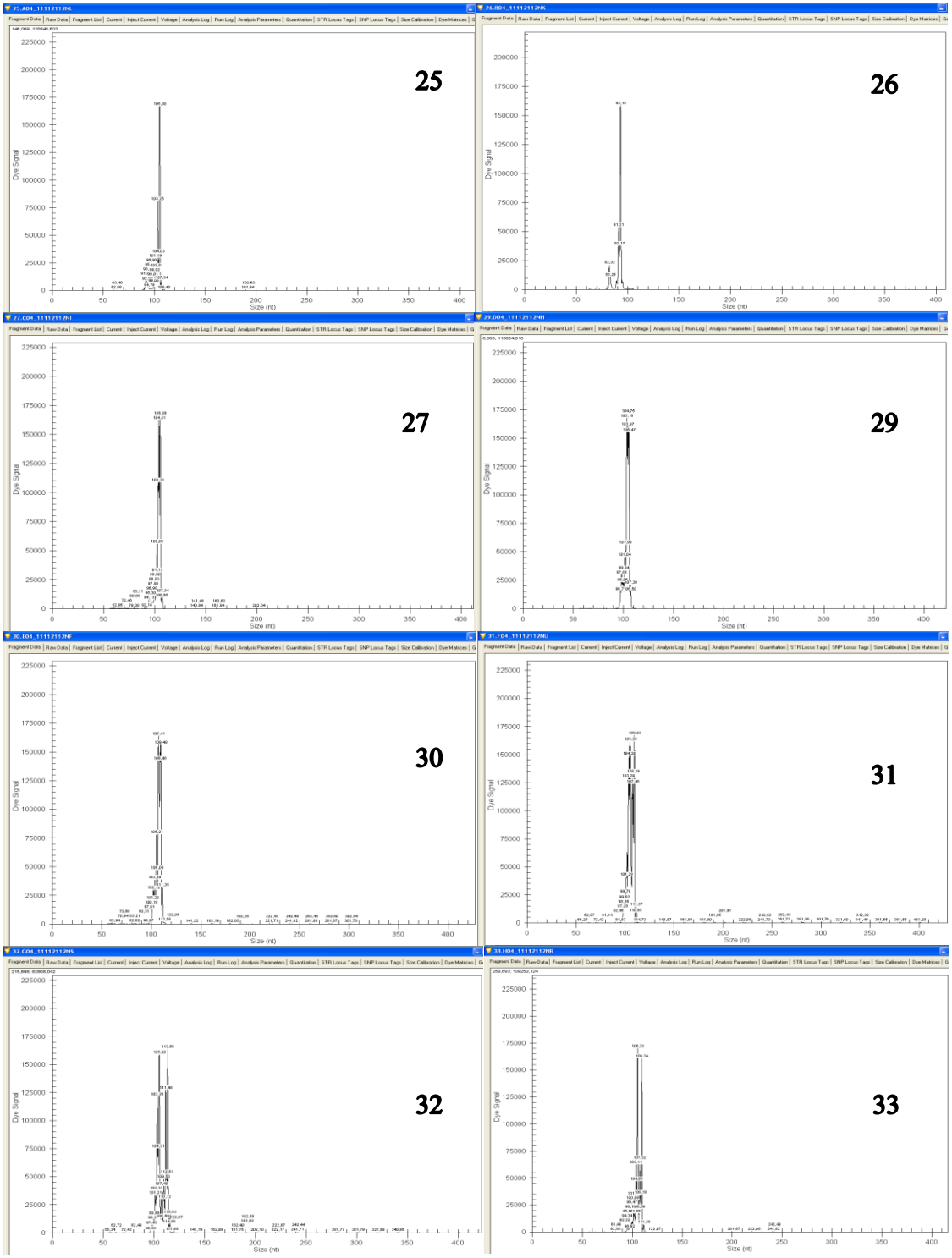


Figure B.4. Fragment analysis graphs of UDP96003 locus for samples 25-27, 29-33.

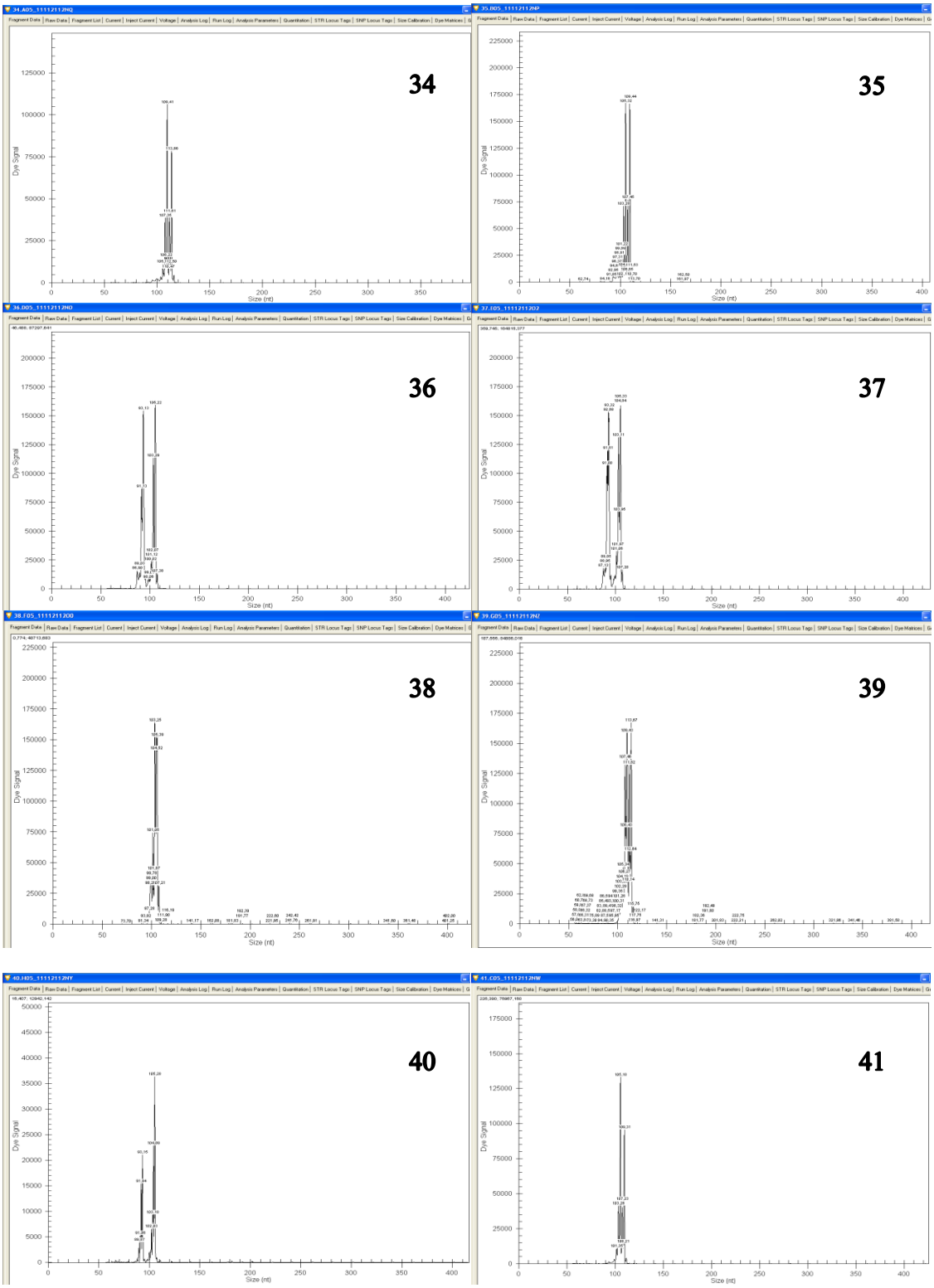


Figure B.5. Fragment analysis graphs of UDP96003 locus for samples 34-41.

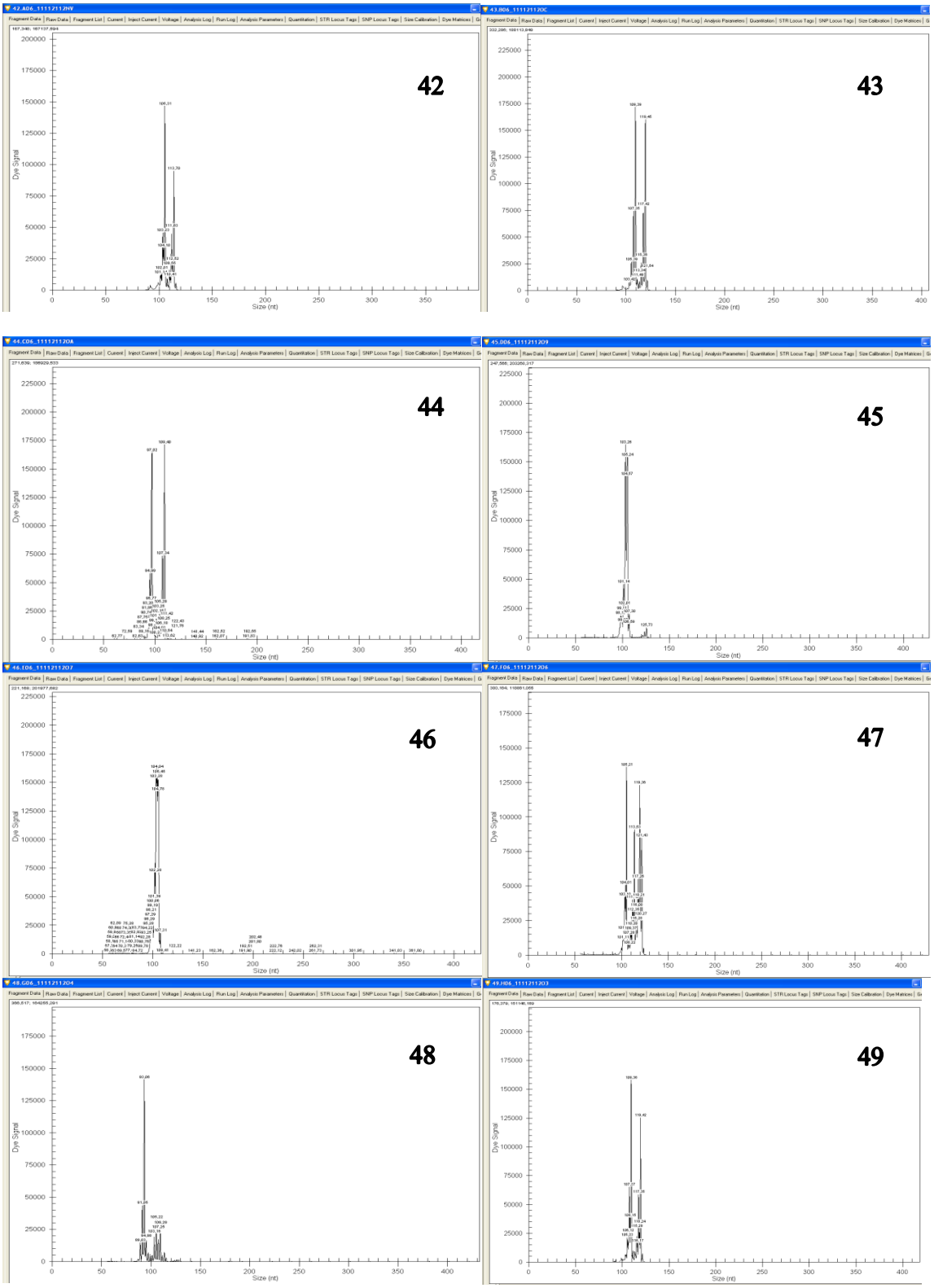


Figure B.6. Fragment analysis graphs of UDP96003 locus for samples 42-49.

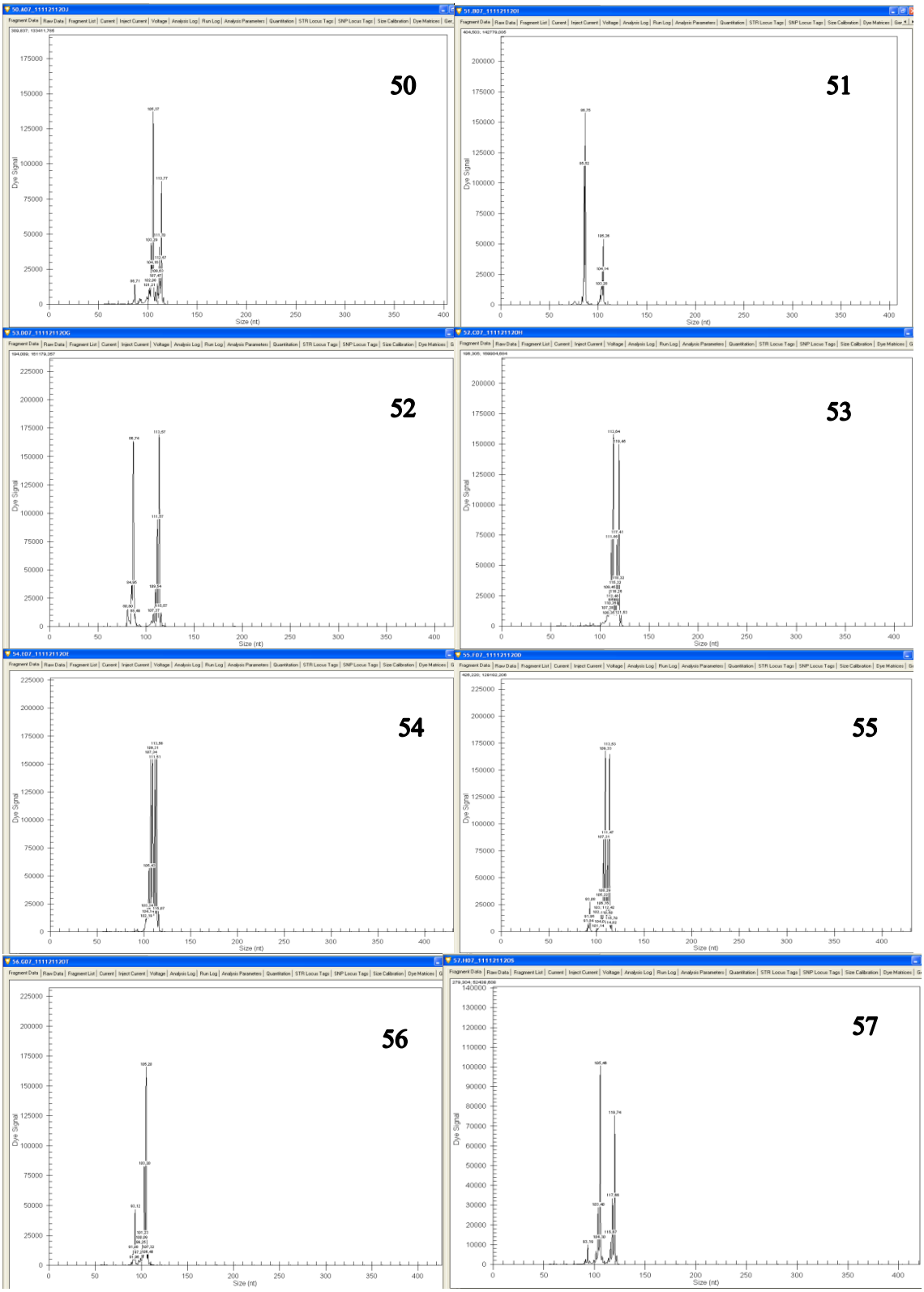


Figure B.7. Fragment analysis graphs of UDP96003 locus for samples 50-57.

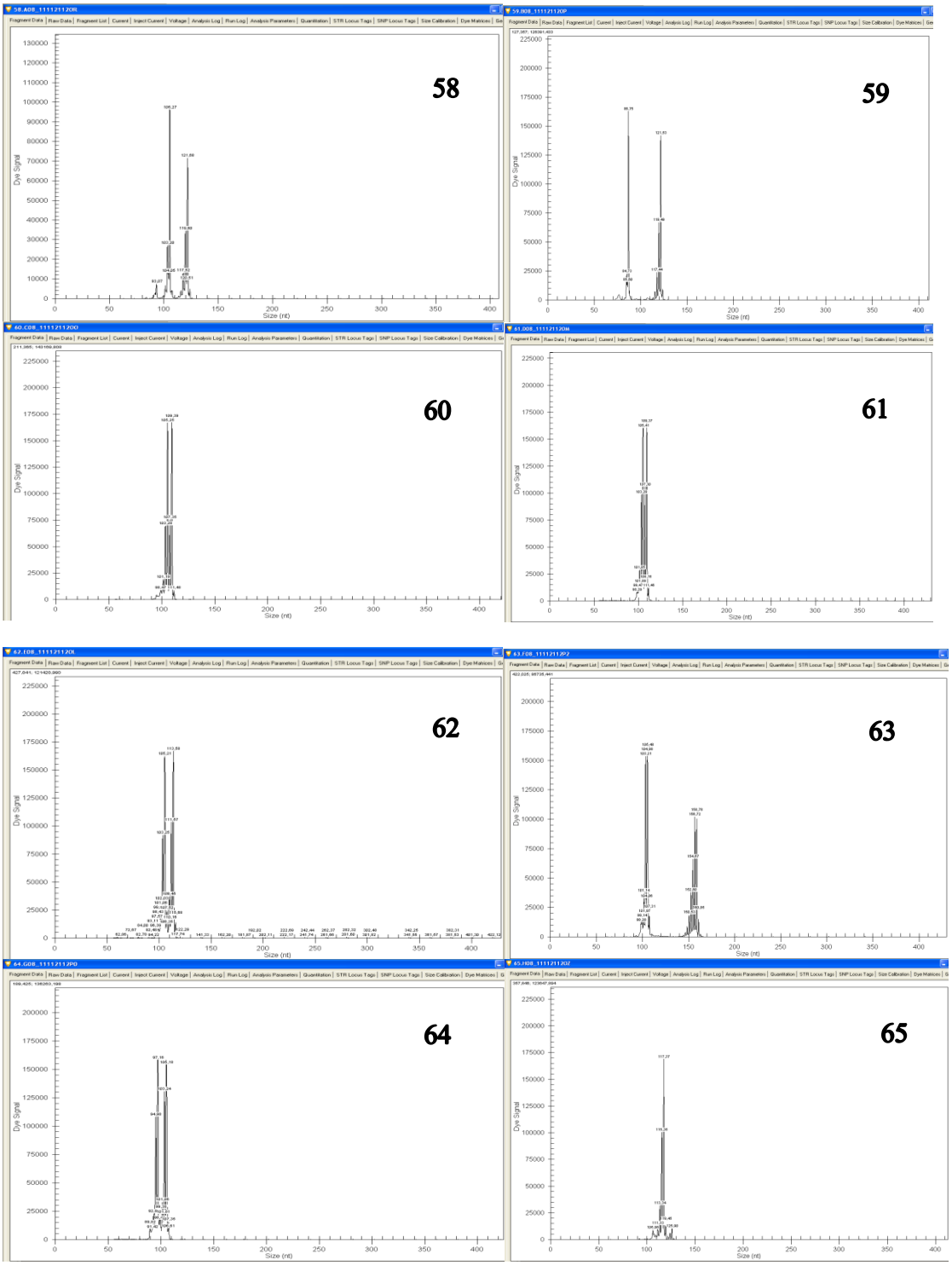


Figure B.8. Fragment analysis graphs of UDP96003 locus for samples 58-65.

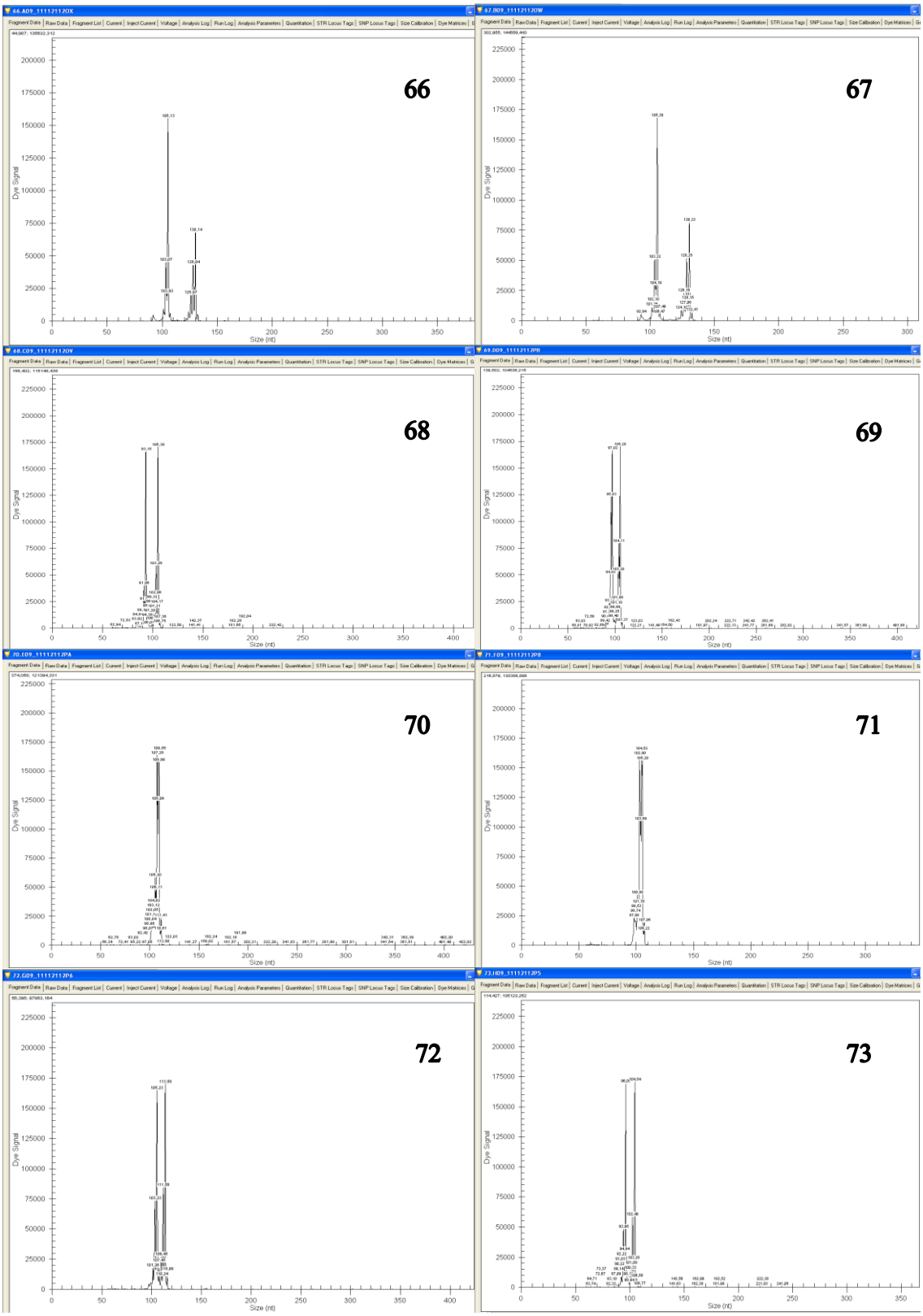


Figure B.9. Fragment analysis graphs of UDP96003 locus for samples 66-73.

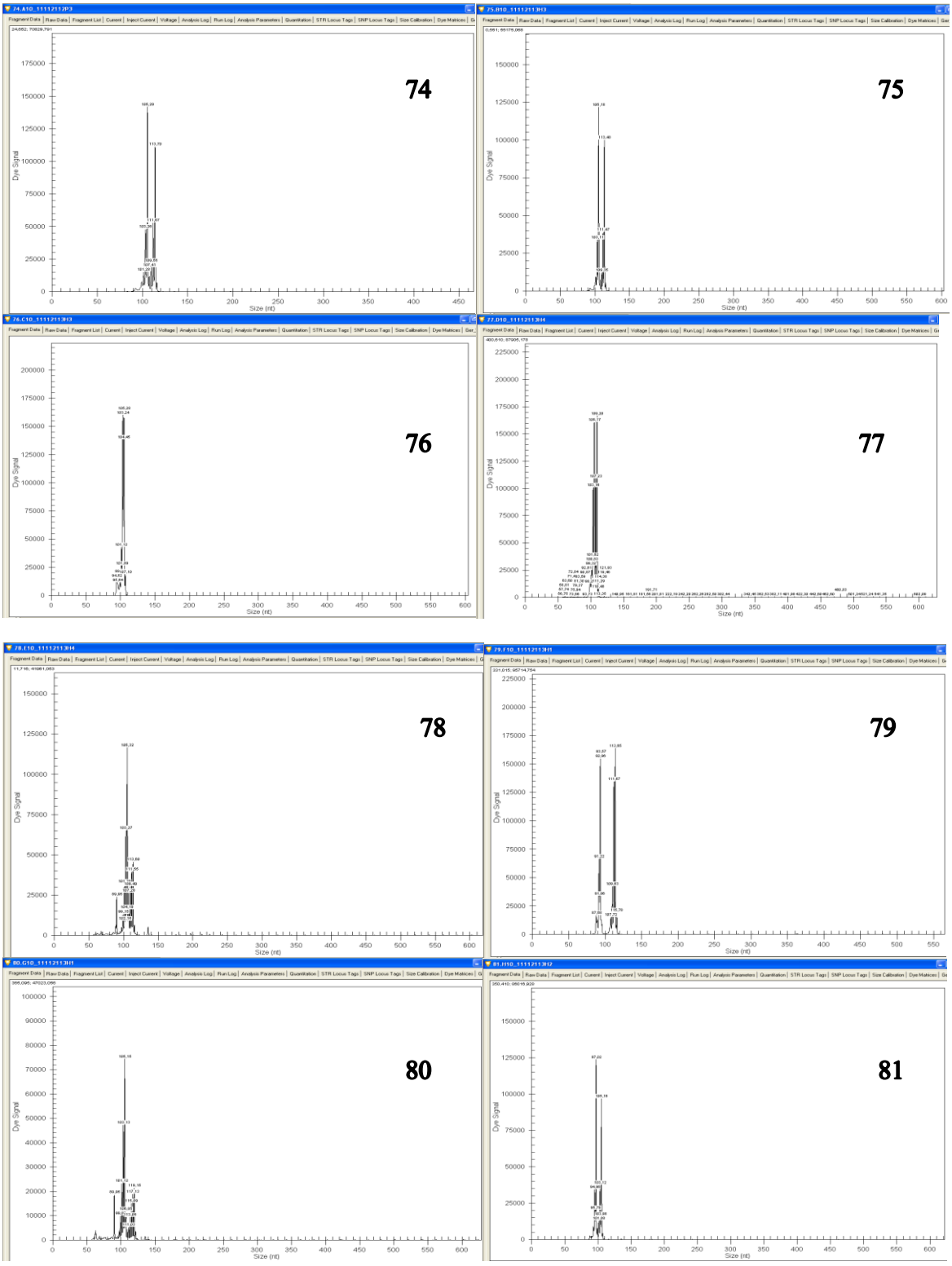


Figure B.10. Fragment analysis graphs of UDP96003 locus for samples 74-81.

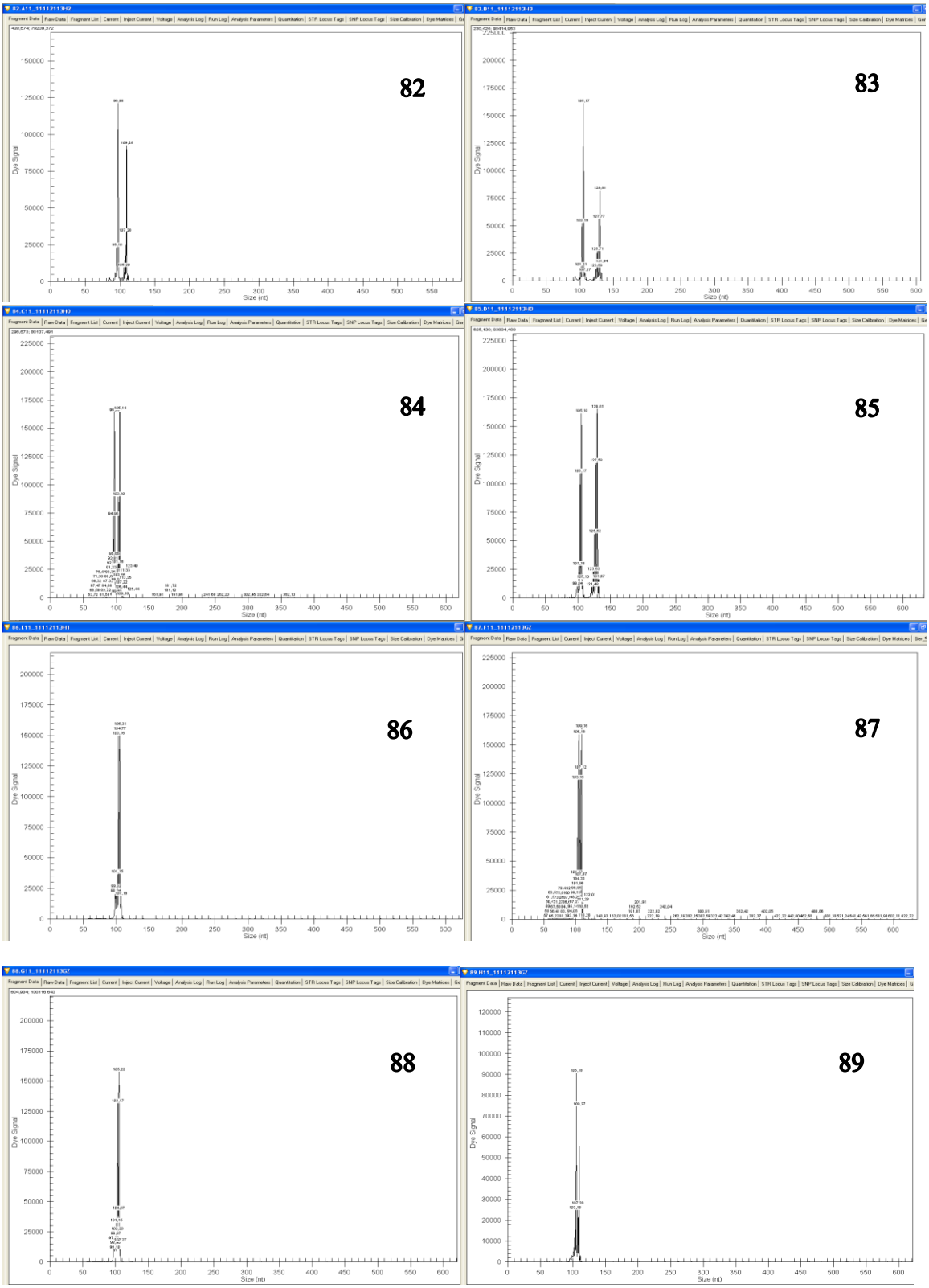


Figure B.11. Fragment analysis graphs of UDP96003 locus for samples 82-89.

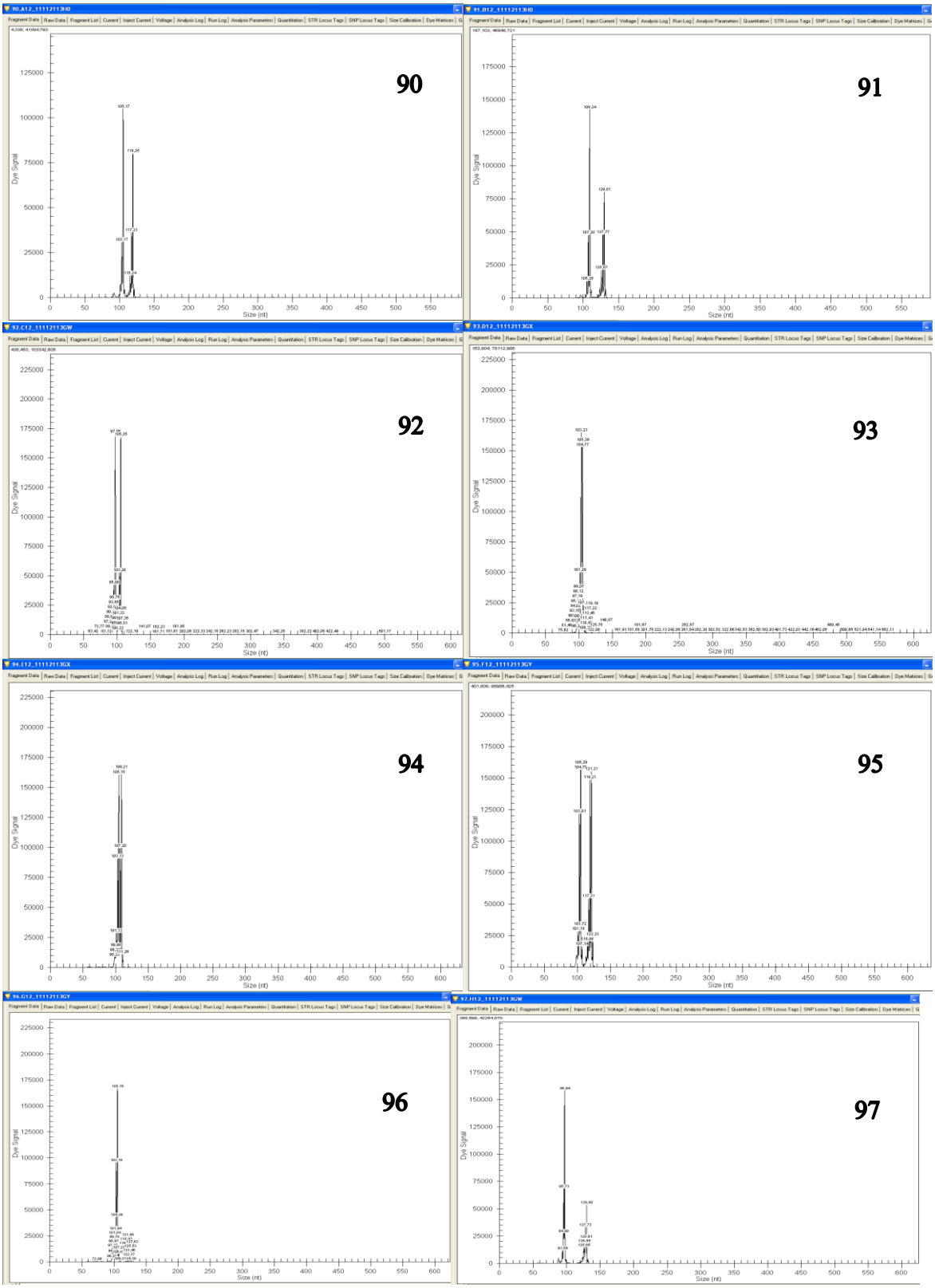


Figure B.12. Fragment analysis graphs of UDP96003 locus for samples 90-97.

# APPENDIX C: FRAGMENT ANALYSIS CHROMATOGRAMS OF THE UDP96005 SSR MARKER LOCUS

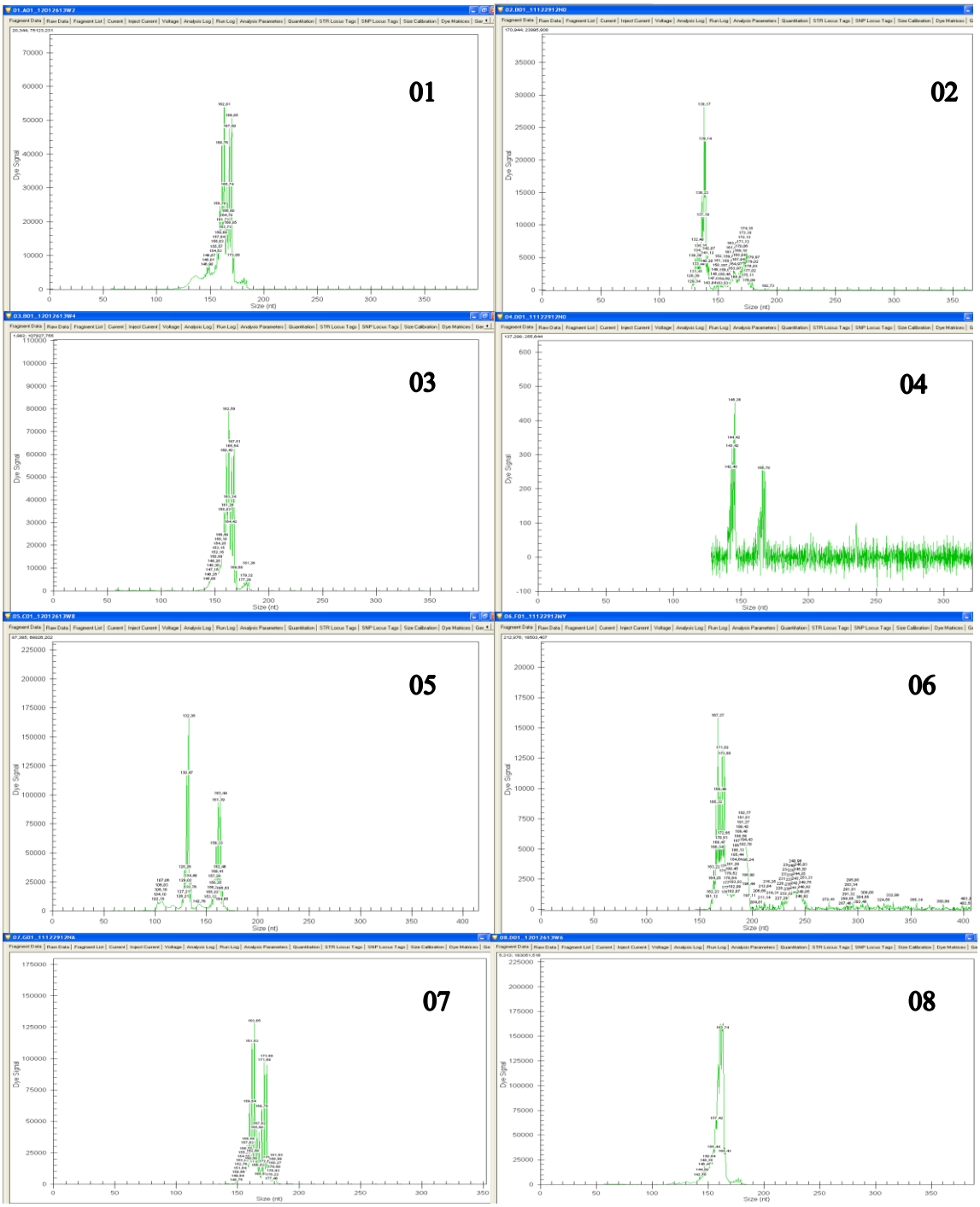


Figure C.1. Fragment analysis graphs of UDP96005 locus for samples 1-8.

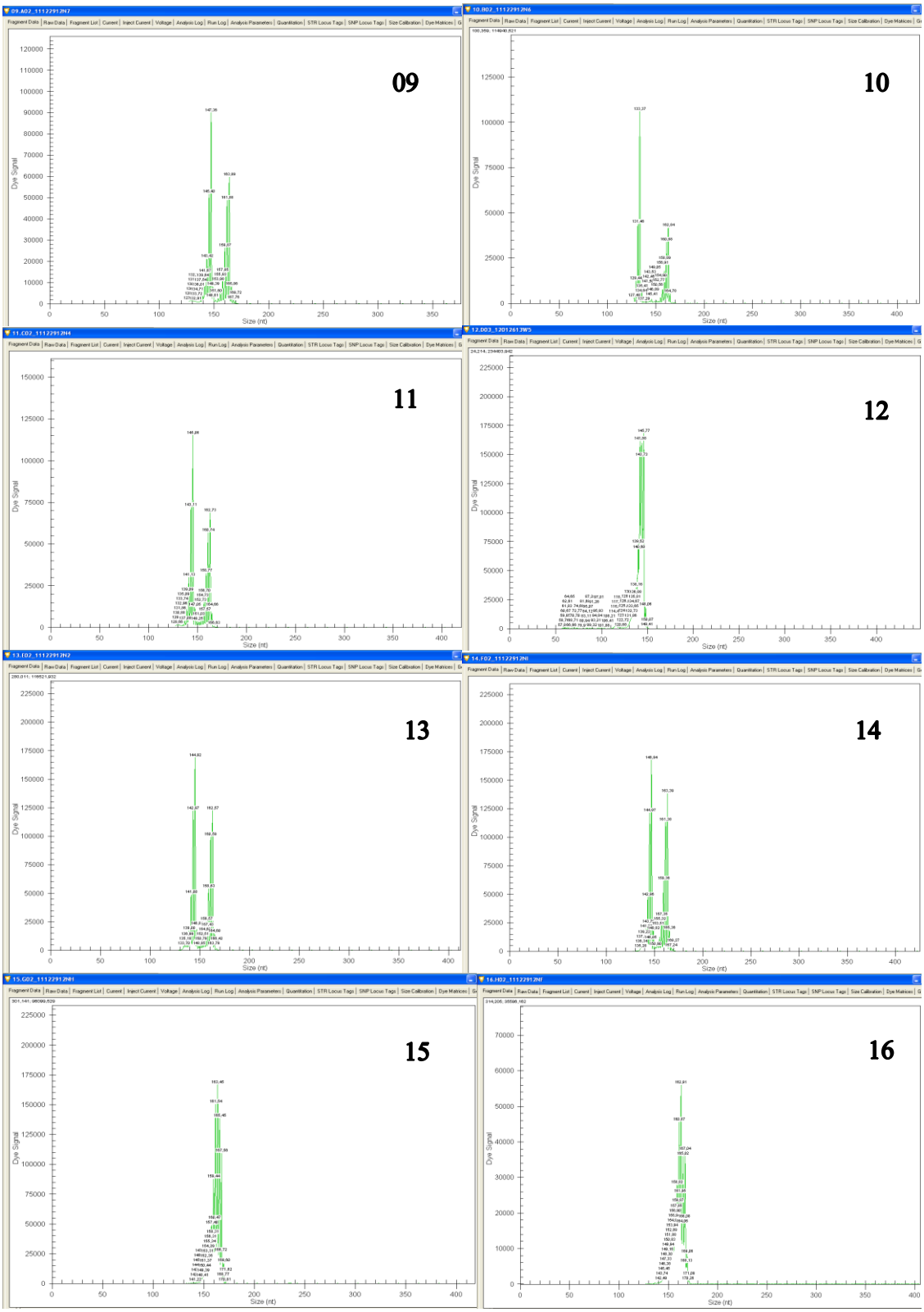


Figure C.2. Fragment analysis graphs of UDP96005 locus for samples 9-16.

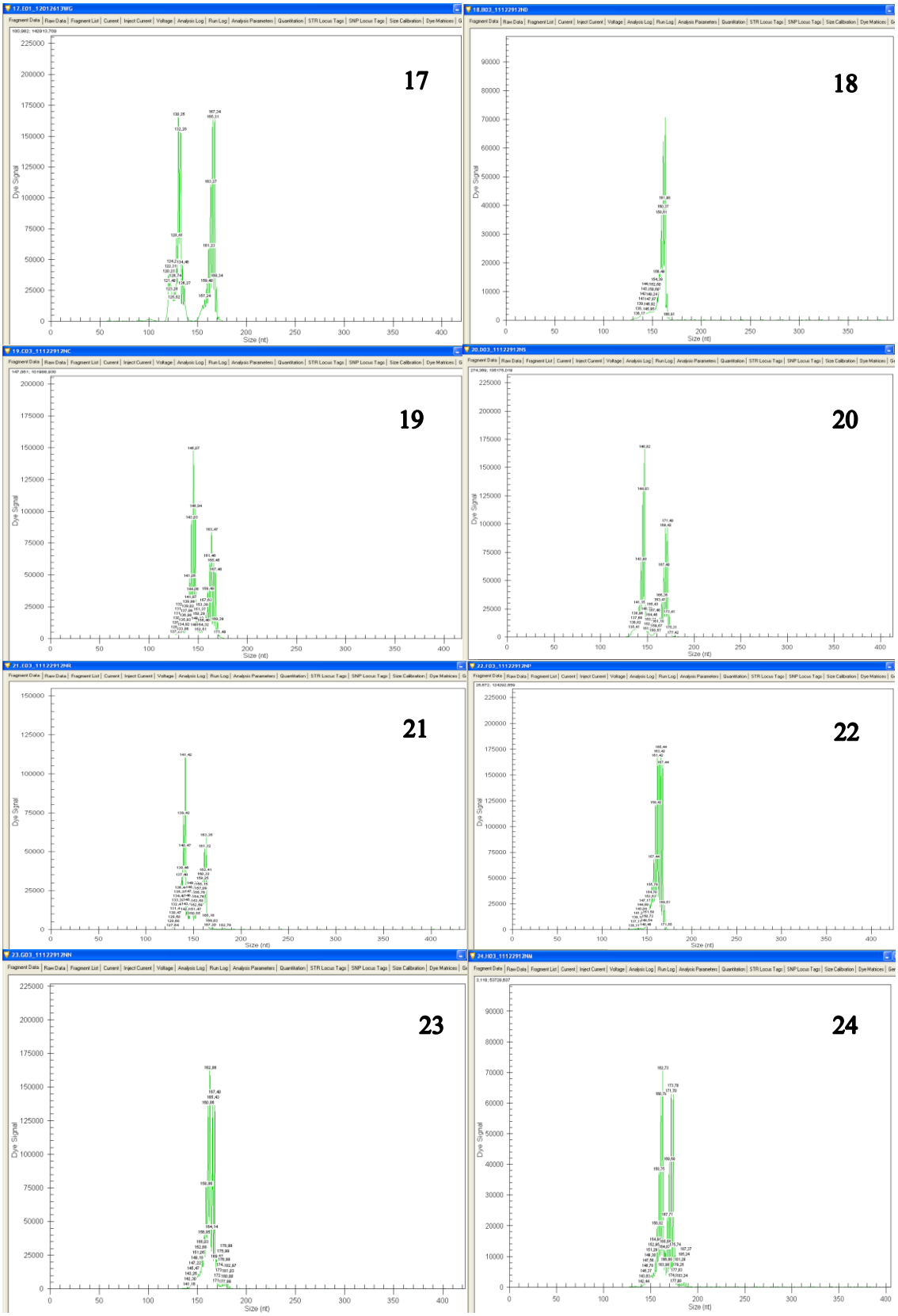


Figure C.3. Fragment analysis graphs of UDP96005 locus for samples 17-24.

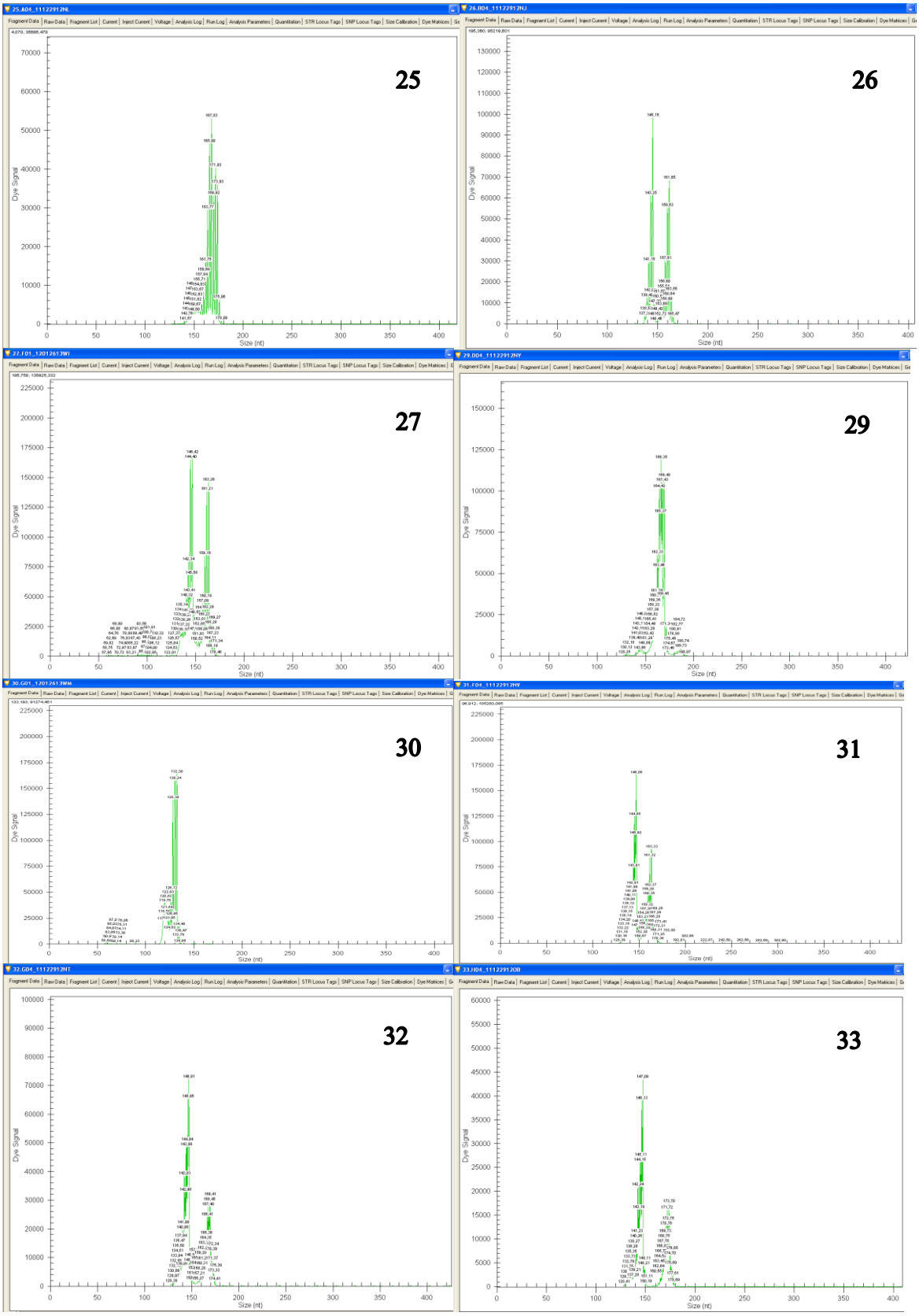


Figure C.4. Fragment analysis graphs of UDP96005 locus for samples 25-27, 29-33.

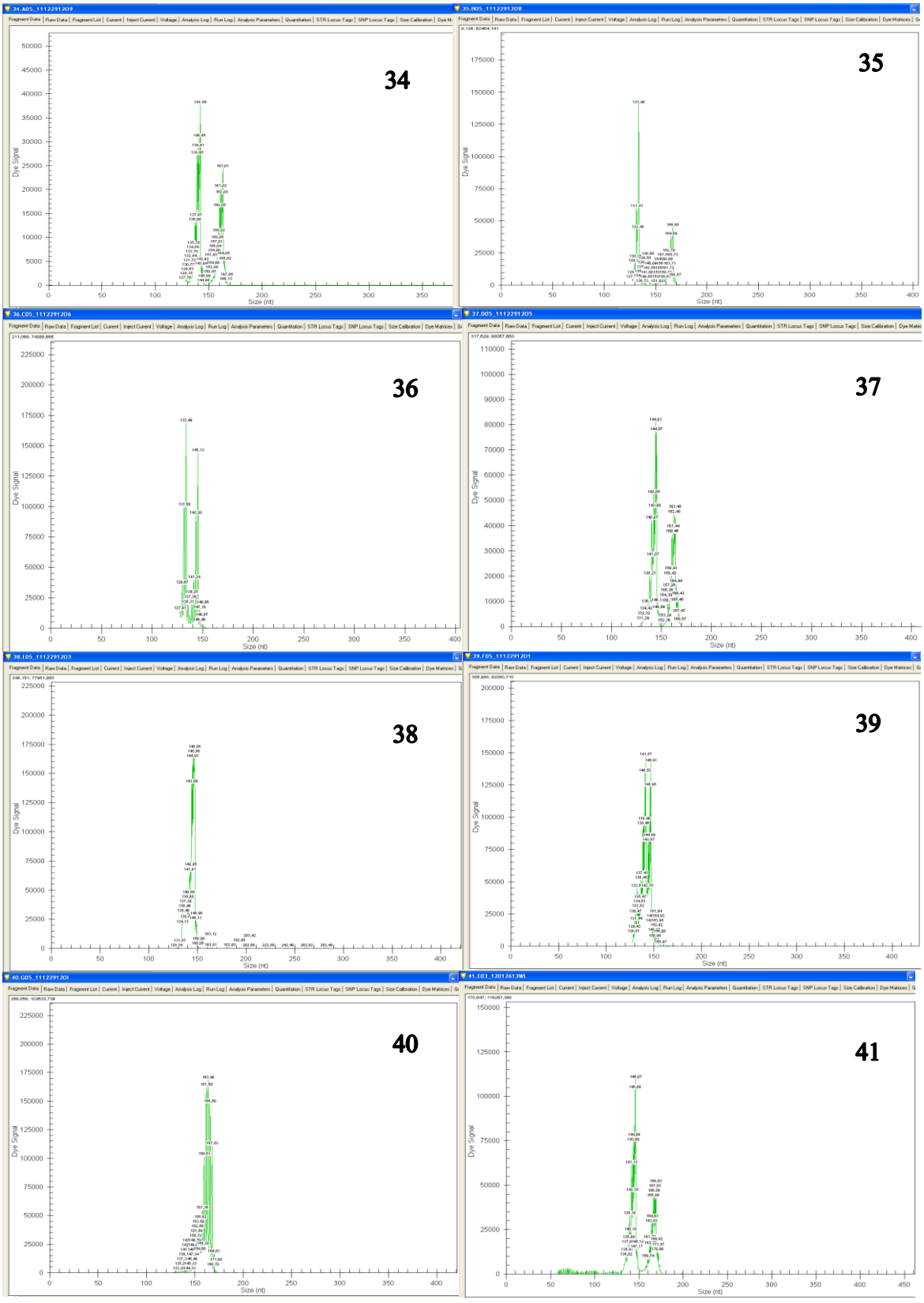


Figure C.5. Fragment analysis graphs of UDP96005 locus for samples 34-41.

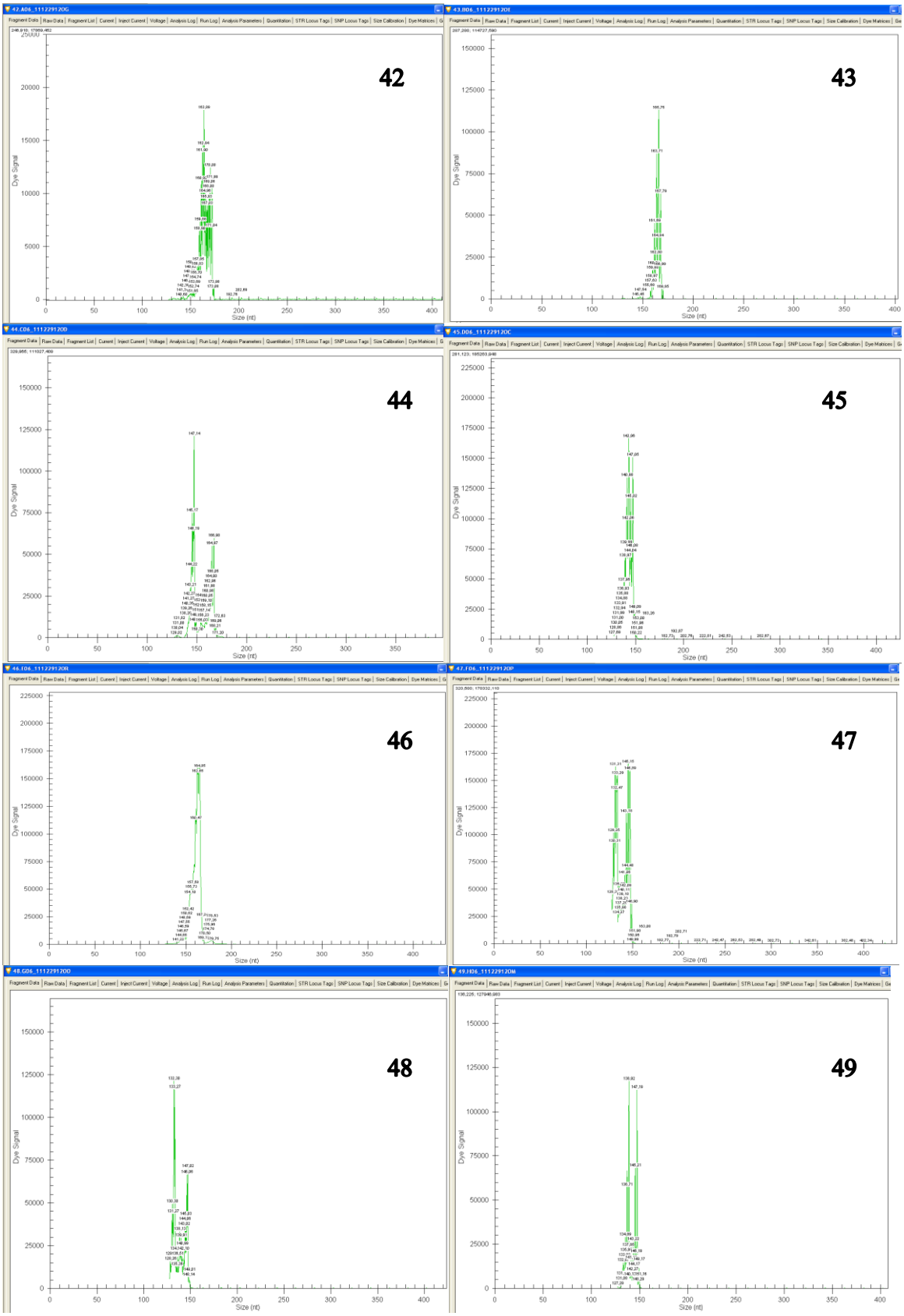


Figure C.6. Fragment analysis graphs of UDP96005 locus for samples 42-49.

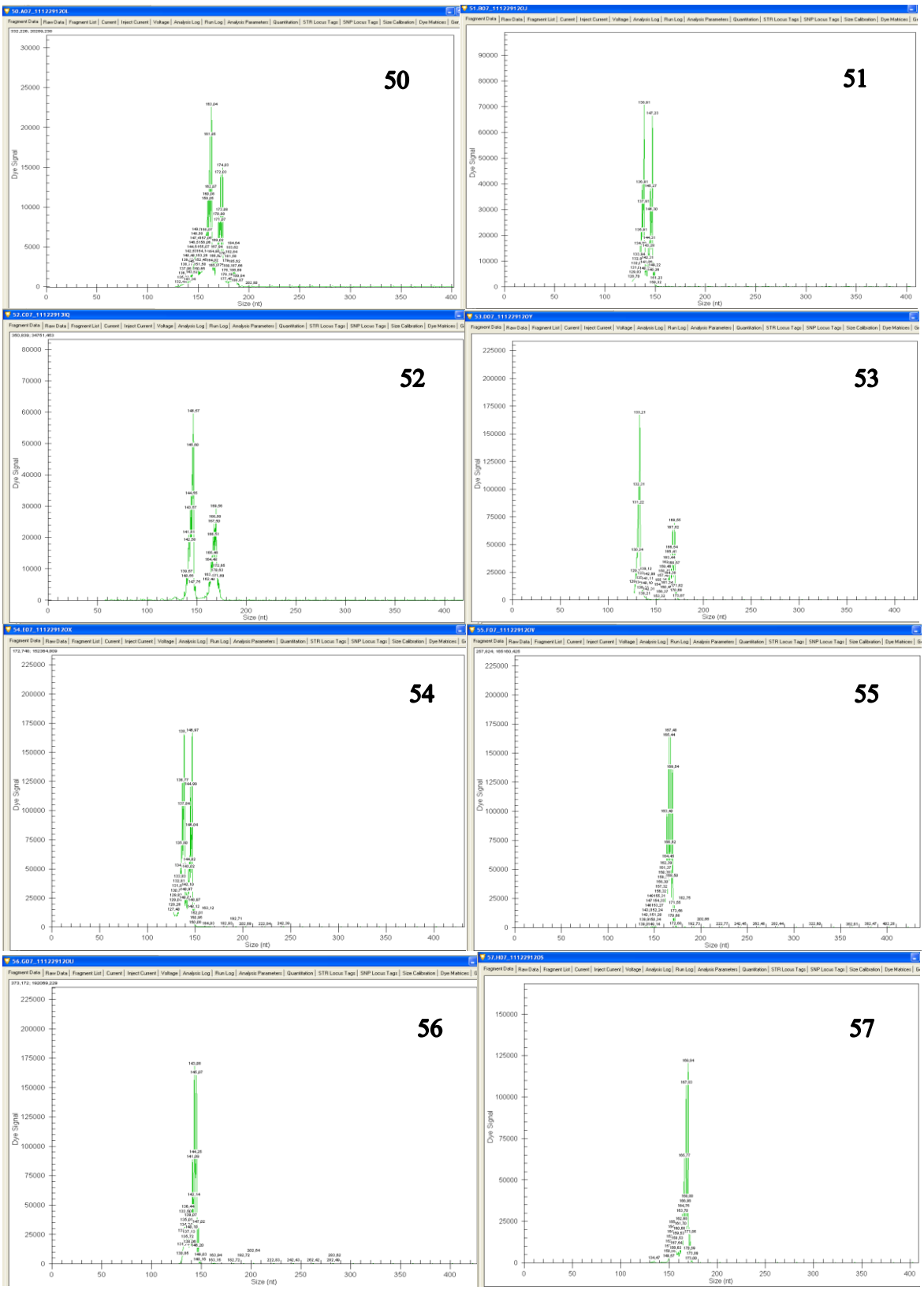


Figure C.7. Fragment analysis graphs of UDP96005 locus for samples 50-57.

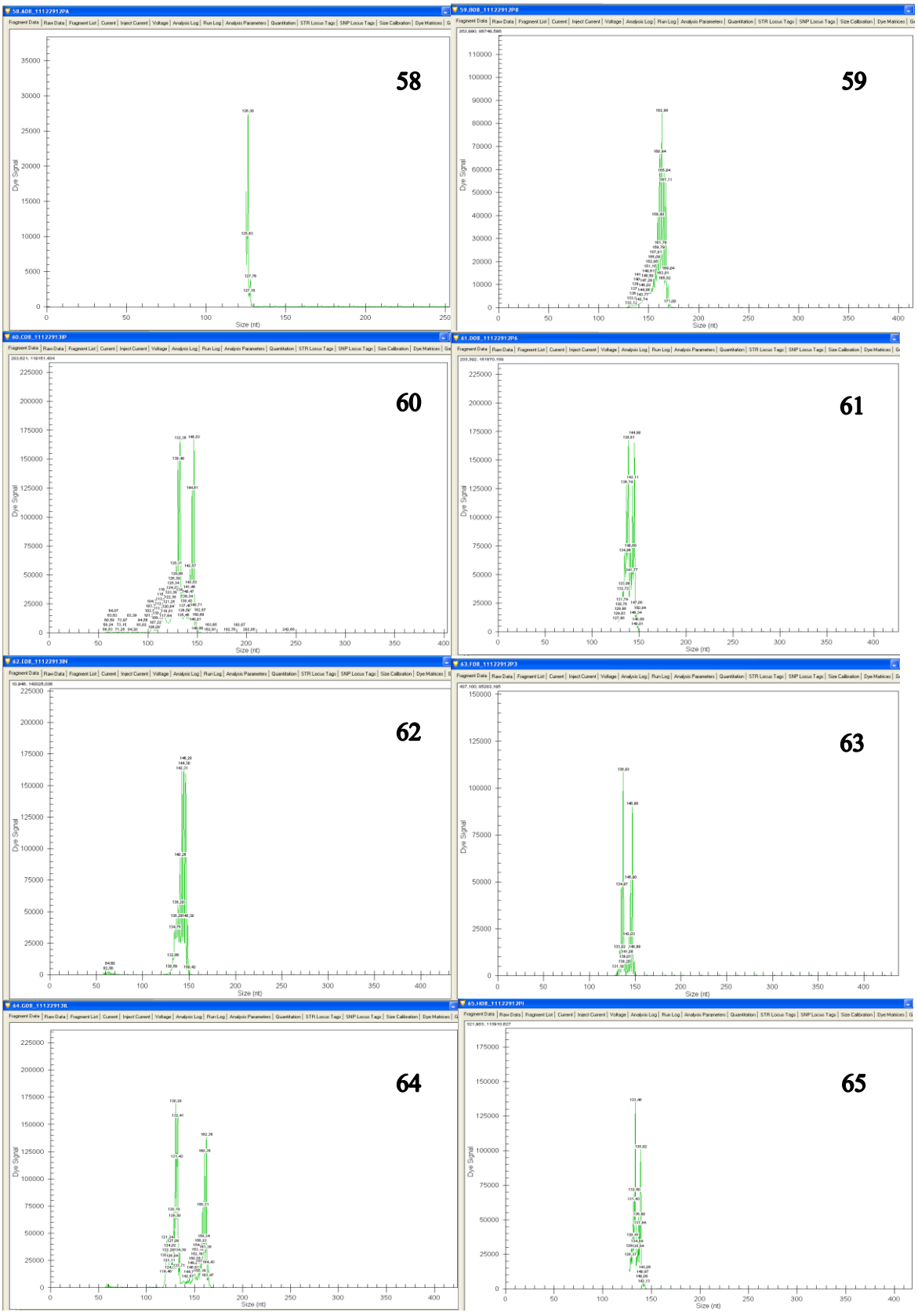


Figure C.8. Fragment analysis graphs of UDP96005 locus for samples 58-65.

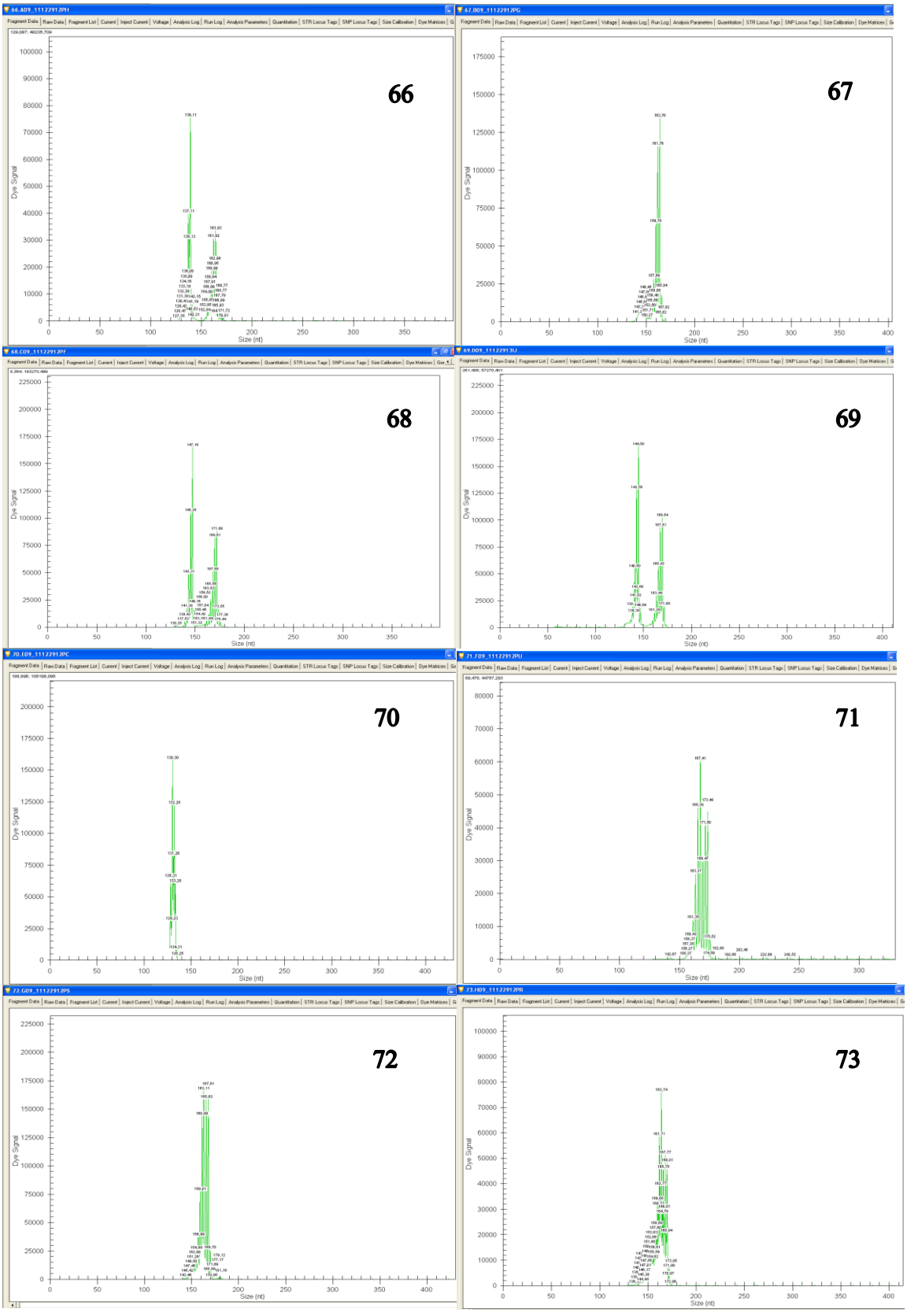


Figure C.9. Fragment analysis graphs of UDP96005 locus for samples 66-73.

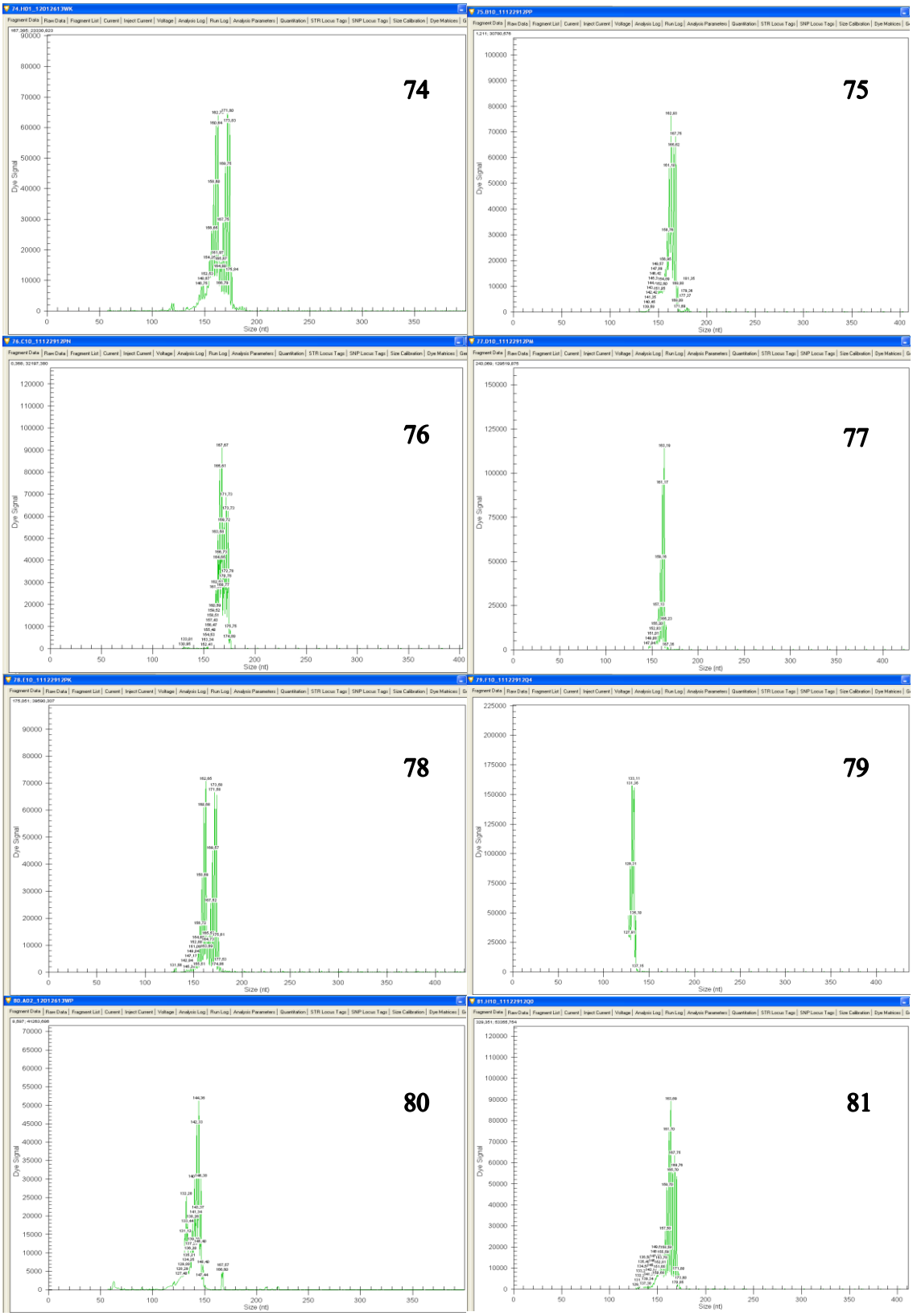


Figure C.10. Fragment analysis graphs of UDP96005 locus for samples 74-81.

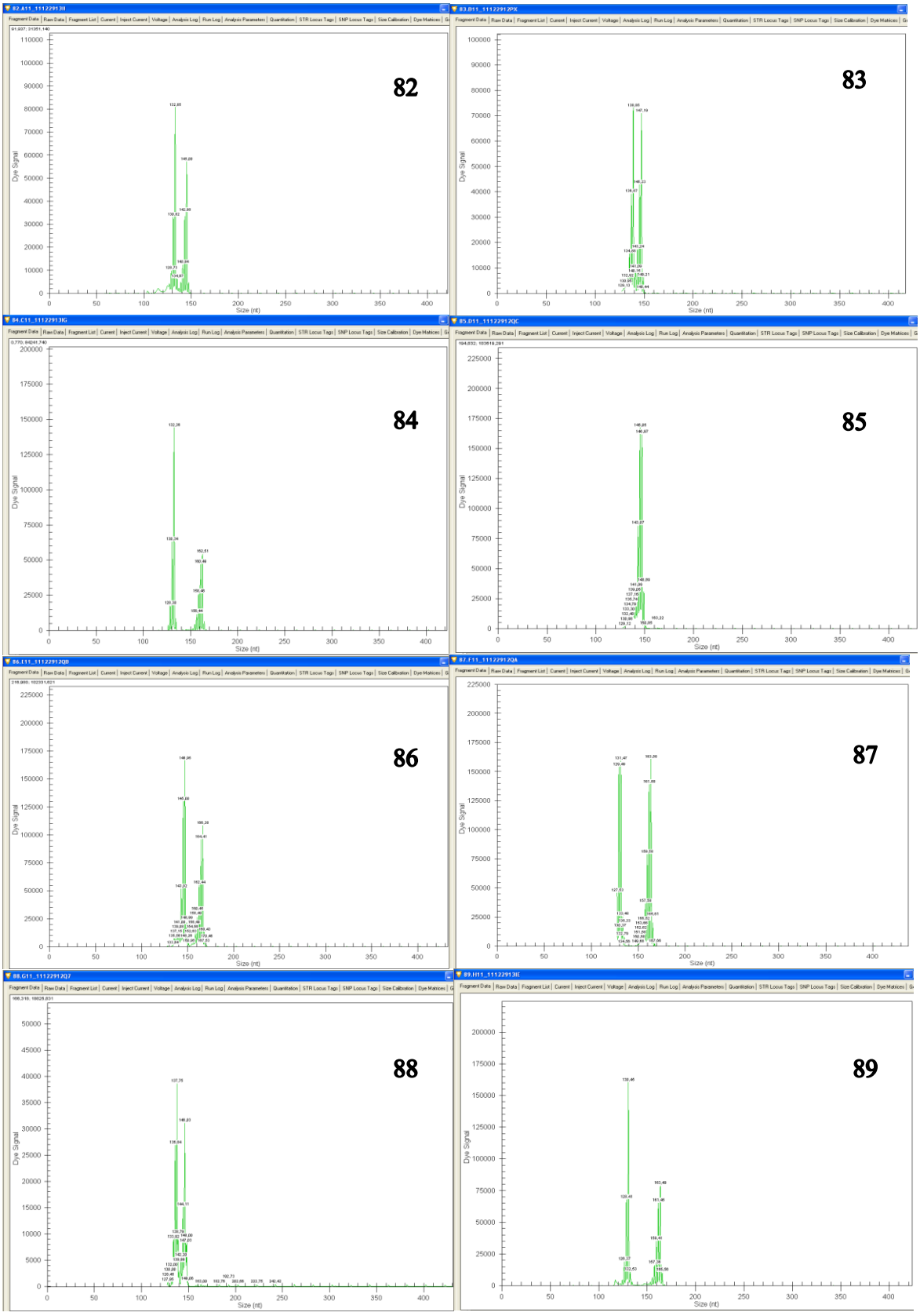


Figure C.11. Fragment analysis graphs of UDP96005 locus for samples 82-89.

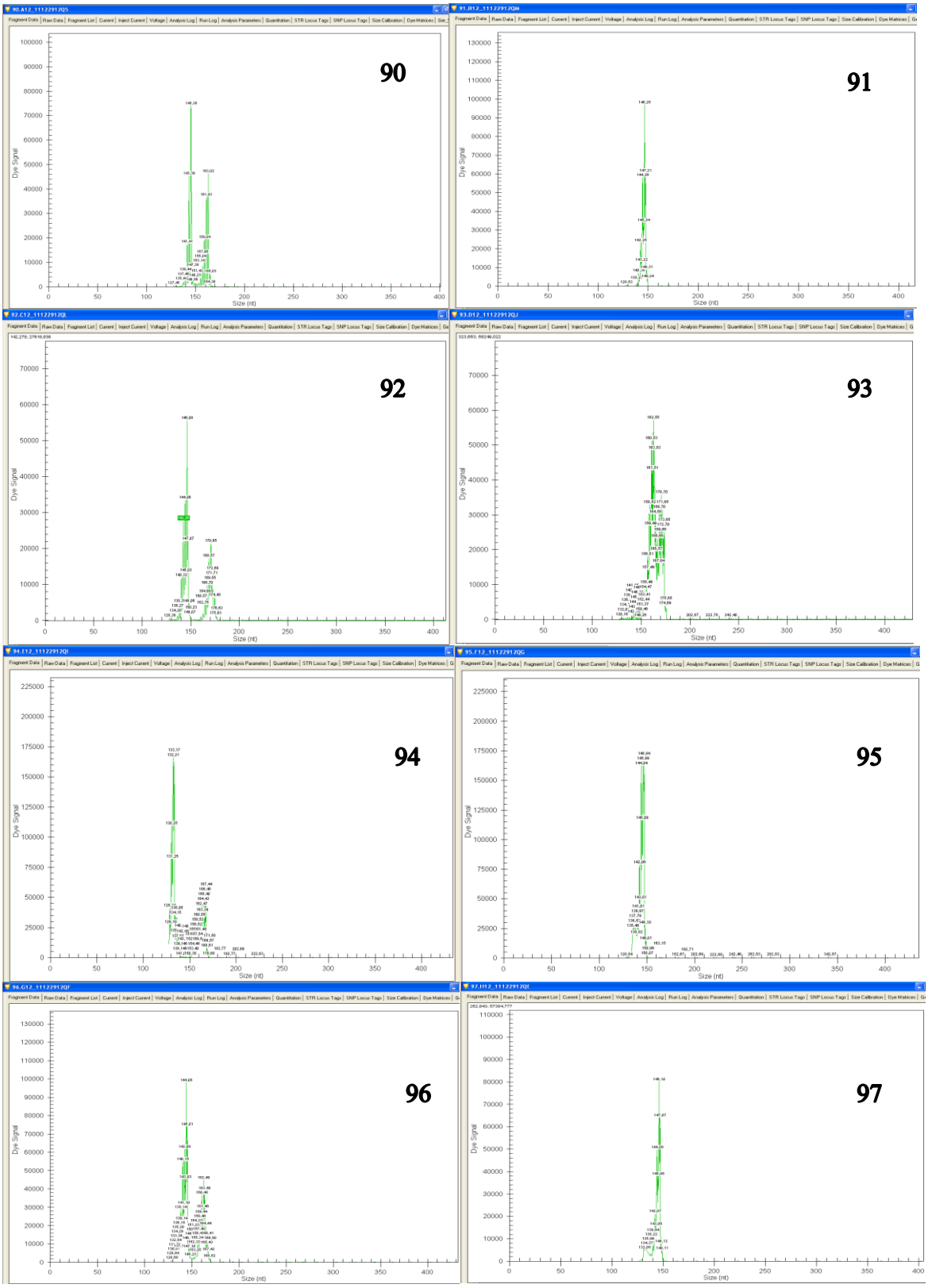


Figure C.12. Fragment analysis graphs of UDP96005 locus for samples 90-97.

# APPENDIX D: THUMBNAILS OF FRAGMENT ANALYSIS CHROMATOGRAMS OF UDP98409 SSR MARKER LOCUS

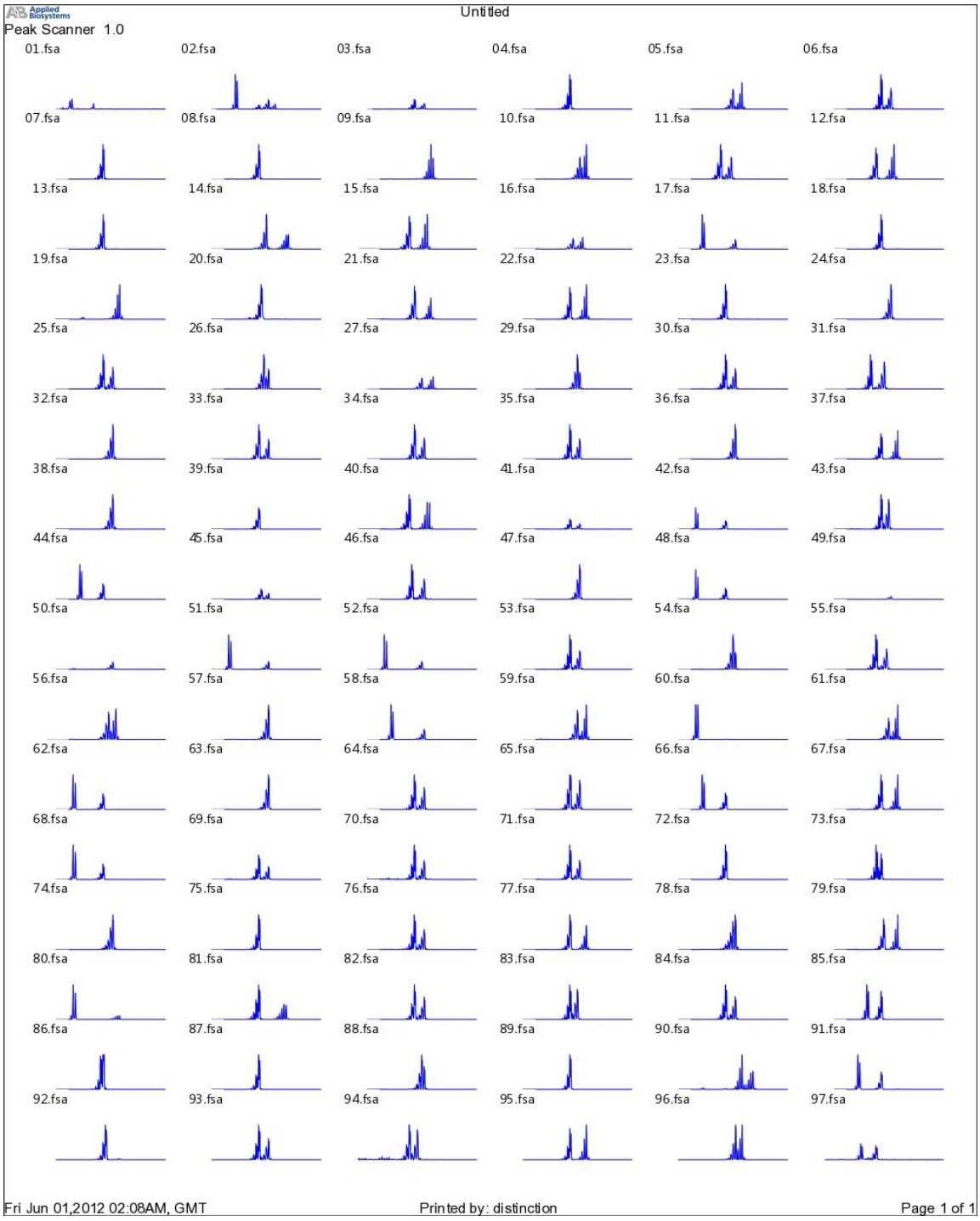


Figure D.1. Fragment analysis thumbnails of UDP98409 locus for all samples.

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