MOLECULAR CHARACTERIZATION OF *BLUMERIA GRAMINIS* F. SP. *HORDEI* USING AFLP MARKERS

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MOLECULAR CHARACTERIZATION OF *BLUMERIA GRAMINIS* F. SP. *HORDEI* USING AFLP MARKERS

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ABSTRACT

MOLECULAR CHARACTERIZATION OF *BLUMERIA GRAMINIS* F. SP. *HORDEI* USING AFLP MARKERS

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Blumeria graiminis f. sp. hordei (powdery mildew) is an obligate biotroph infecting *hordeum vulgare* (barley). It is one of the most devastating pathogens of barley, decreasing barley yield in great extent. In order to decrease barley loss, numerous studies are being conducted for overcoming the disease from the sides of both pathogen and host. However the pathogen is evolving very rapidly preventing the effective use of pesticides such as fungisides or development of resistant barley varieties by crossing race-specific resistance varieties, varieties having R genes, with susceptible but high yield producing varieties. In order to understand the mechanism of pathogen-host interactions, and producing enduring solutions for the problem of yield loss in barley molecular tools need to be used.

In this thesis study, Amplified Fragment Length Polymorphism (AFLP) molecular marker method is used in order to reveal the molecular characterization of Turkish *Blumeria graminis* f. sp. *hordei* varieties collected from Çukurova region in Turkey. Thirty-nine samples were analyzed with eigth universal races, of which virulence genes are studied. AFLP studies were conducted on LI-COR 4300 DNA Analyzer system. Bioinformatics analysis was performed with NTSYS program. By the help

of this Numerical Taxonomic System, similarity, dissimilarity, clustering, dendograms, two-dimensional scatter plots, and three-dimensional perspective plots were obtained. By the light of these analyses Turkish *Blumeria graminis* f. sp. *hordei* varieties together with universal races are grouped into three clusteres.

In conclusion, studying Turkish *Blumeria graminis* f. sp. *hordei* isolates and comparing them with universal races is a unique study in terms of characterizing the Turkish *Bgh* isolates for the first time, and can be used as a frontier study for studying Resistance genes, by reverse genetic tools.

Key Words: powdery mildew, *Blumeria graminis* f.sp. *hordei*, Amplified Fragment Length Polymorphism, AFLP, molecular marker, genetic relationship, molecular characterization.

ÖZ

ARPA *BLUMERİA GRAMİNİS* F. SP. *HORDEİ* İZOLATLARININ AFLP MARKÖRLERİ KULLANILARAK MOLEKÜLER KARAKTERİZASYONU

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Külleme *(Blumeria graminis* f. sp. *hordei)* arpada *(Hordeum vulgare)* hastalığa sebep olan bir zorunlu parazittir. Külleme arpadan elde edilen verimi çok büyük ölçüde azaltan, arpayı en tahrip edici patojenlerdendir. Arpadaki kaybı azaltabilmek ve hastalığı yenebilmek için, gerek hastalığı gerekse konağı inceleyen pek çok çalışma yapılmaktadır. Ancak patojenin çok hızlı evrim geçirebilme özelliği, mantar öldürücü gibi tarım ilaçlarının işlevsel kullanımı ya da ırka-özgü direnç geni taşıyan varyetelerle, duyarlı ancak yüksek verimli varyetelerin çaprazlaması ile dayanıklı ve yüksek verimli ırk elde edilmesi gibi faktörleri etkisiz hale getirmektedir. Patojen ile konağın arasındaki ilişkinin çalışma mekanizmasını anlayabilmek ve arpadaki verim kaybına uzun soluklu çözümler üretebilmek için moleküler yöntemler kullanılmalıdır.

Bu çalışmada, Türkiye'de Çukurova Bölgesi'nden toplanan Türk *Blumeria graminis* f. sp. *hordei* varyeteleri, moleküler karakterizasyonu yapılmak üzere Çoğaltılmış Parça Uzunluk Polimorfizmi (ÇPUP) moleküler markör yöntemi ile incelenmiştir. Otuzsekiz örnek, sekiz virülens genleri önceden çalışılmış, evrensel ırklar ile analiz edilmiştir. ÇPUP çalışmaları LI-COR 4300 DNA Analizör cihazı ile yapılmıştır.

Biyoinformatik çalışmalar NTSYS programı ile yapılmıştır. Bu Sayısal Taksonomi Sistemi'nin yardımı ile benzerlik, başkalık, gruplama, dendogram, iki-boyutlu dağılım grafiği, üç-boyutlu perspektif grafiği elde edilmiştir. Bu bilgilerin ışığında Türk *Blumeria graminis* f. sp. *hordei* varyeteleri üç gruba ayrılmıştır.

Sonuç olarak, Türk *Blumeria graminis* f. sp. *hordei* varyetelerini çalışmak ve onları evrensel ırklarla karşılaştırmak, ilk kez Türk külleme varyetelerinin karakterizasyonu ve direnç genlerinin ters genetik yöntemler ile çalışılması için ön çalışma olması bakımından önemlidir.

Anahtar Kelimeler: külleme, *Blumeria graminis* f.sp. *hordei*, Çoğaltılmış Parça Uzunluk Polimofizmi, ÇPUP, moleküler markör, genetik ilişki, moleküler karakterizasyon.

To my family;
supportive, devoted, and sincere…

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LIST OF ABBREVIATIONS

AFLP : Amplified Fragment Length Polymorphism

*Apa*I : *E. coli* strain that carries the *Apa*I gene from *Acetobacter pasteurianus sub. pasteurianus*

ATP : Adenosine Tri-phospahate

Avr : Avirulence

Bgh : *Blumeria graminis hordei*

cm : Centimeter

°C : Celcius degrees

CTAB : Hexadecyltrimethylammonium bromide

d/n : Day/Night

DNA : Deoxyribonucleic acid

dNTP : Deoxy-nucleotidetriphosphate

g : Gravity force

*Hind*III :Type II site-specific deoxyribonuclease restriction enzyme isolated from *Haemophilus influenza*

HR : Hypersensitive response

hrs : Hours

IRD : Infrared Dye

M : Molar

mA : MiliAmper

mg : Miligram

mL : Mililiter

mM : Milimolar

*Mse*I : *E. coli* strain that carries the *Mse*I gene from *Micrococcus species*

ng : Nanogram

nm : Nanometer

PCD : Programmed Cell Death

PCR : Polymerase Chain Reaction

ppm : Parts per million

*Pst*I : Type II restriction enzyme

R : Resistance

RAPD : Randomly Amplified Polymorphic DNA

RFLP : Restriction Fragment Length Polymorphism

RNA : Ribonucleic acid

RNAse : Ribonucleic acid digesting enzyme

RT-PCR : Reverse transcriptase polymerase chain reaction

SDS : Sodium Dodecyl Sulphate

SSR : Simple sequence Repeats

Taq : *Thermus aquaticus*

T_M : Melting temperature

v/v : Volume/volume ratio

U : Unit

µg : Microgram

µL : Microliter

µM : Micromolar

CHAPTER I

INTRODUCTION

1.1 *Blumeria graminis* f. sp. *hordei* (Powdery mildew)

Blumeria graminis form of species hordei is a fungus causing "powdery mildew" in barley, infection is restricted to *Hordeum*. However relatives of *Blumeria* can cause powdery mildew in a great range of monocotyledonous and dicotyledonous plants; such as barley, wheat, strawberry, squashes, or even oak trees. Disease can cause epidemics and may reduce crop yield from 10% to 36% in temperate regions depending on the severity of the epidemic. It invades the leaves and causes reduction crop yield by affecting the proper formation of the plant parts such as grains, kernel, tillers, heads, and roots. As usual for fungi, it has both mitotic and meiotic life cycles (Ridout, Skamnioti et al. 2006) (Figure 1.1). In asexual phase conidiospores are increased in number by mitosis, this stage takes place on the living plant tissue. Conidiaspores, in haploid form, are divided and spread around by falling apart from the conidia branch. In sexual phase, + and – mating types are required in order to form an "ascus". When the + and – mating types of the spores fused, they form cleistothecia which are the diploid forms. Cleistothecia perform meiosis, it has four spores and after a mitosis "ascus" formation occurs, having eight spores. Cleistothecia are a very protective life form for spores, in order to hibernate during harsh winter conditions. They are generally present in the remaining of the infected plant material. When weather conditions change and temperature increases, ascus formation occurs and the sacs explode in order to infect fresh barley leaves.

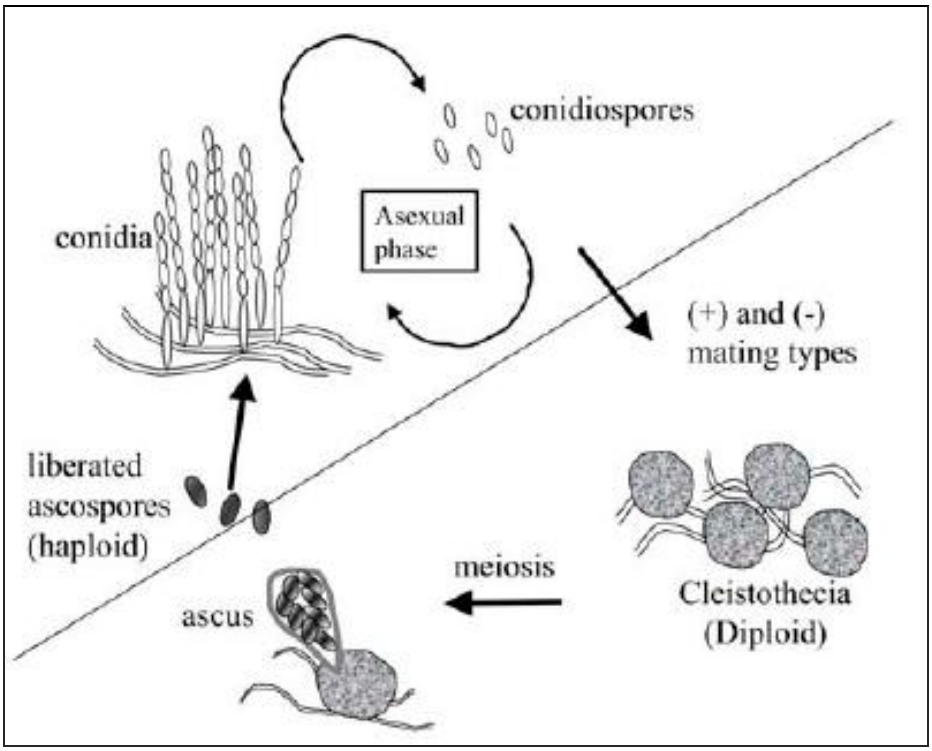


Figure 1.1 Life cycle of *Blumeria graminis.* Haploid and diploid life cycles are represented. The fungi spend its haploid stage on fresh barley plants making it obligate biotroph, and the diploid stage is formed on the plant remaining near the field (Ridout, Skamnioti et al. 2006).

As represented in Table 1.1 *Blumeria graminis* f. sp. *hordei* is an obligate biotroph, it requires living plant material throughout its life cycle. It belongs to the phylum Ascomycota, which are the largest group of fungi (Integrated Taxonomic Information System (ITIS). They are also known as "cup fungi", they got their name from their special sexual apparatus or sac called, "ascus". In each ascus there are eight microspores. By the time the spores mature, the sac bursts by the help of some outside effects like water droplets, or wind, and disperse to the environment. The spores complete their mitotic life cycle on barley leaves and stay near the field as dry leaf material. They infect the next year's seedlings from those remaining parts, completing the cycle. By the help of the rains, spore sacs explode and infect the newly emerging seedlings.

Table 1.1 Nomenclature of *Blumeria graminis f. spc. hordei. Source: Integrated Taxonomic Information System (ITIS)*

Kingdom: Fungi
Phylum: Ascomycota
Class: Ascomycete
Order: Erysiphales
Family: Erysiphaceae
Genus: *Blumeria*
Species: *Blumeria graminis*

Figure 1.2 represents the growth stages of a *Blumeria graminis* f. sp. *hordei* spore. It lands on a barley leaf as conidia, and starting from the second hour it starts to germinate by formation of "primary germ tube", in order to enter the plant cell. Primary germ tube exerts great pressure to the mesophyll cells of the plant and when it tears and enters into the leaf at the eight hour, a secondary structure is formed "haustoria". Haustoria is the special feeding structure of the fungi, and after haustoria formation the fungi grows invasively in order to infect nearby cells as well, by the fifth to seven day after inoculation conidia formation occurs which gives the "powdery" appearance to the barley leaves (Figure 1.3).

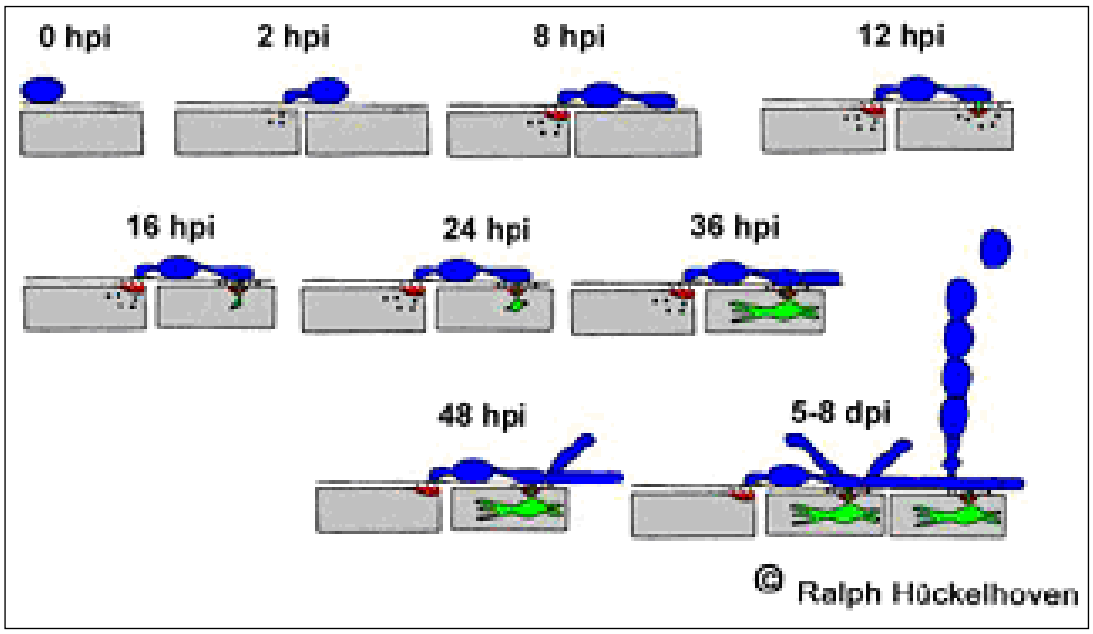


Figure 1.2 General growth diagram of *Blumeria graminis form of species hordei* spores on living barley tissue. Conidia lands on barley leaf at 0 hpi, thrn it starts to form a primary germ tube for entering the cell, after entering mesophyll cells haustoria, a special feeding apparatus is formed and dispersion to nearby cells take place (Ralph Hückelhoven 2005).

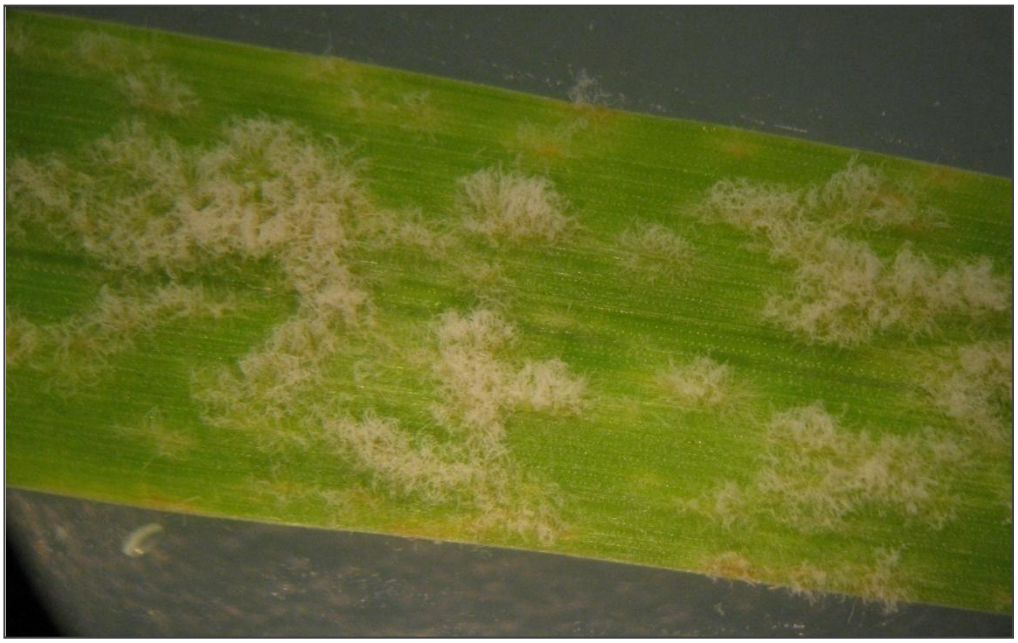


Figure 1.3 The 10^{th} day after inoculation of fresh barley leaves with *Blumeria graminis* spores. The fresh barley leaves are on benzimidazole containing agar medium. Conidia formation results in a "powdery appearance" (Photo is taken by Gülay Dağdaş and Asude Çallak Kirişözü).

1.2 Barley (*Hordeum vulgare*)

Barley is a cereal grain that is cultivated from a wild relative of *Hordeum vulgare (Hordeum spontaneum* C. Koch.) in Fertile Crescent (Zohary and Hopf 1993; Diamond 1998) area, in southeastern part of Turkey, nearly around 8000 BC. Barley (*Hordeum vulgare*) is an economically very important cereal for human consumption such as breakfast cereals or alcoholic beverages, and for animal feed. In 2006, 92.6 million metric tons of barley is produced throughout the world. Turkey comes sixth among the top ten barley producers (Table 1.2).

Table 1.2 Top ten barley producing countries in the world. Quantities are given in metric tonnes. *Source: UN Food and Agriculture Organization (FAO)*

Top Ten Barley Producers, 2006

Rank	Countries	Quantitiy (m.t.)
1	Russian Federation	18153550
2	Germany	11966600
3	Ukraine	11341200
4	France	10412361
5	Canada	9573100
6	Turkey	9551000
7	Spain	8136389
8	United Kingdom	5239000
9	Australia	4257000
10	United States of America	3922660
World total:		**92552860**

1.3 Disease-Pathogen Interactions

Plants do not have an immune system because of this reason they respond to pathogen attack *via* induced resistance mechanisms. They recognize the pathogens by two mechanisms. In the first one plant similar to animals recognizes pathogen attack by detection of special pathogenic factors which are called as effectors. Effectors are pathogen oriented molecules those manipulate host cell structure and function. By this way first plant recognize this protein and produces resistance response, in this case they are called as avirulence factors or elicitors and the second one is they are not recognized by the plant and infection occurs in this case they are referred as virulence factors (Kamoun 2006; Ebbole 2007; Dodds, Rafiqi et al. 2009). There are two types of generally accepted model mechanisms present in order to explain plant pathogen interactions one of them is "gene for gene interaction", and the other one is "guard hypothesis". Gene for gene interaction is first described by H.H. Flor in 1944. As depicted in figure 1.6 this model claims that in plants there are gene products called **R**esistance genes (R), and in pathogen there are **Av**i**r**ulence genes (Avr), when these two gene products interact together in plant, an infected cell recognizes the pathogen and becomes resistant, hypersensitivity response is formed. In any other condition, disease forms, due to lack of R-Avr recognition.

Blumeria graminis f. sp. *hordei* and *hordeum vulgar*e interaction as a well studied model in plant-pathogen interactions. Barley-powdery mildew interaction can be analyzed in gene-for-gene concept as represented in Figure 1.4.

		Host Genotype	
		RR or Rr	rr
Pathogen Genotype	AA or Aa	Resistance	Disease
	aa	Disease	Disease

Figure 1.4 Gene-for gene hypothesis. In host genome there are Resistance genes and in pathogen genome there are Avirulence genes both of which are dominant genes. When their gene products directly come together in the dominant situation resistance occurs, however in any other combination disease takes place.

The second model is the "guard hypothesis". In gene for gene concept plant and pathogen genes are thought to interact directly, however recent studies are showing that this is rarely happening. There are small interacting molecules in between those two molecules forming a cascade in order to develop immune response for pathogen attack. This response is called as **H**ypersensitivity **R**esponse (**HR**) and leads to **P**rogrammed **C**ell **D**eath (**PCD**) by causing oxidative burst of the cell. Callose deposition is another resistance response performed by the attacked cell in order to keep the pathogen inside the cell and prevent further invasion. This mechanism is performed by the cells nearby the pathogen entry site in order to lock the pathogen in a dead zone and protecting the rest of the plant. Moreover, these cells send signals through the signal transduction pathways to nearby cells in order to prevent further pathogen infections. As represented in figure 1.5.a and 1.5.b the guard protein could help the resistance protein in different combinations. It can directly interact with resistance protein to combine with pathogen effector and lead to PCD or it can dissociate a protein complex and lead to PCD, respectively (Heath 2000).

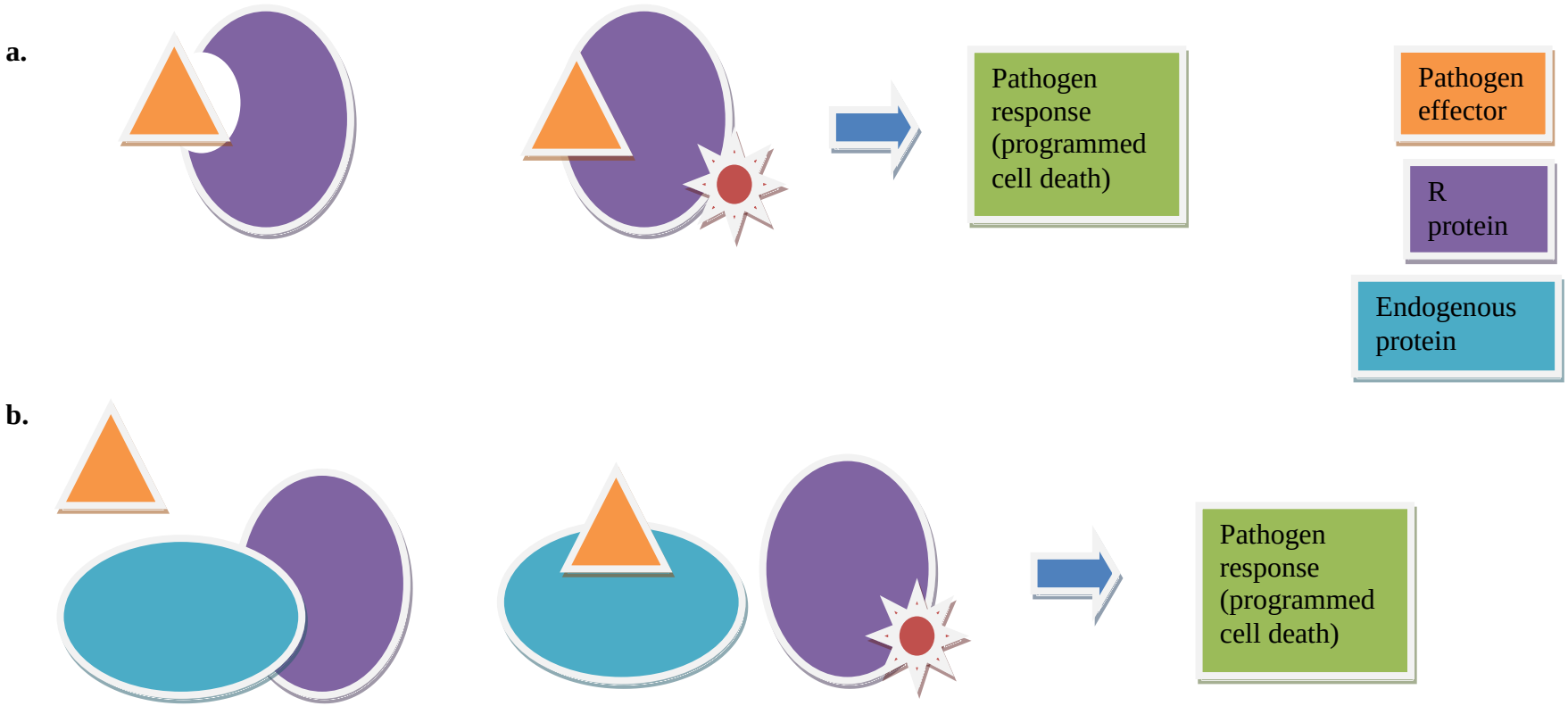


Figure 1.5 Guard Hypotheses. In this model there are small interacting molecules forming cascade like structures in order to prevent pathogen attack. A common and well studied case is Programmed Cell Death (PCD) which is performed for preventing pathogen invasion (Modified from the figure in (Bomblies and Weigel 2007).

1.4 Hardy-Weinberg Equilibrium

Hardy-Weinberg principle is named after two scientists G. H. Hardy and Wilhelm Weinberg. They developed the principle and equation at 1908 independent of each other (D.J.Futuyma, 2008). This principle states that a population stays in equilibrium in terms of allele and genotype frequencies unless an outside disturbance occurs. In other words, two alleles regardless of their previous allele frequencies will have a genotype frequency of $\mathbf{q^2+2pq+p^2}$ after one generation in a random mating population. These allele and genotype frequencies will be same in the following generations unless an outside disturbance affects the population structure. If genotype frequencies in a locus can be assumed in each generation the population can be said to be in Hardy-Weinberg equilibrium. For a population to be in Hardy-Weinberg equilibrium some assumptions should be done:

i. No gene flow occurs meaning there must not be migration either inward or outwards which can easily change the allele frequencies from the standard value.
ii. No mutation occurs. Mutations have the ability to change allele frequency although this process takes time it can chance the frequency.
iii. Random mating occurs; choosing the partner can cause deviations from the equilibrium.
iv. No chance event occurs; the number of individuals in the population must be so big that no chance events can manipulate the allele frequencies.
v. No natural selection meaning every individual in the population has equal chance of survival and reproduction.

The equation is explained by interpreting a Punnet square. In a diploid population the homozygous dominant allele is represented as “A” (p), homozygous recessive allele is “a” (q), and heterozygote individual is “Aa”.

According to Punnet square (Figure 1.6) showing the possibilities in a random mating (egg and sperm comes together and mates in a random way) the ratios are

given as $\mathbf{q^2+2pq+p^2}$ in a equilibrium this ratio is equal to 1 since there is two alleles studied and their frequencies must add up to 100%.

		Sperm	
		$A_1(p)$	$A_2(q)$
Egg	$A_1(p)$	$A_1A_1(p^2)$	$A_1A_2(pq)$
	$A_2(q)$	$A_2A_1(q^2)$	$A_2A_2(q^2)$

Figure 1.6 Punnet square. This diagram shows the possible combinations iwth a sperm and an egg in a random mating situation.

1.5 Molecular Markers

Molecular marker is a piece of DNA or protein that is polymorphic, and indicates one or several loci linked to the genotype of the individual carrying it. Molecular markers are used as molecular probes to reveal the population structure of a group of organisms. An ideal molecular marker should be polymorphic, multiallelic, co-dominant (heterozygote-homozygote differentiation can easily be made), neutral (the specific feature of the marker does not affect the survival chance of the individual carrying it), non-epistatic (genotype can be revealed from the phenotype), unchanged by the environmental fluctuations, and easily reproducible. Roughly molecular markers can be grouped as protein and DNA based markers, and DNA-based molecular marker techniques divide into two as PCR-based and non-PCR based molecular markers. RFLP (**R**estriction **F**ragmant **L**ength **P**olymorphism) for example is a non-PCR (Polymerase Chain Reaction)-based molecular marker. In RFLP, whole genomic DNA is digested with restriction digestion enzymes, and the resultant pieces are hybridized with a probe. Although this technique is very

sensitive, it is very labour intensive. PCR-based techniques can be exemplified as SSR (**S**hort **S**equence **R**epeats), microsatellite, AFLP (**A**mplified **F**ragment **L**ength **P**olymorphism), and RAPD (**R**andomLy **A**mplified **P**olymorphic **D**NA). In these methods PCR is used in order to amplify the starting DNA sample. By this way a very small amount of genetic material would be enough to initiate the experiment. These PCR-based techniques are easier to conduct allowing high throughput analysis.

1.6 Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism is a technique which combines RFLP (**R**estriction **F**ragment **L**ength **P**olymorphism) with PCR-based molecular marker technology. It is a technique firstly described in 1995 by Pieter Vos and his colleagues. The technique contains five steps; the third step may be doubled if the complexity of the genome of the species requires large number of markers.

The steps of the AFLP procedure can be categorized as:

i. Restriction digestion
ii. Adapter ligation
iii. Selective amplification
iv. Gel loading and analysis
v. Phylogenetic analysis

In this molecular marker technique the most important criteria is the quality of the DNA studied. The method is PCR dependent; so it is prone to reduction in amplification specificity if any contaminant is present in the DNA isolate. The concentration of the DNA is not very crucial because selective amplification step can be repeated as needed. However the integrity of DNA is important. Since the first step is the restriction enzyme digestion the comparison of genotypes requires DNA digestion which is not affected by the varying quality of DNA.

The aim of this method comes from the capacity of restriction enzymes to cut every kind of DNA. Restriction enzymes are enzymes used by bacteria or Achaea in order to insert their genome into their hosts. Since every species have restriction enzyme recognition sites, any mutation making this recognition site non-functional, or creating a new recognition site, or any increase or decrease in repeated region in between two restriction enzyme recognition sites creates pieces in different length when digested. Those digested pieces are a very reliable method to differentiate individuals even in the subspecies level (Duim, Vandamme et al. 2001).

AFLP is a dominant marker meaning it does not reveal heterozygosity. While observing a gel image we can only see whether a band is present or absent on the gel. Scoring of the gel image is performed giving 1 if there is a band present, and 0 if there is not a band present on the gel.

AFLP method has a large number of advantages. First of all, the method does not require prior sequence knowledge. Secondly, the method is very robust, reliable, and reproducible. It investigates whole genome for the presence of polymorphisms and it is reproducible. Additionally, many polymorphic bands can be detected in one lane, and many of them can be analyzed on one gel simultaneously. In AFLP number of polymorphism detected per reaction ratio is much higher with respect to other molecular markers. Different markers can be obtained simply by changing the restriction enzyme sites, and additional nucleotides (Blears, De Grandis et al. 1998)

The method also has some disadvantages. The biggest disadvantage is its marker character which creates problems while studying genetic distance, because no such information is obtained by AFLP since heterozygosity or homozygosity cannot be differentiated. The second disadvantage is size homoplasy meaning restriction enzyme digested pieces having same base pair length does not mean that they are the very same fragments, in an AFLP gel same bands among different individuals may belong to different parts from the genome. Another disadvantage is AFLP gives low level of polymorphism. It gives two alleles per locus, however in microsatellite markers many alleles per locus can be observed. In order to study AFLP hundreds of

polymorphic loci are required whereas ten polymorphic loci is enough for microsatellite markers (Bensch and Akesson 2005).

Application areas of AFLP vary in great extend. It can be used in forensic typing, inheritance of economically important traits, genetically inherited disease diagnosis, pedigree and parentage analysis, marker assisted plant breeding, positional cloning for gene of interest, strain identification and characterization in bacteria, fungi, plants, animals (Blears, De Grandis et al. 1998).

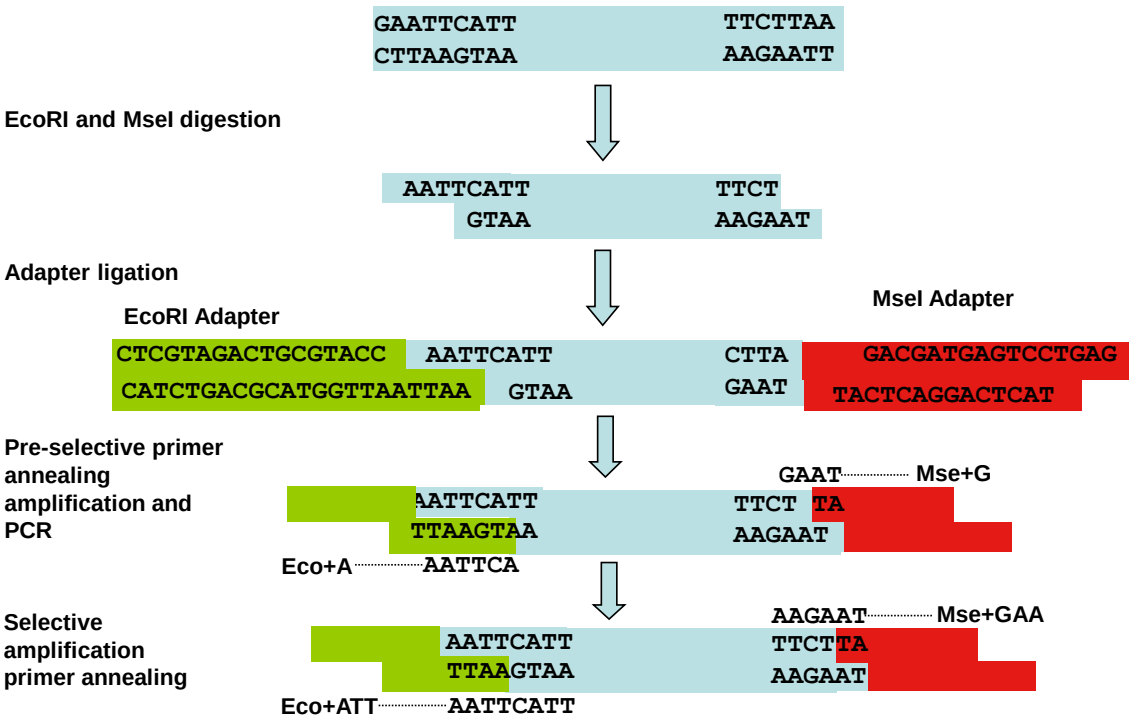


Figure 1.7 Representation of Amplified Fragment Length Polymorphism reaction. Genomic DNA containing *Eco*RI and *Mse*I restriction sites are cut with the enzymes (Restriction Digestion), adapters designed for the cut sites are added (Adapter

Ligation), DNA fragments are reproduced with one and two selective nucleotide added primers (Pre-Selective and Selective Amplifications).

1.6.1 Restriction Digestion

The initial step of AFLP procedure is restriction digestion of high quality DNA. Restriction enzymes are endonuclease enzymes that cut DNA from certain sequences. They may cut the DNA in two different positions, forming sticky or blunt ends. Blunt ends are formed by cutting DNA in same position in both strands; a sticky end is formed by cutting DNA with unequal pieces in two strands. As seen in the Figure 1.7 above genomic DNA is digested with two kinds of restriction enzymes. One of the enzymes is a rare cutter enzyme, a "six base pair recognizing enzyme", and the other one is a frequent cutter enzyme which is a "four base pair recognizing enzyme". Since the frequency of a six to eight base pair sequence to come together is less than that of a four base pair sequence the "frequent cutter enzyme" has more chance to cut the DNA during restriction digestion procedure. There are several reasons of using two different enzymes. First of all frequent cutter enzyme will form small DNA pieces that will be easily seen on denaturing gels, secondly the number of fragments are limited by using a rare cutter enzyme because only the fragments having both enzyme cuts will be selected by the reaction, thirdly using two different enzymes help to label one end of the DNA fragment and prevents formation of "false positives" on the gel which may be formed by labeling of the both ends of the fragment and their appearance as two individual bands on denaturing gels, and the last reason is using two different restriction endonucleases increases the variation obtained by generating more diverse fragments (Vos, Hogers et al. 1995). The complexity of the genome studied and the methylation status of the DNA affect the choice of the enzyme (Bachem, vanderHoeven et al. 1996). Most commonly used rare cutter enzymes can be listed as; *Eco*RI, *Ase*I, *Hind*III, *Apa*I, *Pst*I, and *Tru*I (a cheaper isomer of *Mse*I) and frequent cutter enzymes are *Mse*I or *Taq* I (Bensch and Akesson 2005). Most eukaryotic genomes are AT-rich making MseI enzyme the most commonly used enzyme for AFLP (Blears, De Grandis et al. 1998).

EcoRI restriction digestion enzyme recognition site is:

Source: New England Biolabs Inc.

*Mse*I restriction digestion enzyme recognition site is:

Source: New England Biolabs Inc.

The resultant restriction digested pieces contain three different types of fragments according to the types of enzymes they are cut with; MseI-MseI ended fragments, they also contain the majority of the pieces, estimated to be more than 90%, the second one is MseI-EcoRI ended pieces which are the twice as much the number of the third one, EcoRI-EcoRI ended pieces (Vos, Hogers et al. 1995).

1.6.2 Adapter Ligation

In adapter ligation part, adapters designed for restriction digested parts are used. The adapters have some extension in order to avoid the recombination of the restriction enzyme digested parts by this way restriction digestion and adapter ligation step could be performed in the same test tube.

The structure of the **EcoRI** adapter is:

5-CTCGTAGACTGCGTACC
CATCTGACGCATGGTTAA-5

The structure of the **MseI** adapter is:

5-GACGATGAGTCCTGAG
TACTCAGGACTCAT-5

Double stranded AFLP adapters have a core sequence and enzyme specific region making them specific for the restriction enzyme digested pieces.

1.6.3 Selective Amplifications

Pre-selective amplification step is performed for complex genomes in order to decrease the complexity of the genome. By doubling the selective amplification steps background 'smears' are decreased and repeated restriction fragments that generate different banding patterns are eliminated (Vos, Hogers et al. 1995). Primer design is very crucial for AFLP procedure. AFLP primers are composed of a core sequence, an enzyme specific sequence, and a selective extension regions (Vos, Hogers et al. 1995).

Table 1.3 Structures of AFLP primers

	Core sequence	**Enzyme specific sequence**	**Selective extension**
*Eco*RI	5-GACTGCGTACC	AATTC	NNN-3
*Mse*I	5-GATGAGTCCTGAG	TAA	NNN-3

AFLP primers are designed for double stranded adapter added genomic DNA pieces (Table 1.3). At the end of the adapter ligation step three kinds of pieces are formed which are MseI -MseI, EcoRI-MseI, and EcoRI-EcoRI. Since the EcoRI primer's annealing temperature is greater than the MseI primer, and the repeated region of the MseI primer can easily form a stem loop structure, EcoRI-MseI ended pieces are preferred by the primers.

In pre-selective amplification part primers with one base extension is used. Extention of the primer is an extra nucleotide in the primer used in order to decrease the complexity of the genome. Since there are four nucleotides, additional one nucleotide decreases the complexity by 4^1 for each primer, making a sixteen fold decrease in complexity. Pre-selective amplification products are diluted and used as templates for the selective amplification procedure. In selective amplification three nucleotide extensions are used in order to further decrease the complexity of the genome which decreases the complexity by 4^3 for each primer, in total making two hundred fifty six fold decreases in complexity. In selective amplification touch-down PCR is applied which is done for obtaining the best annealing temperature for the primers because of their structural differences T_M value of EcoRI primer is greater than the MseI primer.

Fluorescently labeled primers take the place of radioactively labeled primers. This makes procedure safer, and timesaving. At the 5' end of the EcoRI primer a fluorescently labeled dye is attached. Labeling of one primer is crucial since this helps visualization of one band in acrylamide denaturing gel, otherwise duplicates labeled at two ends could form bands which are false positives. Fluorescent dye is excited when it hits the laser radiation and is visualized by its characteristic absorption-emission frequencies (Blears, De Grandis et al. 1998).

1.6.4 Gel Loading and Analysis

Gel loading is the last part of the AFLP procedure and 7.5 M urea containing acrylamide gels are used in order to observe DNA pieces differentially cut and labeled with a fluorescent dye. SagaMX software is used in order to score AFLP gels. AFLP bands on the images are scored as +/- by the program which is then converted to 1/0 matrixes.

1.6.5 Phylogenetic Analysis

The data obtained by scoring the gel images, is loaded into phylogenetic analysis programs such as PopGene, Phylip, PAUP, Ntsys, Treecon in order to obtain phylogenetic relationships.

NTSYS program is one of the programs used for construction of linkage maps and it will be depicted in detail since it is used in this thesis study. Dendogram, matrices of genetic distance coefficients from gene-frequency and DNA sequence data (SIMGEND), similarity for quantitative data (SIMQUAL), neighbor joining, principal component analysis (pca), projection, 3D plot, and eigen values are major concepts in this program. SAHN module is used with UPGMA clustering method of calculations in clustering analysis. Simple Matching (SM), Jaccard (J), and DICE coefficients are used for similarity for quantitative data analysis.

The values to be calculated in order to obtain a phylogenetic relationship are as follows:

CPCA (**C**ommon **P**rincipal **C**oordinate **A**nalysis) which fits a single set of eigenvectors to a series of variance-covariance matrixes (Flury 1984, 1988).

EIGEN is composed of Eigenvector and Eigenvalue. This module computes a real symmetric similarity matrix, it is used in the principal components or principal

coordinates analysis by extracting eigenvectors (factors) from a correlation or variance- covariance matrix. The general equation can be represented as:

$$A\mathbf{v} = \lambda\mathbf{v}$$

A indicates a square matrix, λ is eigenvalue and v is eigenvector.

3D plot gives a diagram in 3dimensions view which is used to view the results of principle coordinate analysis (principle component analysis).

Neighbor Joining (NJoin) is a method doing the estimation of phylogenetic trees (Saitou and Nei 1987). This method is basically based on the method of parsimony. If maximum parsimony is considered the probable phylogenetic tree requires the smallest number of evolutionary changes.

Principal Components Analysis (pca) is used when a matrix has a set of variables on a set of objects. In this analysis inter-relationships of variables and objects can be studied. Proximities among the objects can be calculated among objects by using Euclidean distances and among the variables by covariance (correlation).

Projection (PROJ) option gives the usual projection of objects onto one or more factor axes. The data matrix has usually been standardized using the module STAND and the axes are eigenvectors of a matrix of correlations among the variables.

SHAN (Sequential Agglomerative, Hierarchical, and Nested clustering methods (Sneath and Sokal (1973))) module has a variety of clustering methods. UPGMA (Unweighted pair-group method, arithmetic average) is one of the clustering methods given in this module.

SIMGEND (Dendogram, matrices of genetic distance coefficients from gene-frequency and DNA sequence data) calculates a variety of coefficients for genetic data. Nei's standard genetic distance (NEI72) which is the method used for this study calculates the diversification among closely related taxonomic group

(Loeschcke, 1994). The method determines the degree of genetic separation in between species or populations at molecular level.

SIMQUAL (Similarity for Qualitative Data) estimates the similarity and dissimilarity coefficients for qualitative data. Jaccard (J), DICE, and Simple Matching (SM) are most often used coefficients.

1.7 Assessment of Powdery Mildew (*Bgh*) Resistance Genes in Turkish Barley Varieties

Thirty five Turkish barley varieties are investigated for the presence of resistance genes against powdery mildew races with known avr genes (Table 1.4). Since barley is found to be originated from the 'Fertile Crescent' area spanning Southeast part of Turkey (Zhoary 1999, Willcox 1995, Czembor 1996, Zeybek et al. 2008) it is very probable to have barley varieties, landraces, and wild relatives of barley having different types of resistance genes against powdery mildew in Turkey because of being in the center of divergence (Jahoor and Fishbeck 1987, Zeybek et al. 2008).

Table 1.4 Isolates of *Blumeria graminis* f.sp. *hordei* and their infection types on 'Pallas' differential near-isogenic isolates (Kølster *et al*. 1986) and twelve additional cultivars

			Differential test isolates								
No	**Differential isolates**[1]	**R-genes**	**B4(C15)**	**B95(53/01)**	**B100(60/01)**	**B121(26/04)**	**B120(20/04)**	**B97(57/01)**	**B91(98AF066)**	**B21(R86/01)**	**B103(64/01)**
0	Pallas	MLa8	4	4	4	4	4	4	4	4	4
1	P01	MLa1,ML (A12)	0	4	4	4	0	0	0	0	0
2	P02	MLa3,	4	0-1n	0	0	4	4	0-1n	4	0-1n
3	P03	MLa6, MLa14	0	0	0-1n	3n-4	4	4	0	4	4
4	P04B	MLa7, ML(NO3)	0	4	4	3-4	1-2n	3n	4	1n	4
5	P08B	MLa9	0	0	4	4	4	0	4	0	0
6	P09	MLa10, ML (Du2)	0	4	3n	0	4	0	4	0	0
7	P10	MLa12, ML (Em2)	0	4	0-1n	1n	3n-4	4	1n	0-1n	3n-4
8	P11	MLa13, ML (Ru3)	0	0	0	4	0	4	4	0	4
9	P12	MLa22 Mic)	4	0	0	3n-4	0	4	0	4	0
10	P14	MLra	4	4	4	0	4	4	4	4	4
11	P16	MLk	2cn	2cn	4	1-2cn	4	1-2cn	3n-4	1-2cn	3n-4
12	P20	ML	2n	2n	2n	2-3n	2n	4	1-2n	1-2cn	2n
13	P21	MLg,ML (CP)	2-3n	4	4	4	4	4	4	0	4
14	P23	MLla	1-2n	4	4	4	4	2n	4	4	2n
15	P24	MLh	4	4	0	0	4	4	4	4	4
16	ISO2R	MLg	4	4	4	4	4	4	4	1-2n	4
17	SI-1	Sl 1	0	1-2n	3n	0	0	0	0	0	0
18	GUNNAR	MLa3, ML (Tu2)	1-2n	0	0	0	2n	4	2-3n	4	0
19	SV83380	MLab	2n	3n	2-3n	3n	4	4	3n-4	2n	4
20	MELTAN	MLa13, MLı 8lm9),+	0	0	0	4	0	4	0	0	4
21	GOLDİE	MLa 12, U	0	2n	0	4	1n	4	4	0	4
22	STEFFİ	ML (St)	0	0-1n	0-1n	3n	4	2n	4	0	1n
23	HENNİ		0	1-2n	0-1n	4	4	1n	4	0	1-2n
24	PUNTO	MLa3, ML (Tu2), ML (lm9),+	0	0	0	0	1n	4	1-2n	1n	0
25	BENEDİKTE	MLa9, ML(lm9)	0	0	2n	4	0-1n	0	1n	0	0n
26	SCARLETT		0	0-1n	0	3cn	4	2n	4	0	0-1n
27	CARLSBERG	MLa8	4	4	4	4	4	4	4	4	4
28	Bülbül 89 /control		4	4	4	4	4	4	4	4	4

1.8 Aim of the Study

Crop species are very important for human consumption, thus it is important to increase the yield by obtaining high quality cultivars. The most important barley pathogen is *Blumeria graminis* f. sp. *hordei* decreasing barley yield. The most efficient way to overcome the pathogen effect is to understand the population structure of the pathogen. AFLP molecular marker technique is an informative way to examine the virulence genes.

In order to eliminate powdery mildew disease of barley we need to reveal the disease-pathogen interactions. The interaction mechanism is mainly composed of the resistance-virulence gene interactions. By studying local *Bgh* isolates their mutation rate will be understood, their phylogenetic relationships are detected. Comparing the local isolates phylogenetic trees by universal isolates trees we can observe the distribution of the *Bgh* spores through long distances. By this way future experiments can be conducted for investigating the virulence genes.

The present work aimed to reveal population structure, and phylogenetic tree of local *Blumeria graminis* f. sp. *hordei* isolates. Our objective was to apply molecular biology techniques for investigating the relationships between thirty-nine local *Bgh* isolates and eight universal isolates. Molecular marker technique AFLP is the most robust technique for studying filamentous pathogens.

CHAPTER II

MATERIALS AND METHODS

2.1 Obtaining Turkish *Blumeria graminis* f. sp. *hordei* isolates

A controlled field was infected with the Turkish isolates and they were let to be crossed by themselves. The dry leaves containing a great range of spores were collected from the field and taken into the laboratory. In the laboratory agar plates were prepared and second leaves of fresh barley leaves were put onto the plates. A 5 cm part of the dry leaves containing spores were attached to tissue papers, wetted by double distilled water and put onto the fresh barley leaves they were wetted daily. Plates were put into a growth chamber having a period of 18d/6n, and 60 % humidity. After four days dry leaf was removed from the plate and sporulation was observed after a week. Multiple maintenance cycles were performed and in each cycle single colony isolation was performed until pure *Blumeria graminis f. species hordei* isolates were obtained.

2.2 Plant Materials and Growth for Assesment of Powdery Mildew (*Bgh*) Resistance Genes in Turkish Barley Varieties

Thirty-four *Hordeum vulgare L.* cultivars seeds were taken from the Aegean Agricultural Research Institute in Turkey. The list of the tested varieties was given in table represented in Zeybek at al. 2008 (Table 2.2). The seeds were originated from the Aegean and Mediterranean coastal regions. The growth condition in the growth chamber was 20-22 °C, 14/10 hrs day and night period. “Leaf segment test” was performed after second leaf stage (A. Zeybek et al. 2008). The experiments for discovering the resistance genes were performed by comparing the Turkish varieties with ‘leaf segment test’ of nine universal powdery mildew isolates (Lutz et al. 1992,

A.Zeybek et al. 2008). Leaf segment test is a test done with universal powdery mildew isolates those has a defined virulence spectrum (Flor 1942, 1956, Moseman 1959, Zeybek et al. 2008). These isolates' disease resistance and/or susceptibility results are used as reference values for Turkish barley varieties. The lesions on the leaf are checked according to the 0-4 scale of Welz 1988 (Zeybek et al. 2008) (Table 2.1). The leaves giving symptoms in between 0-2 were classified as resistant, whereas leaves in giving symptoms in between 3-4 were named as susceptible (Zeybek et al. 2008).

Table 2.1 Description of infection types (IT) and symptoms (Welz 1986, Zeybek 2008)

IT	Symptoms
0	No visible symptoms (immunity)
1	Negrotic flecks, usually minute;no mycelial growth; no sporulation; (hypersensitivity)
2	Frequent clorosis; reduced mycelial growth; no or very scare sporulation
3	Moderate mycelial growth, moderate sporulation; sometimes chloriss
4	Profuse sporulation of well developed colonies and sometimes green islands

Table 2.2 Registration number, variety name, and botanical name of thirty-four Turkish barley varieties used in Assessment of powdery mildew (*Bgh*) resistance genes in Turkish barley varieties (Zeybek et al. 2008)

Number	Registration number	Variety names	Botanical name
1	TR41009	Zafer 160	Hordeum vulgare vulgare
2	TR41010	Yeşilköy 387	Hordeum vulgare vulgare
3	TR41011	Gemici 7243	Hordeum vulgare vulgare
4	TR41012	Kaya 7794	Hordeum vulgare distichon
5	TR45288	Tokak 157/37	Hordeum vulgare distichon
6	TR45289	Cumhuriyet 50	Hordeum vulgare distichon
7	TR45290	Yerçil 147	Hordeum vulgare distichon
8	TR50882	Hamidiye 85	Hordeum vulgare distichon
9	TR50883	Obruk 86	Hordeum vulgare distichon
10	TR50884	Anadolu 86	Hordeum vulgare distichon
11	TR50885	Bülbül 89	Hordeum vulgare distichon
12	TR57795	Efes-1	Hordeum vulgare distichon
13	TR57796	Efes-2	Hordeum vulgare distichon
14	TR57797	Efes-3	Hordeum vulgare distichon
15	TR57786	Şahin 91	Hordeum vulgare distichon
16	TR57790	Yea. 793.12	Hordeum vulgare distichon
17	TR68592	Bornova 92	Hordeum vulgare nutans
18	TR68593	Şerife hanım 98	Hordeum vulgare
19	TR69697	Vamık hoca 98	Hordeumvulgare agriacrithom
20	TR69698	Akhisar 98	Hordeum vulgare agriacrithom
21	TR69699	Süleyman bey 98	Hordeum vulgare nutans
22	TR69700	Bilgi 91	Hordeum vulgare
23	TR72333	Beyşehir 98	Hordeum vulgare
24	TR72334	Konevi 98	Hordeum vulgare
25	TR72338	Başgöl	Hordeum vulgare
26	TR72340	Çıldır-02	Hordeum vulgare
27	TR72342	Avcı-2002	Hordeum vulgare
28	TR72343	Yesevi-93	Hordeum vulgare
29	TR72344	Orza-96	Hordeum vulgare
30	TR72345	Aydan hanım	Hordeum vulgare
31	TR76583	Özdemir 05	Hordeum vulgare
32	TR76584	İnce 04	Hordeum vulgare
33	TR76585	Kalaycı 97	Hordeum vulgare
34	TR76586	Erginel 90	Hordeum vulgare

2.3 Pathogen isolates

Nine isolates of *Blumeria graminis* (DC.) Golovin ex Speer *f. sp. hordei* Em. Marchal (synamorph Erysiphe graminis DC. f. sp. hordei Em. Marchal) were propagated and used as differential isolates in the experiments. The isolates were provided by Dr. M.S. Hovmøller (Royal Agricultural and Veterinary University, Denmark) they were chosen according to the differences in virulence spectra on Pallas near isogenic isolates (Kolster et al. 1986), and they are taken as single spore isolates on susceptible barley cultivar 'Carlsberg' fresh leaves (Zeybek et al. 2008).

2.4 Disease assessment

Leaf segments those are two to three cm long were cut from middle part of the primary leaf of 12 day old seedlings, laid on benzimidazole containing agar plates (35 ppm benzimidazole in 1.5 % agar) in order to obtain disease evaluation. Inoculation of the leaves was performed by the settling towers. The agar plates were put into growth chambers at 17-18 °C with 12 hrs of day and night period. The resultant leaves were scored after ten days according to the '0-4 scale' of Welz (1986). The leaves having 0-2 scale were regarded as resistant whereas leaves having 3-4 scales were called as susceptible (Zeybek et al. 2008)

2.5 Maintenance of the isolates

Blumeria graminis f. species hordei isolates were grown in growth chambers. Since the fungi were obligate biotroph it required living tissue throughout its life cycle. Barley plants (*Hordeum vulgare*) were grown in pots in growth chamber having 18hr day / 6 hr night periods and 60 % humidity. They were harvested at the 10^{th} day when their second leaves were seen and cut into 2-3 cm pieces onto agar plates, their blades should stay in upwards position for infection to occur effectively. Agar plates were prepared by putting 7g of agar medium onto 450 mL of double distilled water and heating at 110 $^{\circ}$C for 10 minutes and melting the agar. After the mixture

came to the room temperature 50 mL of 1 % Benzimidazole (1 g of Benzimidazole in 1000 mL of double distilled water) was poured onto the mixture and delivered into Petri dishes. Since Benzimidazole was a plant growth regulator it helped to prolong the lifetime of barley leaves. And plates were infected with different isolates by blowing out an infected leaf onto the healthy leaves through homemade inoculation towers. Sporulation was seen after 3 days. The procedure was repeated in every 10 days in order continue the recirculation and by this time interval spores were collected in eppendorf tubes and stored in -80 $^{\circ}$C until they were used in CTAB DNA isolation.

2.6 CTAB DNA Isolation

CTAB procedure was applied to the cleistothecia collected from barley leaves inoculated with 39 different *Blumeria graminis f. species hordei* isolates collected from Cukurova region in Turkey and with additional 8 universal races of which virulence functions are known. The 0.5 g of cleistothecia was collected into the eppendorf tubes and grinded with liquid nitrogen. After grinding 0.5 mL of CTAB buffer (preheated to 65 $^{\circ}$C), and 5 µL β-mercaptaethanol were added into the eppendorf tubes and 15 µL of proteinase K (20 mg/mL, Fermentas) is added in order to get rid of the proteins from the degrading cleistothecia structures. The mixture was put into water bath at 65 $^{\circ}$C for 1 hour. 2x CTAB buffer was prepared according to (Saghaimaroof, Soliman et al. 1984). In order to prepare 200 mL of 2x CTAB buffer; 4 g of 2 % CTAB (Hexadecyltrimethylammonium bromide), 100 mM Tris of pH 8 (10 mL/mL), 20 mM EDTA (ethylenediaminetetraacetic) (8 mL), 1.4 M NaCl (8.2 g/mL), 2 % PVP 40 (2.0 g/mL), 0.2 % Beta-mercaptoethanol (added just prior to use) (2 µL/mL solution) was used. Following, equal volume chloroform (0.5 mL) was added. The solution was vortexed and centrifuged at 15200 g, at 18 $^{\circ}$C for 20 minutes. The aqueous supernatant was transferred into a new tube and equal volume of isopropanol was added. The mixture was centrifuged at 15200 g at 4 $^{\circ}$C, for 30 minutes. DNA was visualized at the bottom of the test tube after this step. The supernatant was poured and the tube was washed with 0.5 mL 70 % ethanol and

spinned for 5 minutes at 15200 g. After this step, samples were air dried. DNA was resuspended in 40 µL double distilled water.

2.7 Concentration Measurements of Genomic DNA

DNA concentrations were obtained by Nano Drop on Nano Drop ND-1000 Spectrophotometer device using ND-1000 v3.1.2 program. The measurements were taken according to the instructions. A_{260}/A_{280} values are also considered for determining the purity of the samples.

2.8 Purification of DNA with RNAse Treatment

RNAse treatment was carried out on the DNA isolates in order to obtain high quality DNA. QIAGEN brand RNAse having lot number of 127153085 with 100 ng/µL was used for digesting possible contaminating RNA. The enzyme was diluted so that 20 ng/µL are applied to every sample. In the last step dilutions were performed in order to keep as same concentrations of each sample. All the samples were diluted to 40 ng /µL and 5 µL was taken from each sample (200 ng for AFLP).

2.9 LI-COR 4300 DNA Analyzer and Amplified Fragment Length Polymorphism (AFLP)

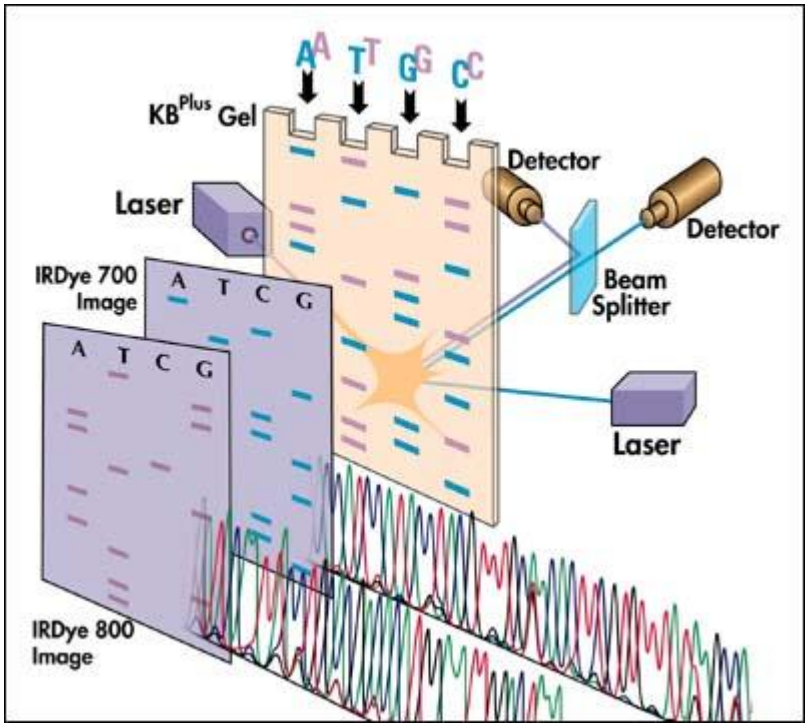


Figure 2.1 Working principle of LI-COR 4300 DNA Analyzer. The device have 2 different detectors emitting 700 and 800 nm laser beams this system helps to made two readings at the same time, from the same channel. *Source LI-COR Biosciences*

LI-COR is an automated gel fragment analysis system. It has two different laser beams that can read at different absorption spectrums. While the gel is running through the 7.5 M acrylamide denaturing gel, in front of laser scanner, two photodiode detectors detect fluorescence. '700 Channel Laser Source' emits at 685 nm, and '800 Channel Laser Source' emits at 785 nm (Figure 2.1). Since there is a 100 nm difference in between two lasers, detectors can detect each of the lasers independently at the same time. The image is collected in real-time and can be visualized by SagaMX software.

The procedure of Amplified Fragment Length Polymorphism included five parts which were; Restriction Digestion, Adapter Ligation, Pre-Selective Amplification, Selective Amplification, and Gel Loading. All the samples apart from 10X reaction buffer and Taq DNA polymerase were present in the kit provided by LI-COR (IRDye Fluorescent AFLP Kit for Large Plant Genome Analysis).

2.9.1 Restriction Digestion

In restriction digestion 5X reaction buffer (2.5 µL), [50 mM Tris-KCl,(pH 7.5, 50 mM Mg-acetate-250 mM K-acetate)], EcoRI/MseI enzyme mix(1µL), [1.25 units/µL each in 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 1mM DTT, 200 µg/mL BSA, 50% (v/v) glycerol, 0.15 % Triton X-100], Template DNA (5 µL), and deionized water 4 µL was put into a PCR tube on ice. Deionized water was adjusted so that the total volume of the mixture will be 12.5 µL. Template DNA was adjusted so that 200 ng of DNA is present in the final mixture. The mixture was mixed gently, centrifuged briefly and incubated at the 37 $^{\circ}$C for 2 hours. After the mixture was incubated it was put in 70 $^{\circ}$C for 15 minutes in order to make restriction enzymes nonfunctional, and then it was incubated on ice. In order to simplify the procedure the PCR tubes were put into PTC100 Thermalcycler with the program of first cycle 2 hours of incubation, following 15 minutes of 70 $^{\circ}$C, and 4 $^{\circ}$C soak.

2.9.2 Adapter Ligation

Adapter mix (12.5 µL) [EcoRI/ MseI adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-Acetate, 50 mM K-acetate] and T4 DNA Ligase (0.5 µL) [5 units/ µL in 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 50 mM KCL, 200 µL/ mL BSA, 50% (v/v) glycerol] enzyme were put into the pervious tube on ice. The combined volume is 25 µL. The mixture was pipetted and centrifuged briefly and incubated at 20 $^{\circ}$C for two hours. After incubation 1:10 dilution was performed by adding 10 µL of the mixture into a new tube and adding 90 µL of TE buffer (10 mM Tris-HCL (pH 8.0), 1.0 mM EDTA) and then the mixture was mixed well.

2.9.3 Pre-Selective Amplification

20.0 µL AFLP Pre-amp primer mix, 2.5 µL PCR reaction buffer (10 X) (100 mM Tris-HCL (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl), 0.5 µL Taq DNA polymerase (5 units/ µL) , and 2.5 µL of 1:10 diluted ligation mixture from the previous step was added into a PCR tube on ice. The total volume was 25.5 µL. PTC100 Thermal Cycler was used in order to perform the pre-amplification. The kit was optimized for Taq DNA polymerase and 10 X reaction buffer (100 mM Tris-HCl, 15 mM MgCl2, 500 mM KCl, pH 8.3 (20°C)) from Roche Molecular Biochemicals, catalogue numbers are 1146173, and 1271318 respectively. The mixture was mixed gently by pipetting up and down and then centrifuged briefly. The PCR program used was as in Table 2.3.

Table 2.3 PCR conditions for pre-selective amplification.

Step	**Temperature (°C)**	**Time**	
1	94	30 seconds	20 cycles
2	56	1 minute	
3	72	1 minute	
4	4	soak	

After performing this PCR 1:40 dilutions was done by taking 5 µL of the pre-amplification mixture and 195 µL of double distilled water. The remaining mixture was stored in -20 °C for future studies.

2.9.4 Selective Amplification

Since the volumes dealed with were very small master mixes were prepared. The first master mix was Taq DNA Polymerase working mix, the mixture prepared was enough for 33 reactions.

2.9.4.1 Taq DNA Polymerase Working-Mix

158.0 µL of deionized water, 40.0 µL of 10 X Amplification Buffer (100 mM Tris-HCl, 15 mM MgCl2, 500 mM KCl, pH 8.3 (20°C)) (Roche Molecular Biochemicals, Cat. No.1271318), and 2.0 µL of Taq DNA polymerase (5 units/µL) (Roche Molecular Biochemicals, Cat. No. 1146173) was put together, mixed gently, centrifuged briefly. For every sample 200 µL/33 reactions ≈ 6.01 µL of this mixture was required.

2.9.4.2 Selective Amplification Mix

2.0 µL of MseI primer (containing dNTPs) (Table 2.4), 0.5 µL of IRDye 700 labeled EcoRI primer A (1 µM) (Table 2.5), 0.5 µL of IRDye 800 labeled EcoRI primer B (1 µM) (Table 2.6), and 2.0 µL of diluted pre-amp DNA was added onto the ≈ 6.01 µL of Taq DNA Polymerase working mix prepared as above. The total volume was 11.0 µL for each sample.

The basic design of MseI primers was **GATGAGTCCTGAGTAA** additional three nucleotides were added at the end of the primers. In the kit provided by LICOR Biosciences the list of the primers are as in Table 2.4.

Table 2.4 MseI primers used in selective amplification reaction.

Primer name	Primer Sequence
Primer M-CAA	GATGAGTCCTGAGTAACAA
Primer M-CAC	GATGAGTCCTGAGTAACAC
Primer M-CAG	GATGAGTCCTGAGTAACAG
Primer M-CAT	GATGAGTCCTGAGTAACAT
Primer M-CTA	GATGAGTCCTGAGTAACTA
Primer M-CTC	GATGAGTCCTGAGTAACTC
Primer M-CTG	GATGAGTCCTGAGTAACTG
Primer M-CTT	GATGAGTCCTGAGTAACTT

The basic design for the EcoRI primers were **GACTGCGTACCAATTC** and additional three nucleotides were added at the end of the primers. In the kit provided by LICOR Biosciences the list of the primers given was as in Table 2.5 for IRDye 700 primers and as in Table 2.6 for IRDye 800 primers.

Table 2.5 EcoRI primers used in selective amplification reaction with IRDye 700 labelled fluorescent dyes.

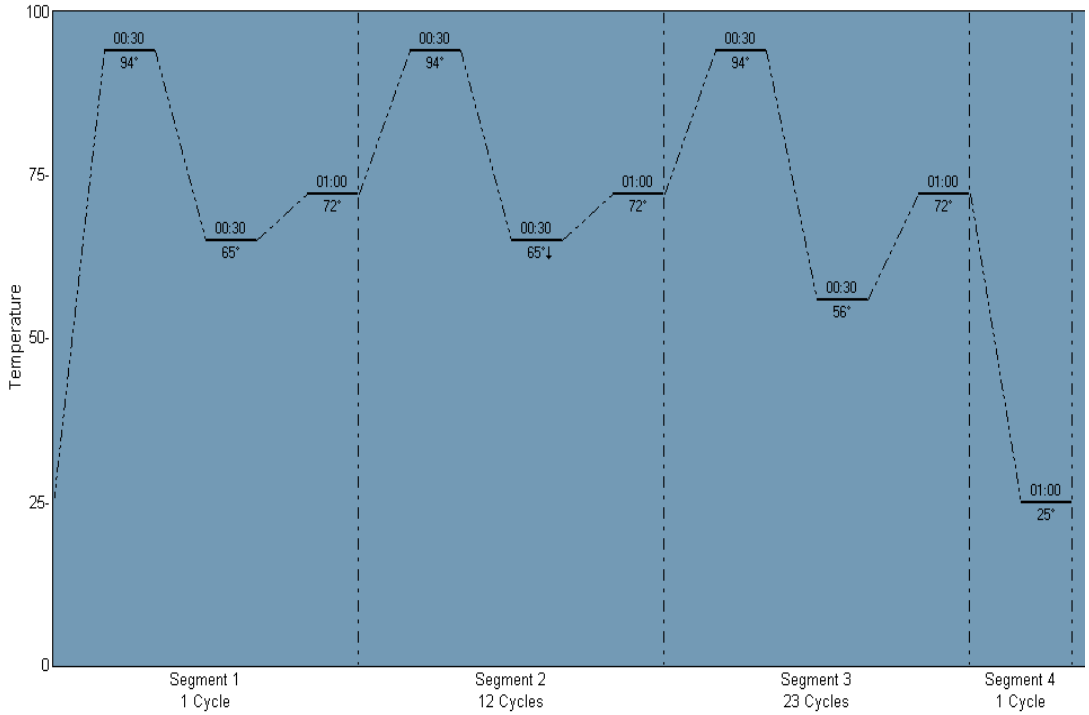
IRDye 700 Labelled Primer name	IRDye 700 Labelled Primer Sequence
Primer E-AAC	GACTGCGTACCAATTCAAC
Primer E-AAG	GACTGCGTACCAATTCAAG
Primer E-ACA	GACTGCGTACCAATTCACA
Primer E-ACT	GACTGCGTACCAATTCACT

Table 2.6 EcoRI primers used in selective amplification reaction with IRDye 800 labelled fluorescent dyes.

IRDye 800 Labelled Primer name	IRDye 800 Labeled Primer Sequence
Primer E-ACC	GACTGCGTACCAATTCACC
Primer E-ACG	GACTGCGTACCAATTCACG
Primer E-AGC	GACTGCGTACCAATTCAGC
Primer E-AGG	GACTGCGTACCAATTCAGG

The mixture was centrifuged briefly so that all the reagents were settled to the bottom of the tube. The procedure was established in the Stratagene Mx3005P Real Time Thermal Cycler (Figure 2.2).

Figure 2.2 Touchdown PCR of *Blumeria gramins f. species hordei* samples at selective amplification step.



After the PCR, 5 µL of Blue Stop Solution was added into each well and tubes were centrifuged briefly and tubes were denatured for 3 minutes at 94 °C and after this time interval they were loaded immediately.

Molecular weight standards were used in order to make analysis of the data easier. 50-700 base pair size standards were composed of 18 IRDye labeled DNA fragments in 90 % formamide solution with bromophenol blue. Size standard was loaded into the wells for 48 well combs, 1 µL for the first well and 1 µL for the last well (after all the samples were loaded) were required. Size standard (2 µL) was put into a 0.2 µL centrifuge tube and it was denatured at 95 °C for 1-2 minutes, and then soaked at 4 °C for 10 minutes. The image of the size standard starting from 50 base pair to 700 base pair was seen below in Figure 2.3. The size standard was provided by LI-COR Biosciences for IRDye 700 and IRDye 800 labeled fluorescent dyes independently (Catalogue Numbers 4200-60, and 4000-45 respectively).

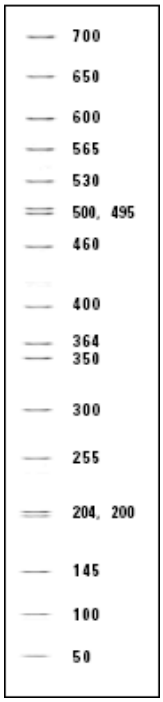


Figure 2.3 LICOR Biosciences IRDye 700 and 800 labeled size standards.

2.9.5 Preparation of 6.5 % Acrylamide Denaturing Gel

In order to prepare gel matrix for 6.5% acylamide denaturing gel, 40% Acrylamide-Bisacrylamide solution was prepared. In order to obtain a 200 mL stock solution 76 grams of Acrylamide, and 4 grams of Bisacrylamide was mixed together and double distilled water is added until the solution volume is 200 mL, and it is transferred in and mixed well. The reaction was performed in dark since the Acrylamide-Bisacrylamide mixture was light sensitive. Gel matrix was also prepared from the stock solution. For preparation of the stock 600 mL of 6.5% acrylamide denaturing gel, 120 mL of 5X TBE Buffer [Tris-Boric Acid-EDTA (108g;55g;9.3g)], 120 mL of 40% acrylamide solution, 252 g of urea was put onto a mixer and mixed with a stirrer, and the final volume was increased to 600 mL by adding of double distilled water.

Dust could cause improper gel formation so cleaning of the glass plates are very important. Glass plates were cleaned very well by brushing with 10% SDS (10 g of Sodium dodecyl sulphate in 100 mL double distilled water), and then plates were washed with double distilled water. Plates were then dried with tissue paper and cleaned two times with ethanol, and two times with distilled water.

Gel preparation was performed adding 30 mL of gel matrix as prepared above, and 225 µL of 10% APS (0.1 g ammonium persulfate in 1 mL double distilled water) was added and the solution was mixed well, and 22.5 µL of TEMED (N, N, N', N'-Tetramethylethylenediamine, AppliChem) was added and mixed evenly and then poured into the glass plates slowly without formation of air bubbles. 20 mL of gel was enough to cover the required area in between glass plates, the comb was placed and the remaining 10 mL was poured onto the comb for preventing its contact with air which delays the polymerization. Gel was left to dry for at least 1.5 hours for equal polymerization.

2.9.6 Gel Loading

The voltage of the LI-COR 4300 DNA Analyzer was set to 1500 Volt, the power was set to 40Watt, and the current was set to 40 mA, and the temperature was set to 45 $^{\circ}$C. The scan speed was set to 2 for the model 4300. After the gel is put into LI-COR 4300 DNA Analyzer, Saga Generation Software was started and programmed for the primer pairs used. After programming was finished the gel was pre-run for 30 minutes for preparing gel ready for the loading of the samples. The wells were flushed for removing the urea precipitates. Samples were loaded into each well (1 μL). The first and the last wells were loaded with size standards. The electrophoresis was kept for 3.5 hour; the image was collected in real-time.

2.9.7 Gel Analysis and Dendogram Formation

Gels were analyzed for the presence or absence of a band in the same row for every forty-seven sample isolates, on each gel for all the AFLP primer combinations studied indicating a locus or an allele. 47 samples were compared with each other by LICOR's imaging program SAGA MX, this program labels + for presence of the allele and – for the absence of the allele. However, in some situations personal interference was required for some loci. Sixteen gels were analyzed. In each gel, best sixty polymorphic loci were scored. In two of the gels, the number of polymorphic bands was recorded as sixty-two and sixty-five. Each sample had at least 60 polymorphic bands in each gel and they were grouped one after another starting from the first gel to the sixteenth gel and the same procedure was applied to each individual. The resultant excel file contained 47 samples each having 967 polymorphic bands one after another. The recorded numbers were uploaded into the NTSYS (Numerical Taxonomy and Multivariate Analysis System). The program was accepting the "excel files" so the data obtained was converted to the required file format and program was opened. In the program there were two sub-windows; first, **NTedit** program was opened. Following instructions were applied: **File→Import Excel→Using OLE**. While the file was importing, the cursor was

functionless and until it regained its function waiting was required. Then the output file was uploaded with **File→Save file as** and the file was saved with an NTS extension. This sub-window was closed and the second sub-window was opened namely **NTSYSpc**. **Similarity→SimQual** was chosen accordingly, then another window was opened. In the input file the previously saved file was loaded and coefficient was chosen as **Jaccard** and **DICE** for five gel analysis and **Simple Matching** for sixteen gel analysis. This difference is due to the very high number of polymorphic loci studied the program could not attempt to perform the sixteen gel analysis with Jaccard method. Output file was given a name and **Compute** option was chosen. By this way a second file was obtained. The small window is then closed. In the last part **Clustering→SAHN** options were chosen and in the input-file place the second file saved was loaded, output-tree file was named and clustering method was chosen as **UPGMA** and in case of ties option was chosen as **WARN**. After this computation the dendogram was formed. In order to obtain a sharper dendogram five of the gels were used in which a high degree of polymorphism is observed. Both of the dendograms were compared with each other. Neighbour Joining tree was also acquired and compared with the dendogram obtained. In order to obtain **NJoin**-tree, NJoin program was chosen under the heading of **"Clustering"**. In case of ties option was chosen as **WARN**. The input-file used was the second file obtained from the SIMQUAL window. **Principle Component Analysis (PCA)** was also performed. **Similarity** sub-window was chosen, **Interval data** module was opened. Input file was the first data file obtained from **NTedit** window, and coefficient was chosen as **CORR** (correspondence). Then **Ordination** sub-window was opened, **Eigen** module was selected. Input-file is the correlation file obtained above and degrees of freedom value is n-1 where n represents the species number studied. The result of this module gives the three dimensional view of the samples studied which is called as principle component analysis.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Total DNA Isolation from Powdery Mildew Isolates (*Bgh*)

DNA isolation was performed by CTAB DNA isolation method (Saghaimaroof, Soliman et al. 1984) and the concentrations were obtained by Nano Drop ND-1000 Spectrophotometer. The exact concentration values have to be known in order to obtain significant banding pattern in AFLP because any concentration difference may cause misscoring in the gel image.

DNA concentrations are presented in Table 3.1. The samples were diluted so that every sample contains 40 ng/µL of DNA, and 5 µL of DNA from those diluted samples are used for reactions (200 ng per sample). This step is critical, since the concentration fluctuations among samples concentrations could cause unequal amount of amplification which can affect reproducibility of the experiments.

Table 3.1 DNA concentrations of *Blumeria graminis f. species hordei* spores. "Loading order" is represented as L.order., I.Con. is "initial concentration", Name is "name and/or number of the samples".

L. order	I. Con.	Name	L. order	I. Con.	Name
1	156.8 ng/µL	B4C15	25	58.9 ng/µL	21(2)
2	240.6 ng/µL	B21	26	98.9 ng/µL	22
3	243.3 ng/µL	B91	27	53.5 ng/µL	24
4	213.5 ng/µL	B95	28	111.6 ng/µL	35(2)
5	212.1 ng/µL	B100	29	502.2 ng/µL	36
6	118.0 ng/µL	B103	30	178 ng/µL	43
7	154.8 ng/µL	B120	31	259.1 ng/µL	46
8	218.5 ng/µL	B121	32	311.5 ng/µL	52
9	41.5 ng/µL	1	33	80.2 ng/µL	53
10	230.3 ng/µL	4	34	365.0 ng/µL	55(2)
11	243.5 ng/µL	5	35	41.8 ng/µL	56(2)
12	372.9 ng/µL	6	36	353.9 ng/µL	57
13	24.7 ng/µL	7	37	362.8 ng/µL	58
14	177.8 ng/µL	8	38	165.6 ng/µL	59
15	330.9 ng/µL	9	39	146.3 ng/µL	60
16	499.1 ng/µL	11	40	133.0 ng/µL	61(2)
17	38.5 ng/µL	12	41	250.4 ng/µL	64
18	160.9 ng/µL	13	42	36.2 ng/µL	66(2)
19	587.9 ng/µL	15	43	136.6 ng/µL	68(2)
20	399.7 ng/µL	15(2)	44	373.1 ng/µL	69
21	99.9 ng/µL	17	45	127.4 ng/µL	71(2)
22	31.8 ng/µL	17(2)	46	193.5 ng/µL	72
23	26.2 ng/µL	20	47	774.4 ng/µL	76
24	163.1 ng/µL	20(2)			

***** The samples names with "B" are the differentially universal isolates/races with known virulence.

3.2 Evaluation of Polymorphic Loci

In sixteen AFLP gels with different primer combinations 967 polymorphic loci were detected (Figures 3.1-3.16). Detection of the gels was performed by forming 0-1 matrixes in which 0 indicates the absence, and 1 indicates the presence of the allele (Tables 3.3-3.18). In the SagaMX program provided by LI-COR, the gels were scanned in real time and they were scored at the end of the run by giving + if there is a band and – if there is not band, and the program inserts an empty box (□) when it cannot differentiate whether the presence or the absence of the band. The gels and scoring were examined by eye and for every gel at least sixty polymorphic loci were detected. In some gels there was a high degree of polymorphism (Figures 3.9, 3.10, 3.13, 3.14, 3.15). The polymorphisms from these gels were also analyzed separetly and the genetic relationship data were compared to the data resulting from all the primer set combinations.

Table 3.2 Gels studied under the name of the primer sets. “S. No” indicates gel number, “primer pair” explains which primer pair is studied in each gel, and “P. Loci scored” is the polymorphic loci scored from the gel. Gel images and matrixes presented below are in the same order with this table.

S. no	Primer pair	P. loci scored
1	M-CAC_E-AAG	60
2	M-CAC_E-ACT	60
3	M-CAC_E-ACA	60
4	M-CAC_E-AGC	60
5	M-CAT_E-AAC	60
6	M-CAT_E-ACG	60
7	M-CAG_E-ACC	60
8	M-CAG_E-AGG	60
9	M-CTA_E-ACC	62
10	M-CTA_E-AGG	60
11	M-CTA_E-AAC	60
12	M-CTA_E-AGC	60
13	M-CTC_E-AAC	60
14	M-CTC_E-ACG	60
15	M-CAT_E-ACA	60
16	M-CAT_ E-AGG	65

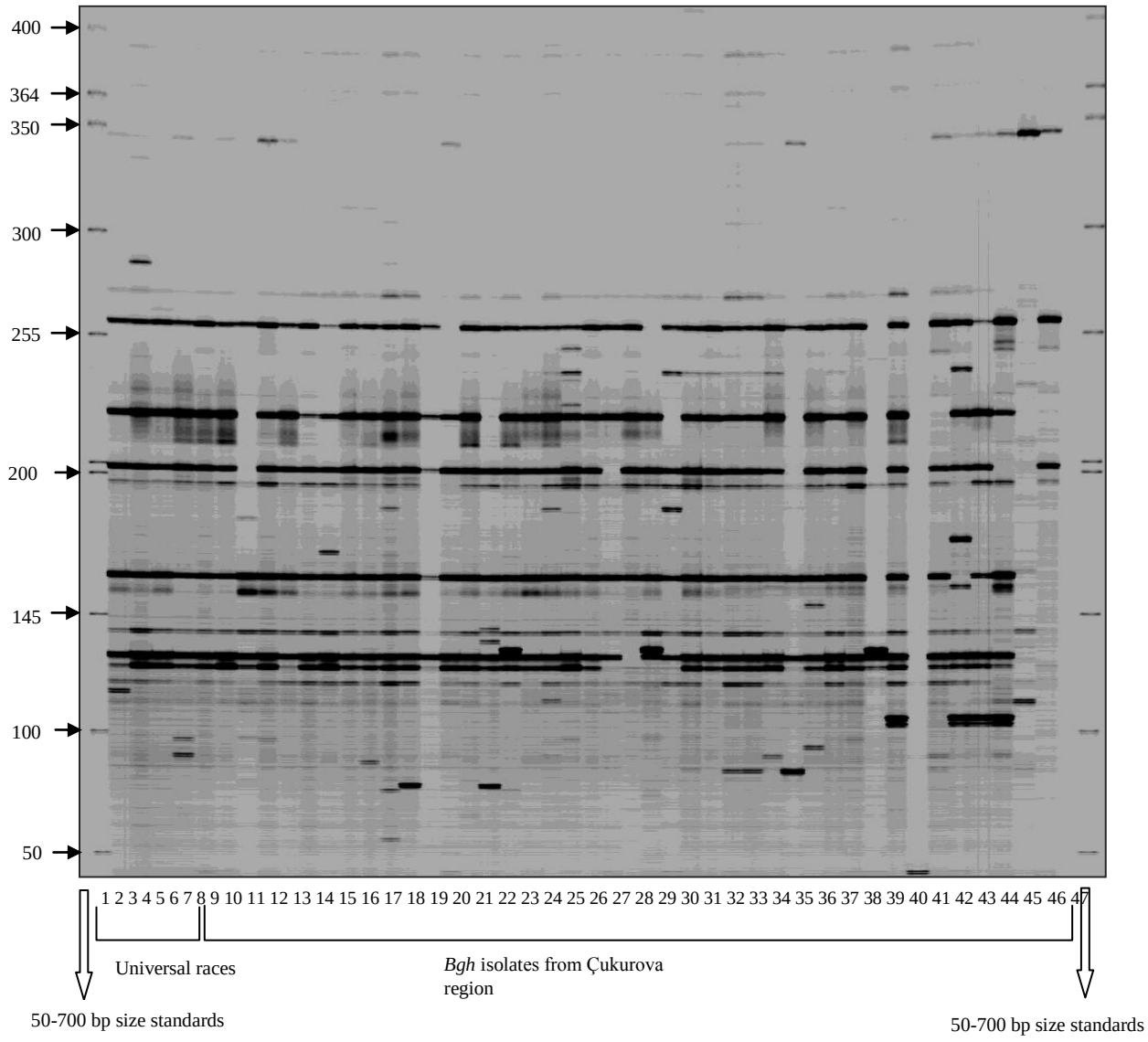


Figure 3.1 M-CAC/E-AAG primer combination, read at 700 nm channel. The number of polymorphic loci scored was 60.

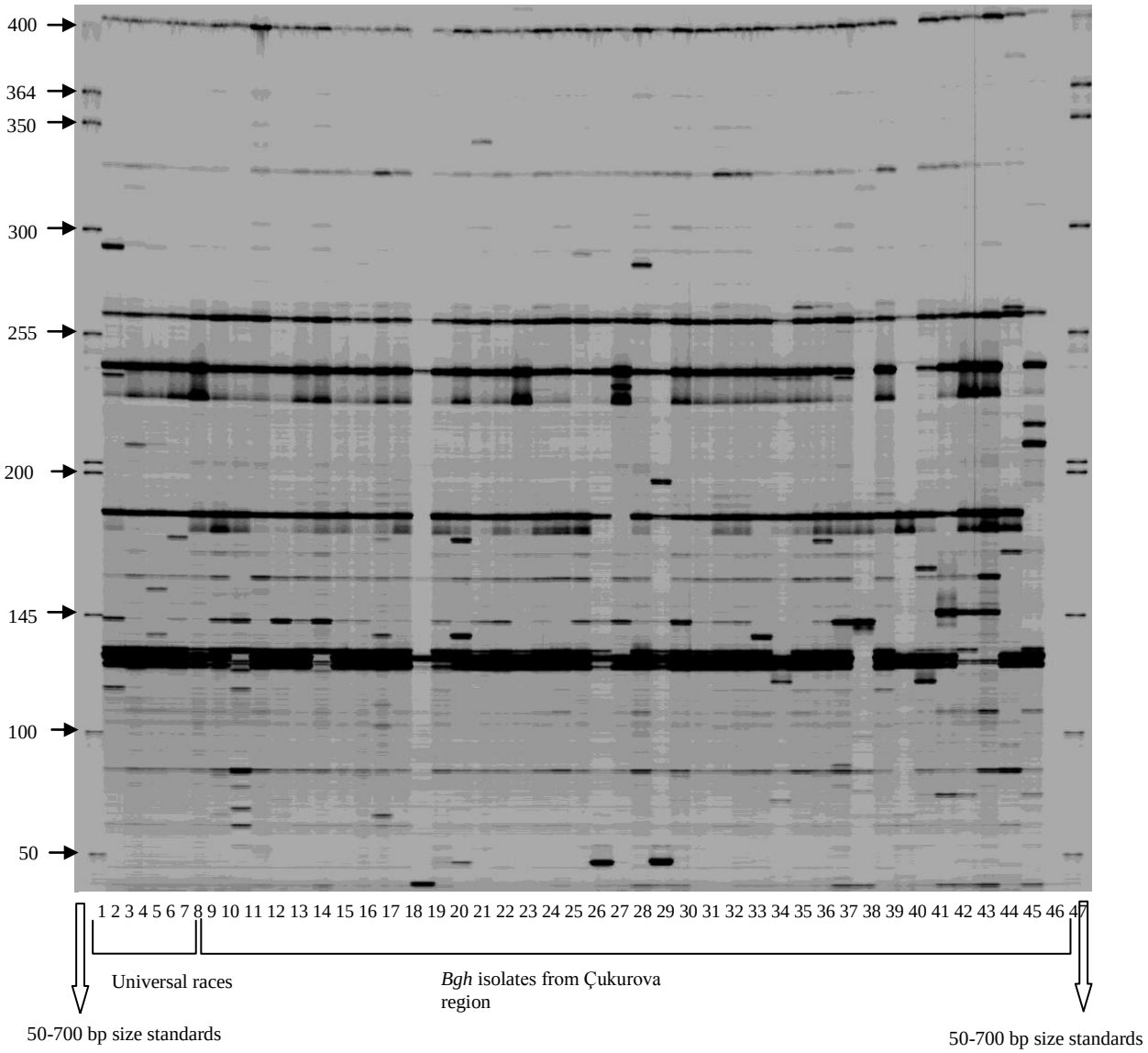


Figure 3.2 M-CAC/E-ACT primer combination read at 800 nm channel. The number of polymorphic loci scored was 60.

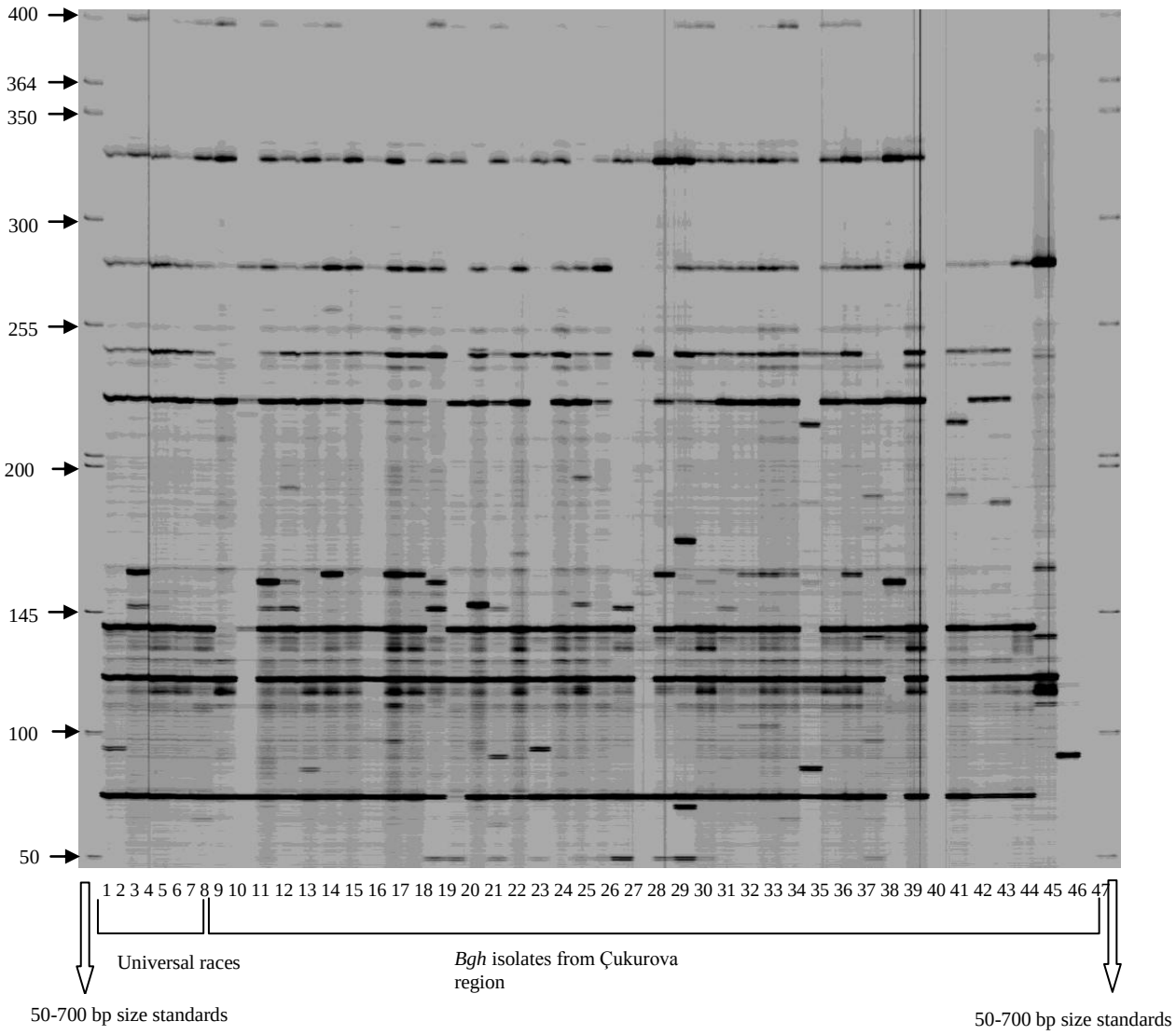


Figure 3.3 M-CAC/E-ACA primer combination read at 700 nm channel. The number of polymorphic loci scored was 60.

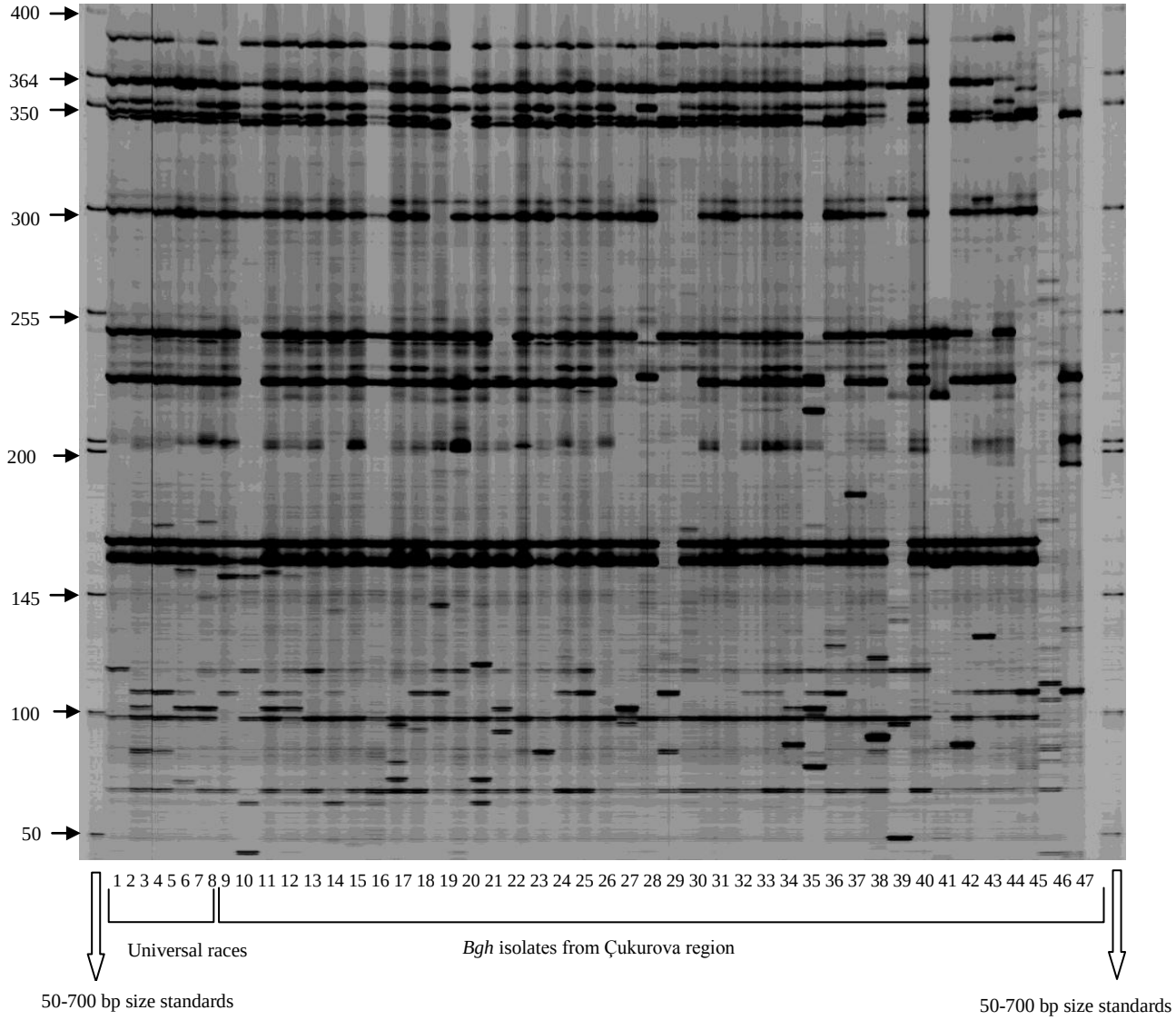


Figure 3.4 M-CAC/E-AAG primer combination read at 700 nm channel. The number of polymorphic loci scored was 60.

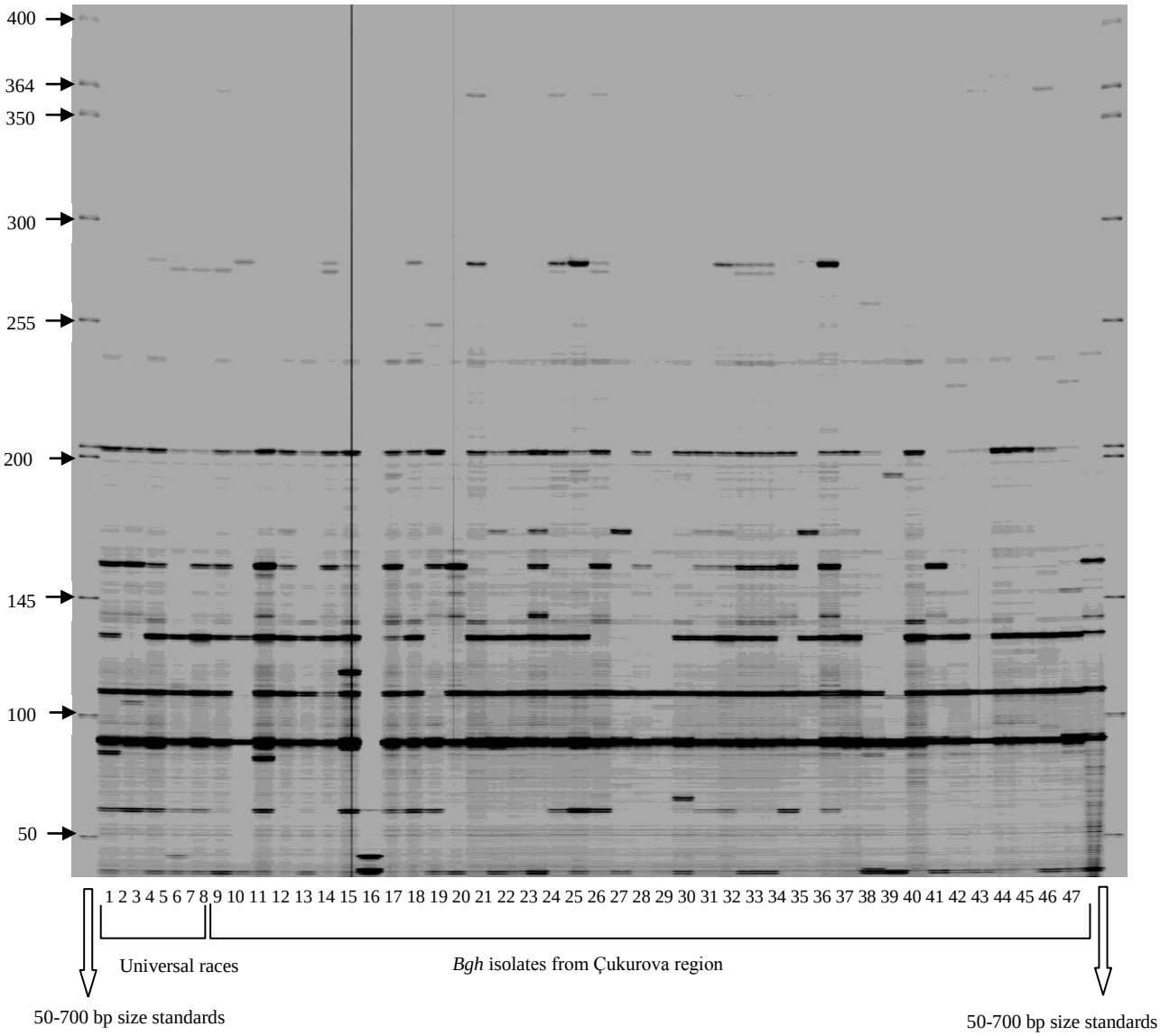


Figure 3.5 M-CAT/E-AAC primer combination read at 700 nm channel. The number of polymorphic loci scored was 60.

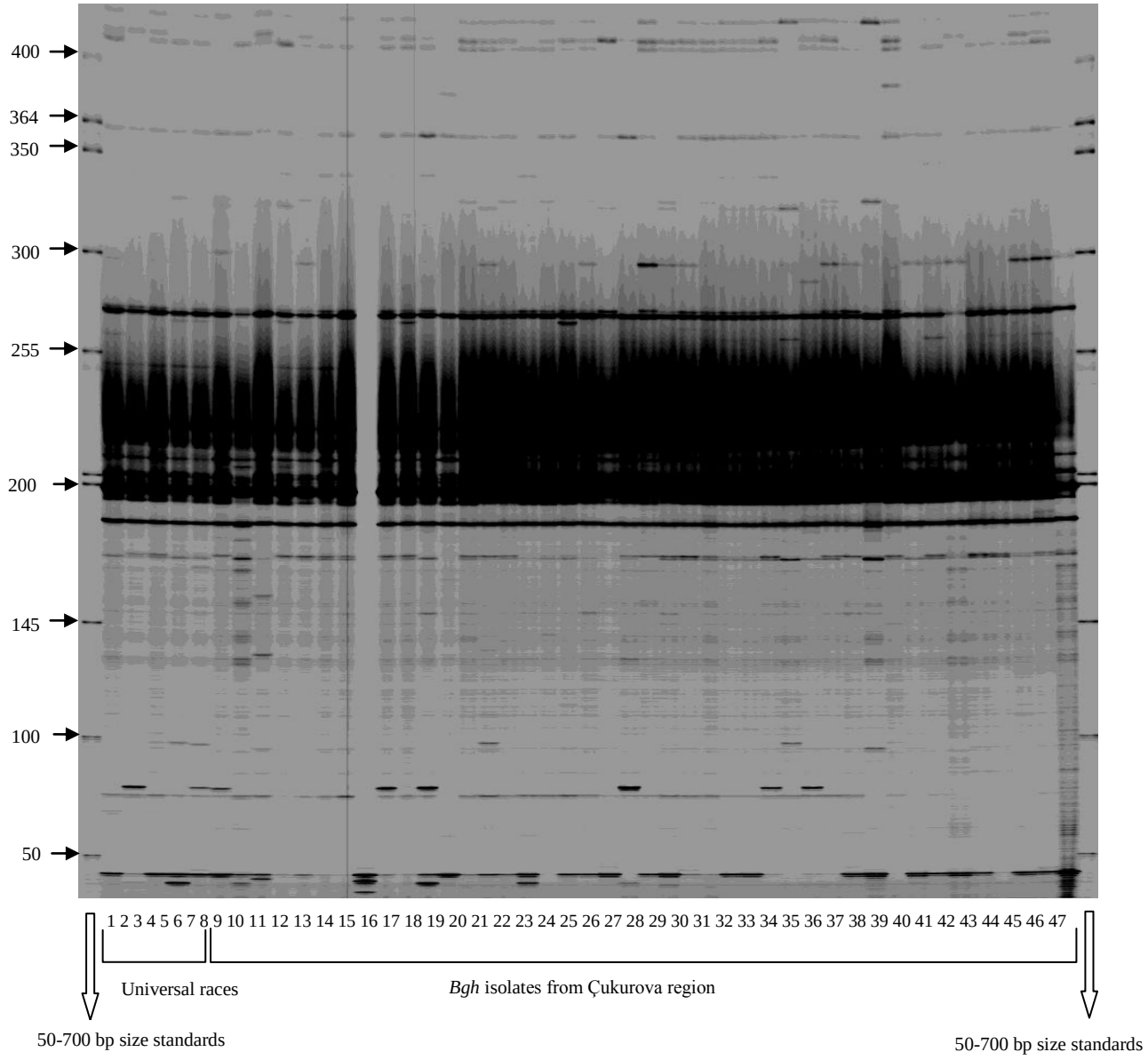


Figure 3.6 M-CAT/E-ACG primer combination read at 800 nm channel. The number of polymorphic loci scored was 60.

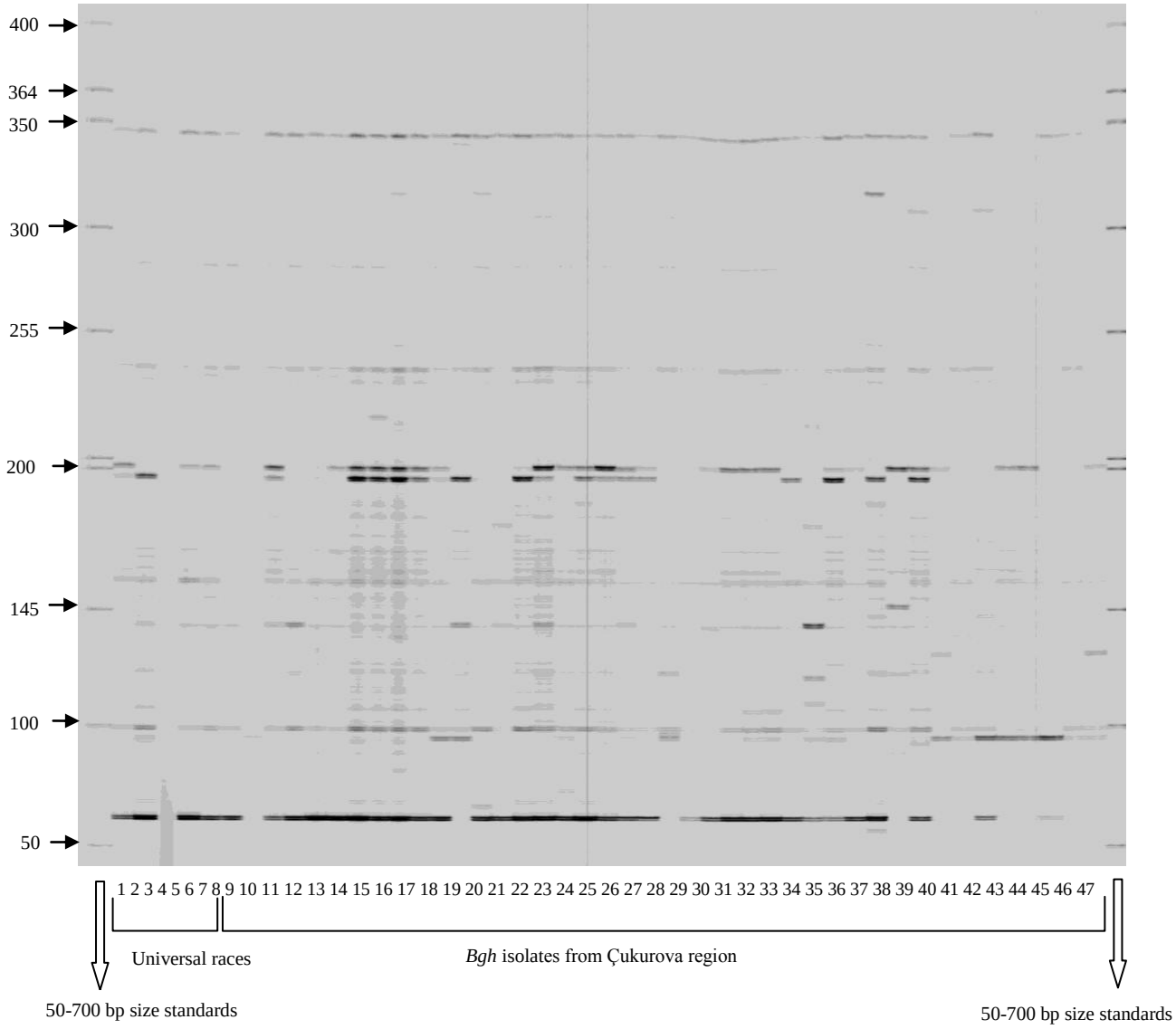


Figure 3.7 M-CAG/E-AAC primer combination read at 700 nm channel. The number of polymorphic loci scored was 60.

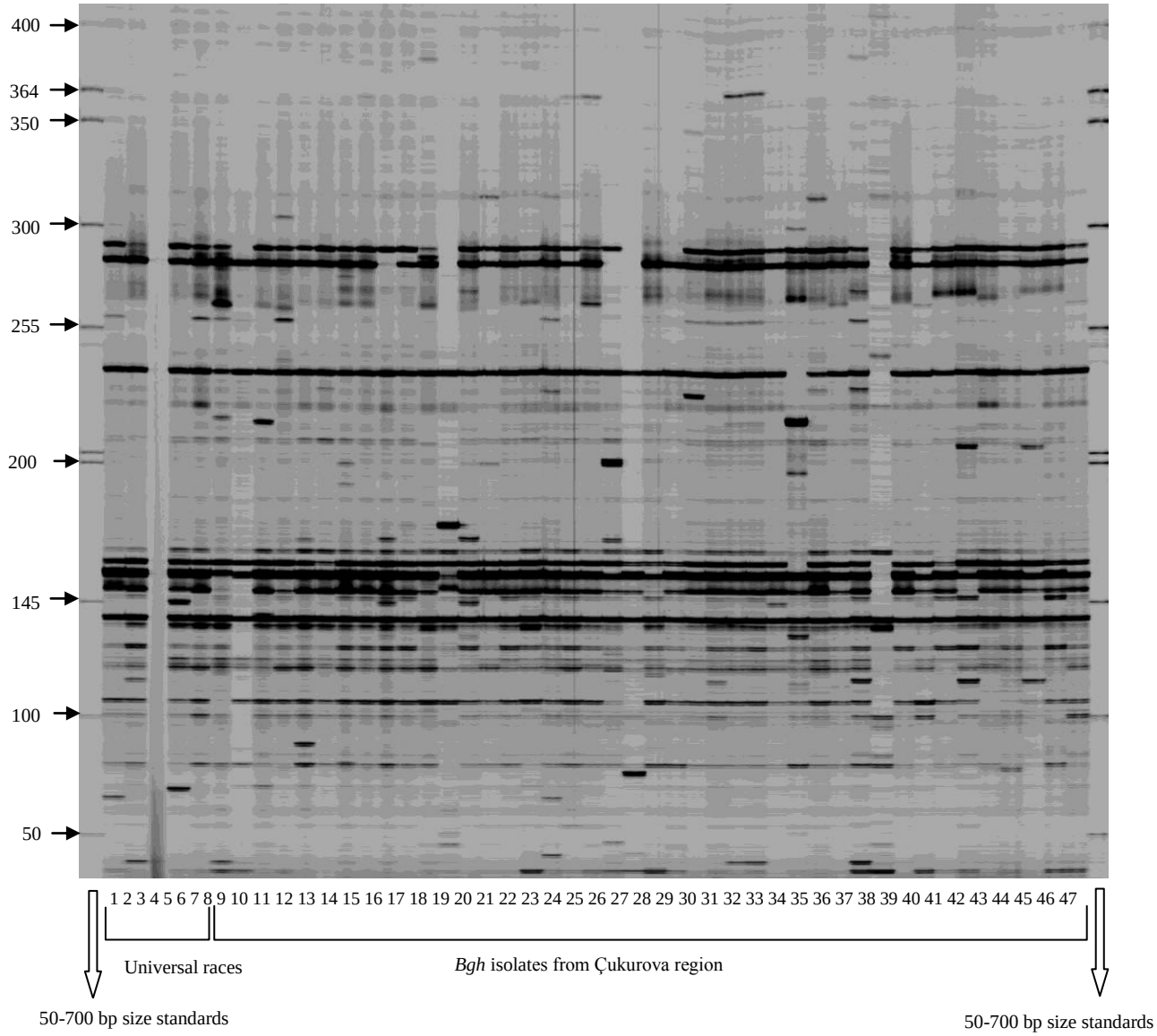


Figure 3.8 M-CAG/E-AGG primer combination read at 800 nm channel. The number of polymorphic loci scored was 60.

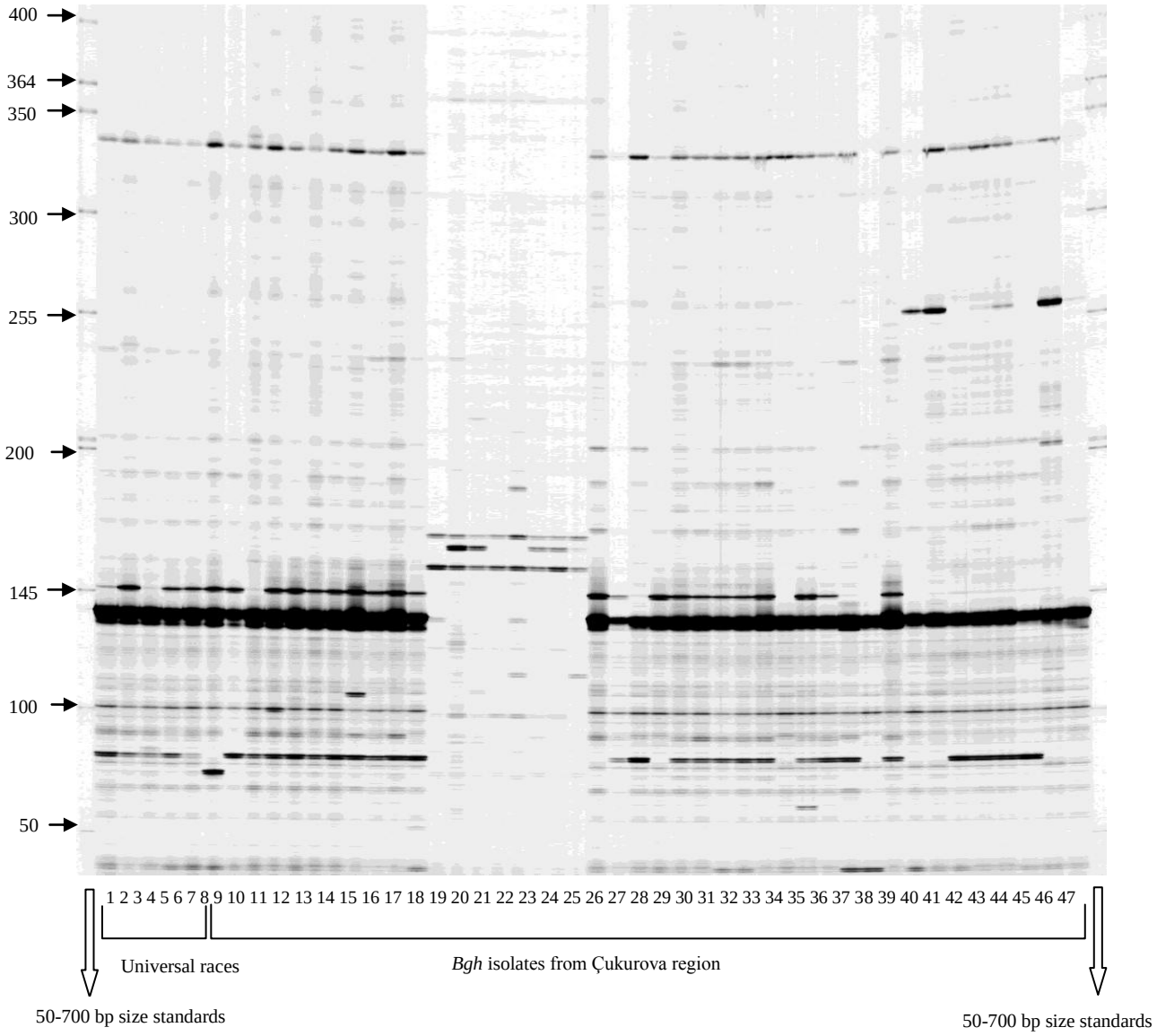


Figure 3.9 M-CTA/E-ACC primer combination read at 700 nm channel. The number of polymorphic loci scored was 62.

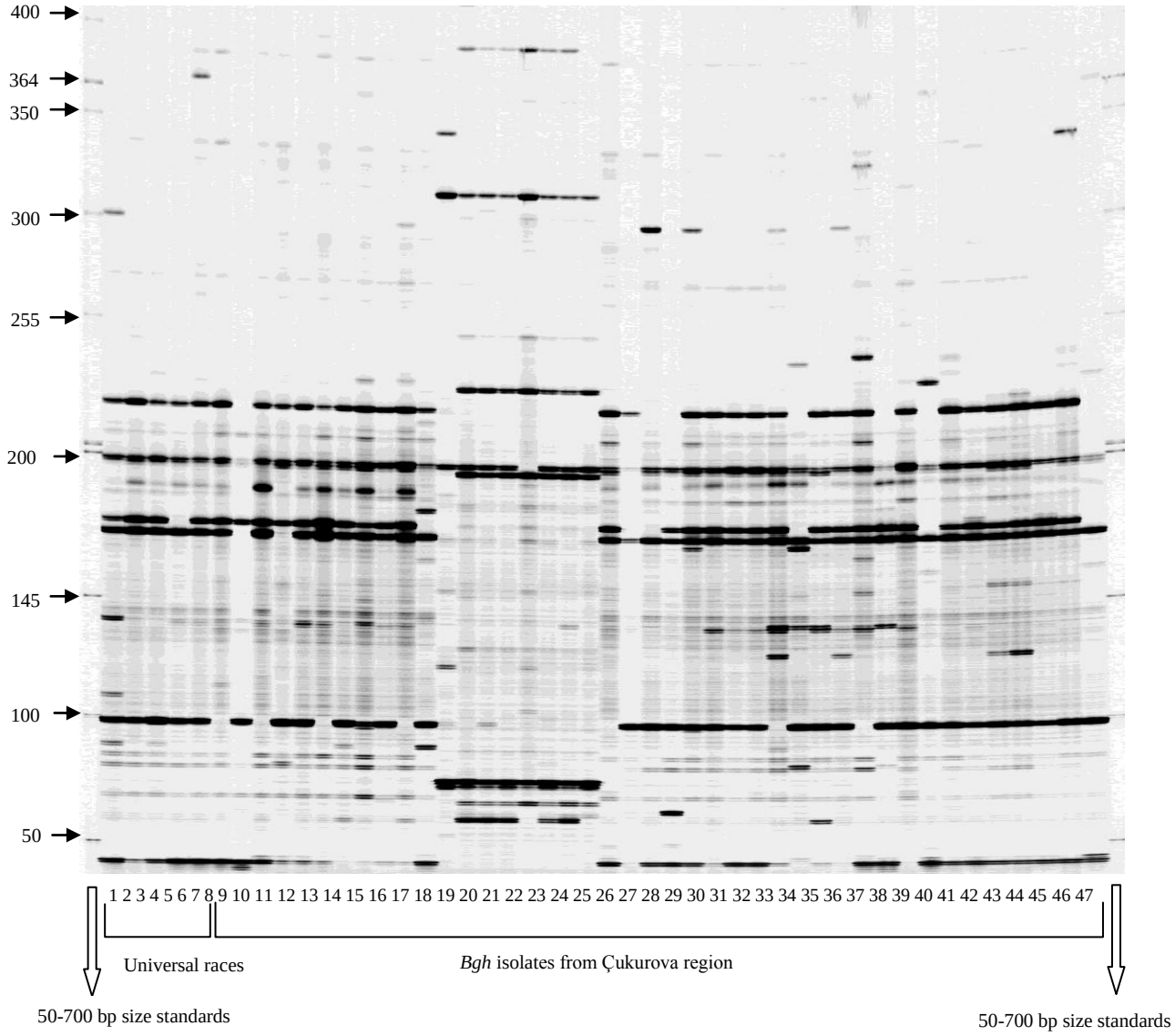


Figure 3.10 M-CAA/E-AGG primer combination read at 800 nm channel. The number of polymorphic loci scored was 60.

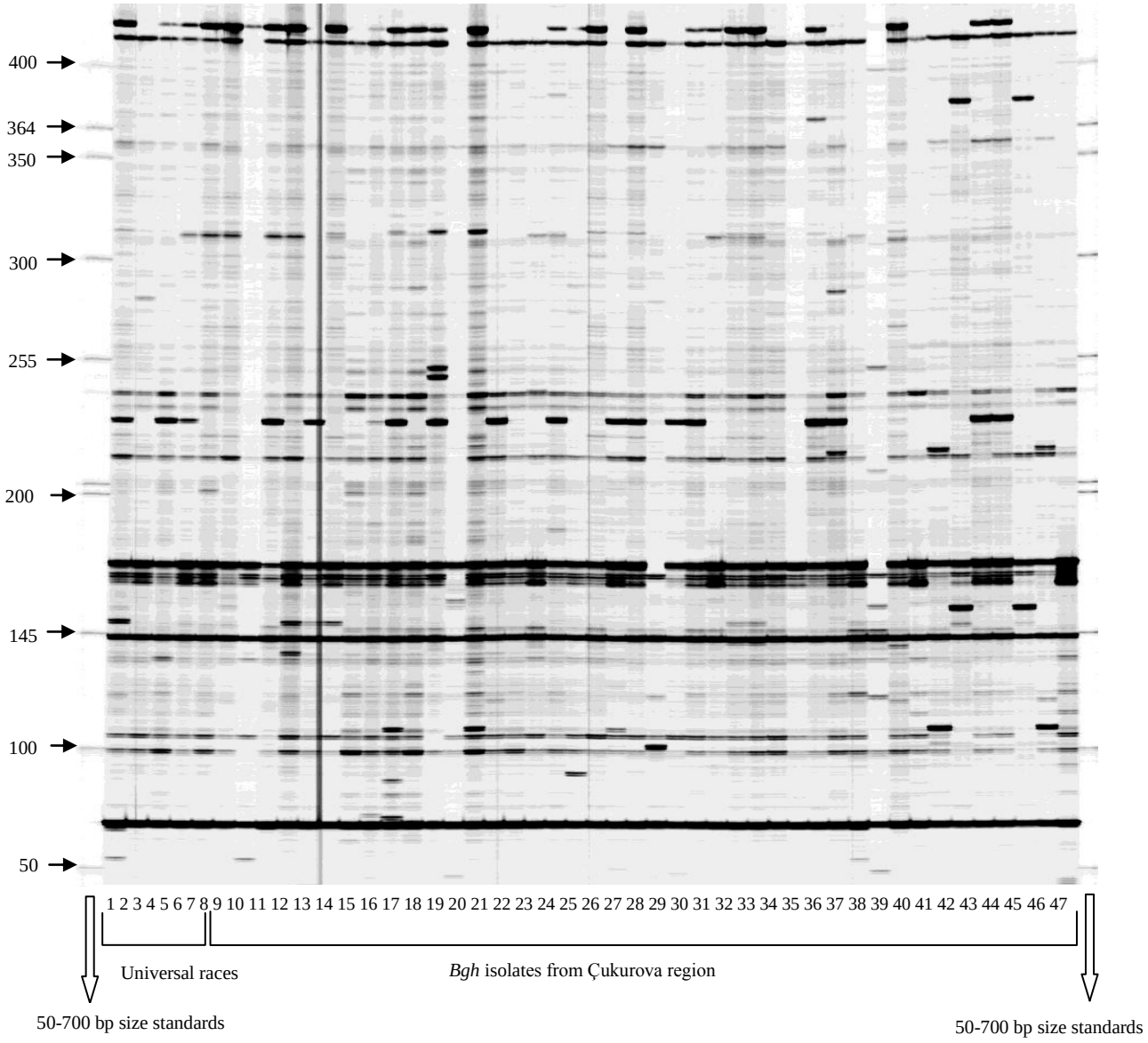


Figure 3.11 M-CTA/E-AAC primer combination read at 700 nm channel. The number of polymorphic loci scored was 60.

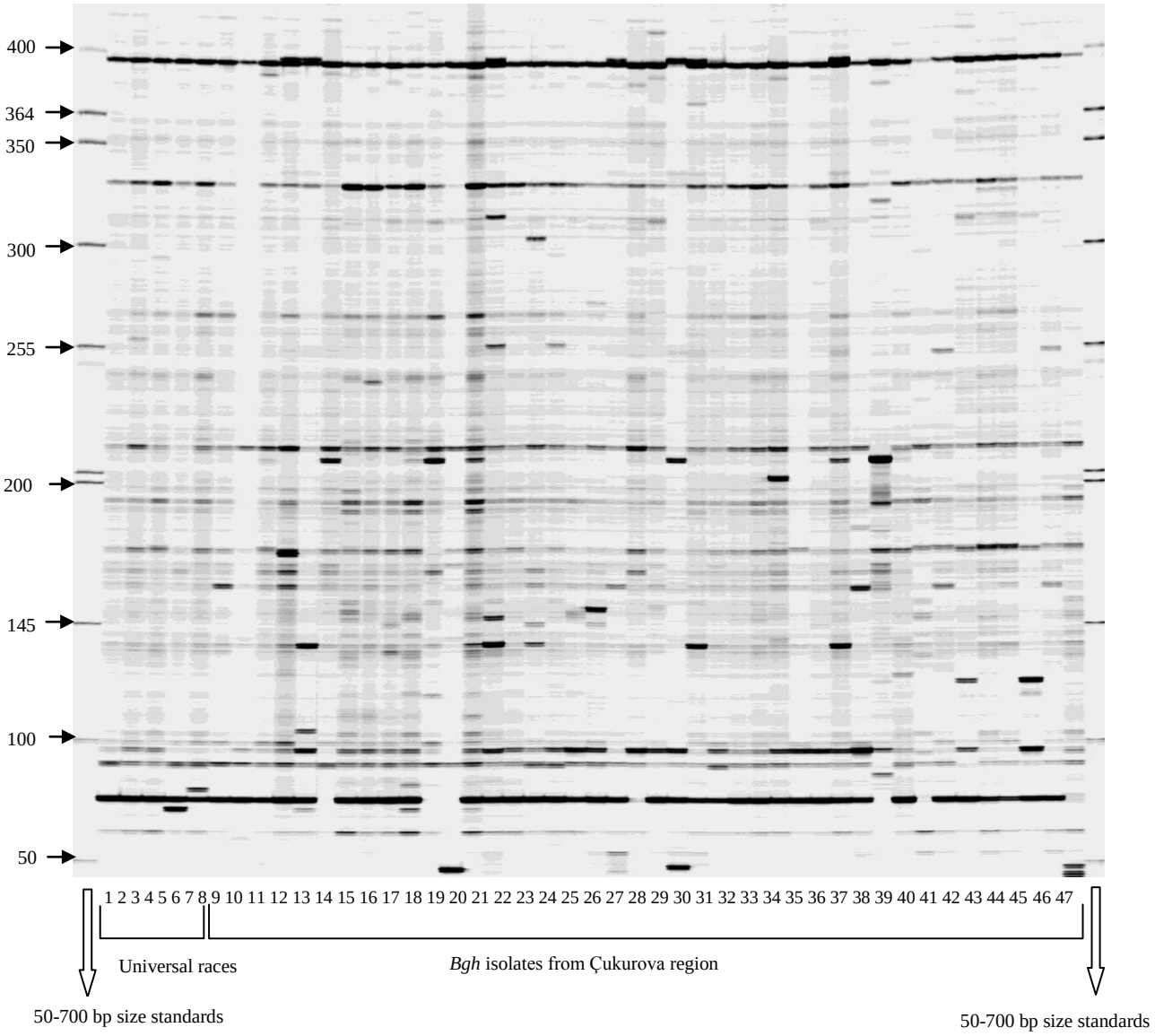


Figure 3.12 M-CTA/E-ACG primer combination read at 800 nm channel. The number of polymorphic loci scored was 60.

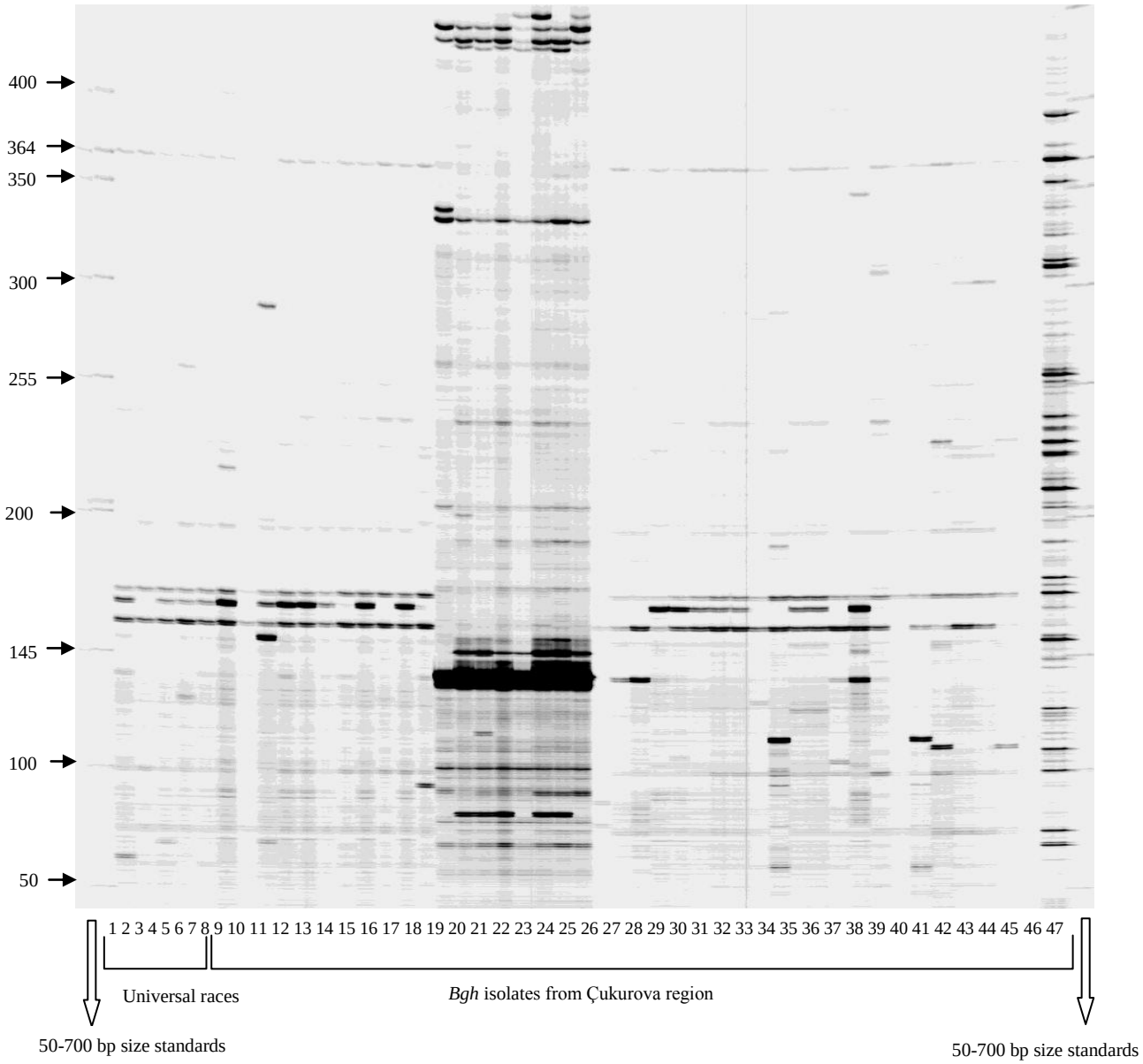


Figure 3.13 M-CTC/E-AAC primer combination read at 700 nm channel. The number of polymorphic loci scored was 60.

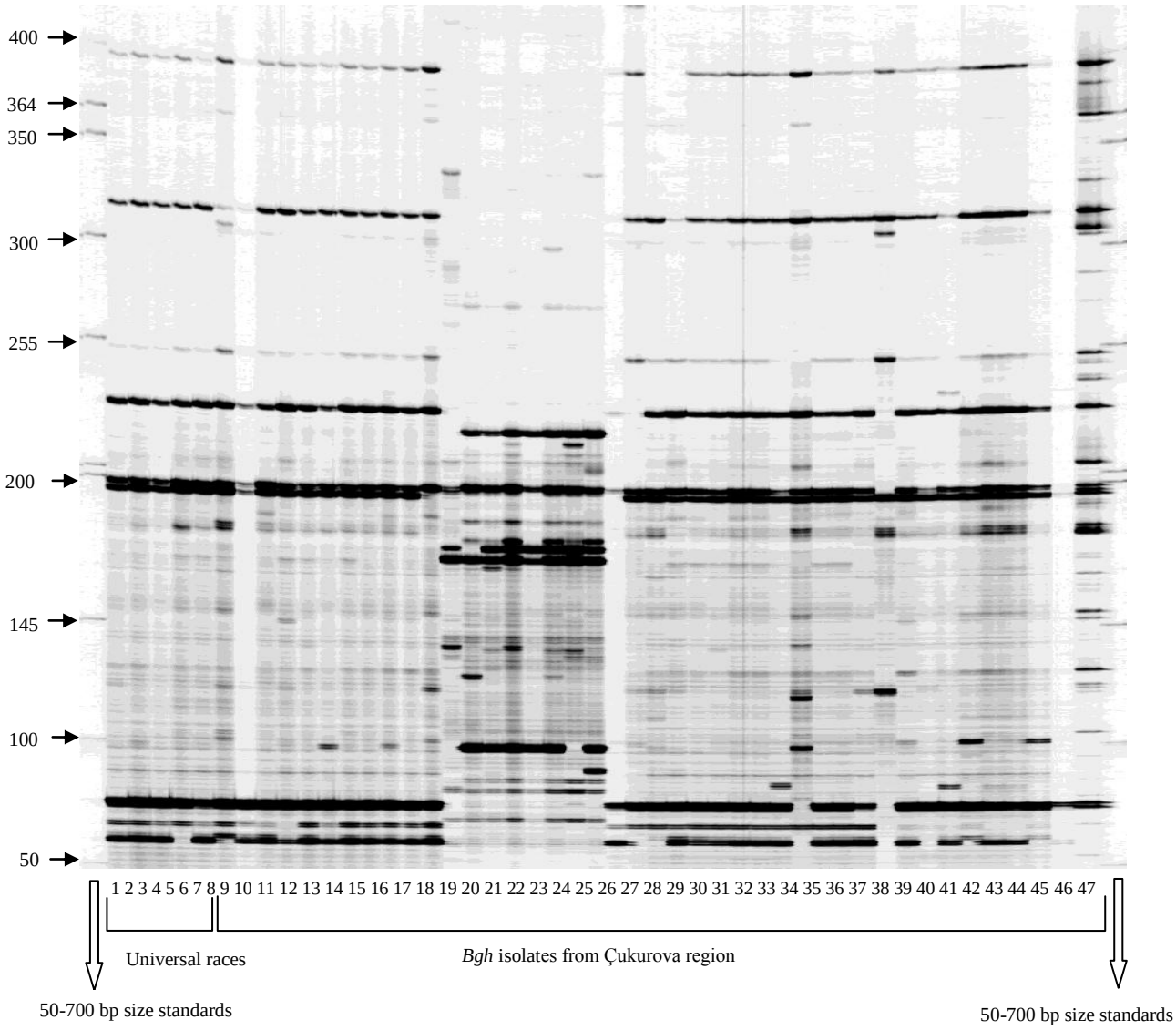


Figure 3.14 M-CTC/E-ACG primer combination read at 800 nm channel. The number of polymorphic loci scored was 60.

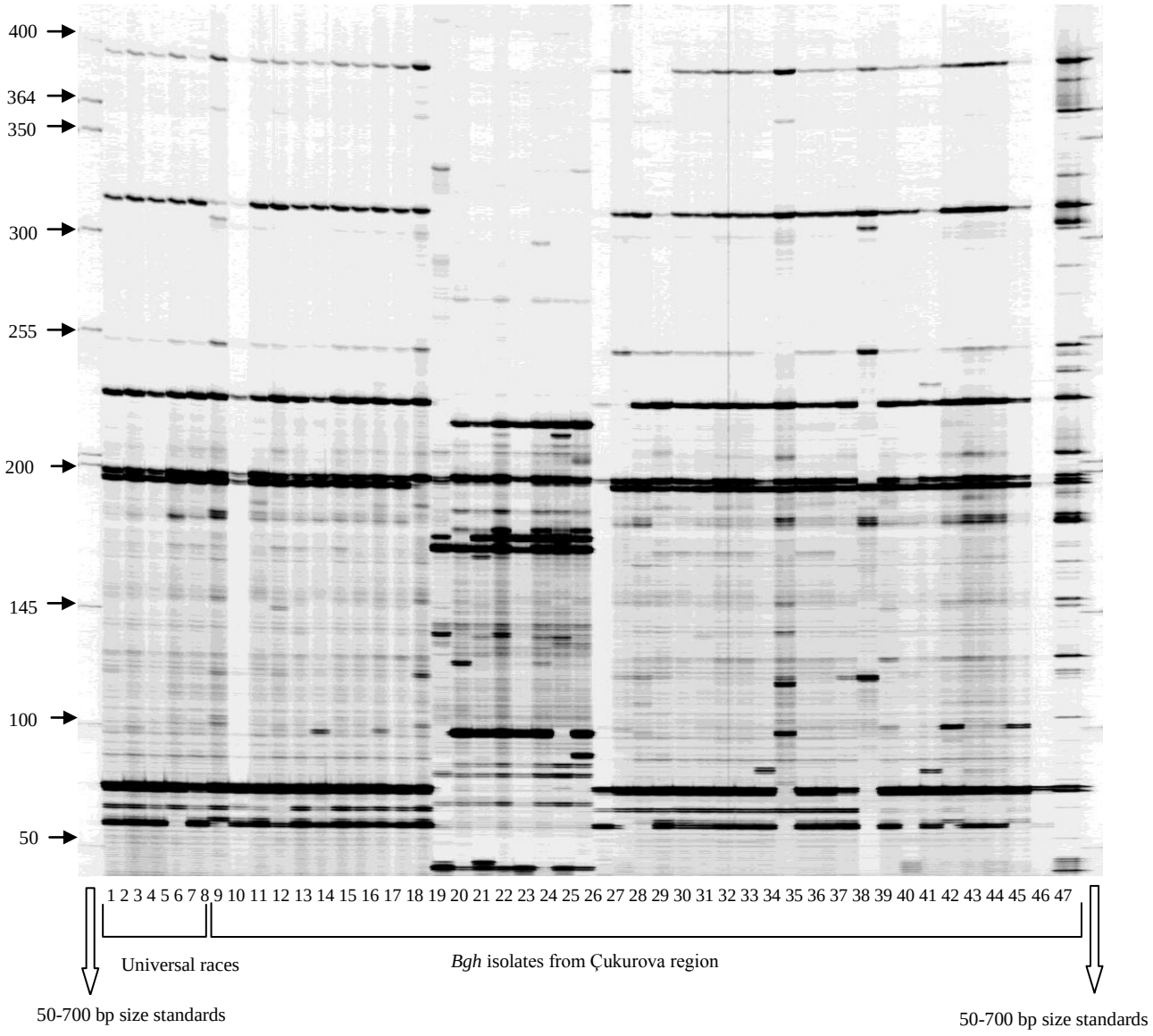


Figure 3.15 M-CAT/E-ACA primer combination read at 700 nm channel. The number of polymorphic loci scored was 60.

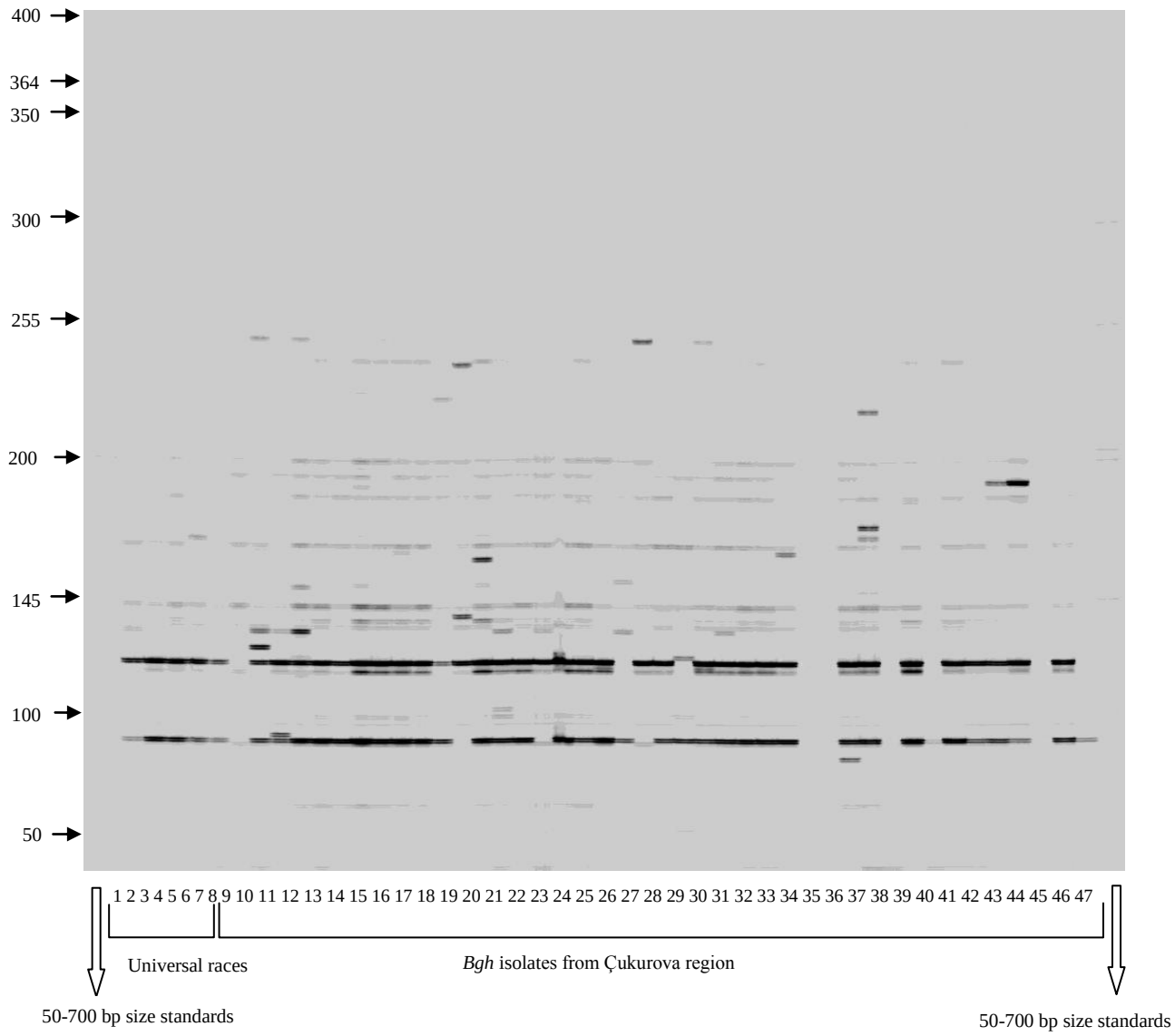


Figure 3.16 M-CAT/E-AGG primer combination read at 800 nm channel. The number of polymorphic loci scored was 65.

3.3 Phylogenetic Analysis with NTSYS

The detected loci were analysed with NTSYS program, and with versions 2.02 and 2.1. First of all the gels were analysed by eye. All the analysis was performed for sixteen primer combinations and five primer combinations independently. Because among sixteen gels analysed five of the gels showed a great number of polymorphism (Figures 3.9, 3.10, 3.13, 3.14, and 3.15). Pyhlogenetic tree, Neighbour Joining tree, two and three dimensional principle coordinate analysis were performed for sixteen and five primer pair containing sets independently. All the analysis was performed by NTSYS.

According to dendogram done by using UPGMA (Simple Matching module) method and by using all sixteen gel images Figure 3.17 is obtained. In this figure there are two general roots. One of them was isolates from Çukurova region and all other forty-six samples were in the other root. This means that isolate 76 is a very distant relative to all the other isolates. The remaining of the dendogram is divided into two general branches one of them is the universal race B120, meaning that B120 is like isolate 76 a distant relative of the samples studied. In general universal isolates are clustered together apart from B91 which is a relative of Turkish isolates 55(2) is the closest relative, isolates 46 and 52 are seemed to be very similar to each other.

In Figure 3.18 dendogram was formed by using UPGMA (DICE) method and five primer combinations. In this dendogram B120 is again the most distant relative of the samples. However in this five primer combinations, isolates 6, and 11 seems to be closely related with the universal races, they are clustered together. Samples 68 and 69 are also very similar to each other. If we compare this dendogram with the previous one; there are three general branches diverging by B120 alone, and two additional roots, and in this dendogram universal races seems to be interfered with Çukurova isolates more than the previous one.

In Figure 3.19 dendogram was formed by using UPGMA (JACKARD) method and five primer combinations. This dendogram was quite similar to the previous one obtained with the DICE method; however the coefficients of dissimilarity varied to some extent.

Dendogram formed by using Neighbour Joining method with sixteen primer combinations were shown in Figure 3.20. This method is based on the "least parsimony principle". In other words this is a particular situation of minimum evolution method (Rzhetsky and Nei 1992) and the method requires an unbiased distance measure (Tateno, Takezaki et al. 1994). There are some differences in clustering in between the sixteen and five primer combinations containing Neighbour Joining methods. Whereas two Neighbour joining dendograms formed by five primer combinations and with different modules (DICE and JACCARD) seemed to be very similar to each other in other words they are consistent with each other (Figures 3.20, 3.21,and 3.22).

In Figure 3.23 two-dimensional representation of the five primer combination is calculated. It is clearly seen that two groups are formed. In Figure 3.24 two-dimensional representation of the sixteen primer combination is characterized and four groups are formed in this case.

In Figures 3.25 and 3.26, three-dimensional representations of the sixteen and five primer combinations are shown. Two and three-dimensional representations are consistent with each other.

The first two Eigen values for five-primer combinations are 34.35% and 9.81 and for 41.22%, and 5.16% for sixteen primer combinations (Figures 3.23, and 3.24). These results indicate that our analysis was accurate.

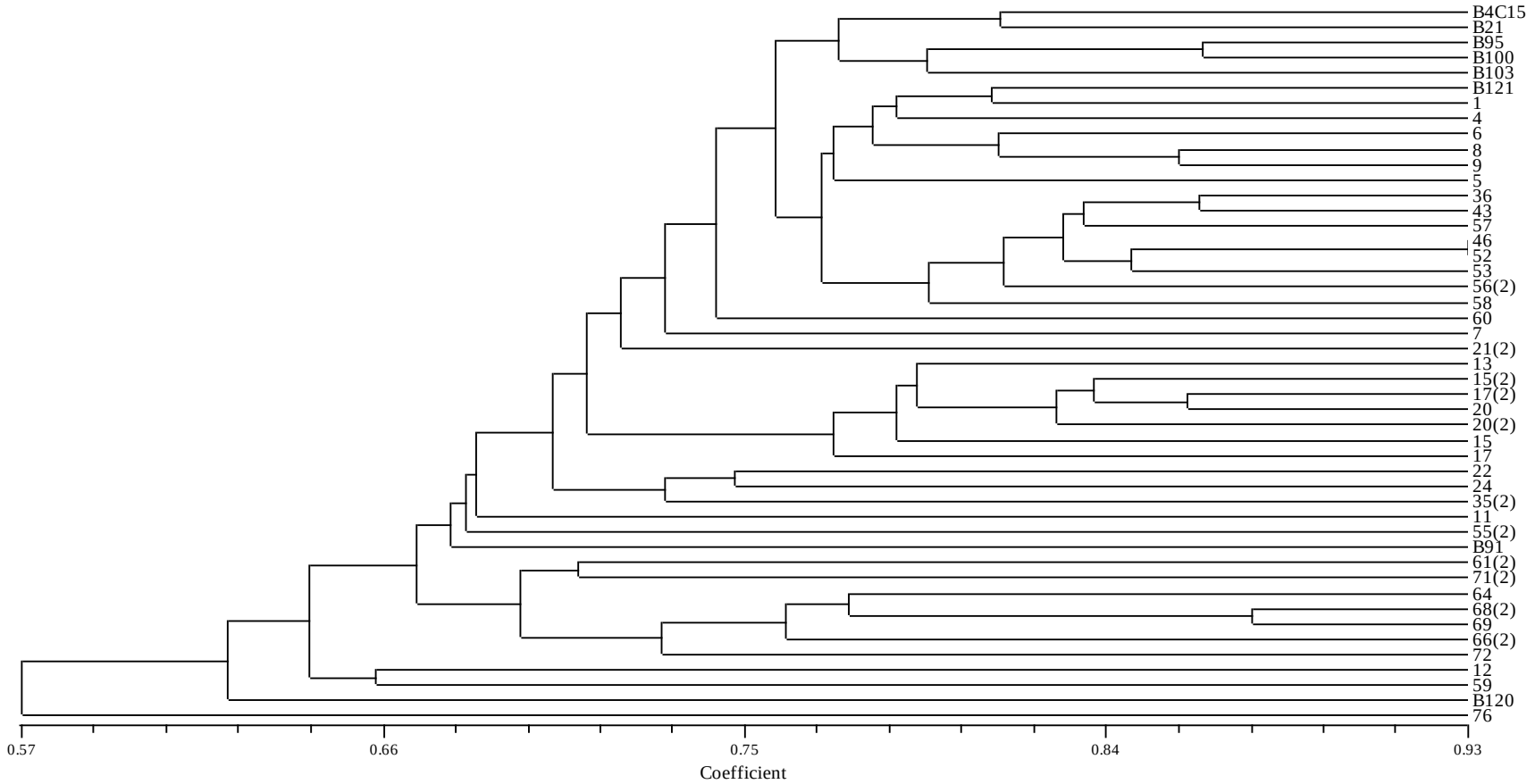


Figure 3.17 Dendogram of individuals based on Nei's (1972) genetic distance and UPGMA done *via* Simple Matching method by NTSYS 2.02 and 2.2 Sixteen matrixes made from different primer combinations were used at least sixty different polymorphic loci were detected in order to form matrixes, making up 967 loci totally.

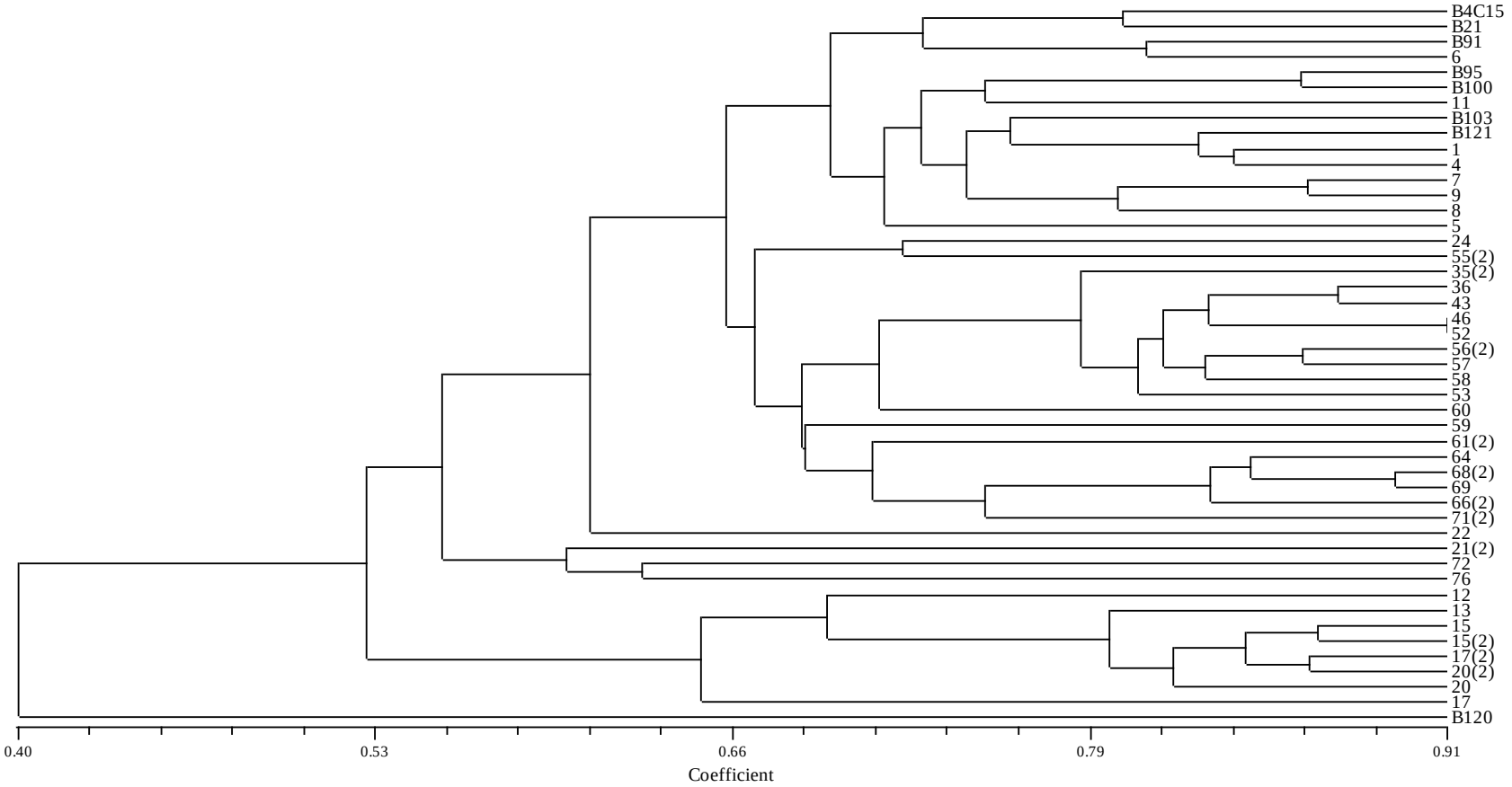


Figure 3.18 Dendogram of individuals based on Nei's (1972) genetic distance and UPGMA done *via* DICE method by NTSYS 2.02 and 2.2. Five matrixes made from different primer combinations were used, and at least sixty different polymorphic loci were detected in order to form matrixes, making up 302 loci totally.

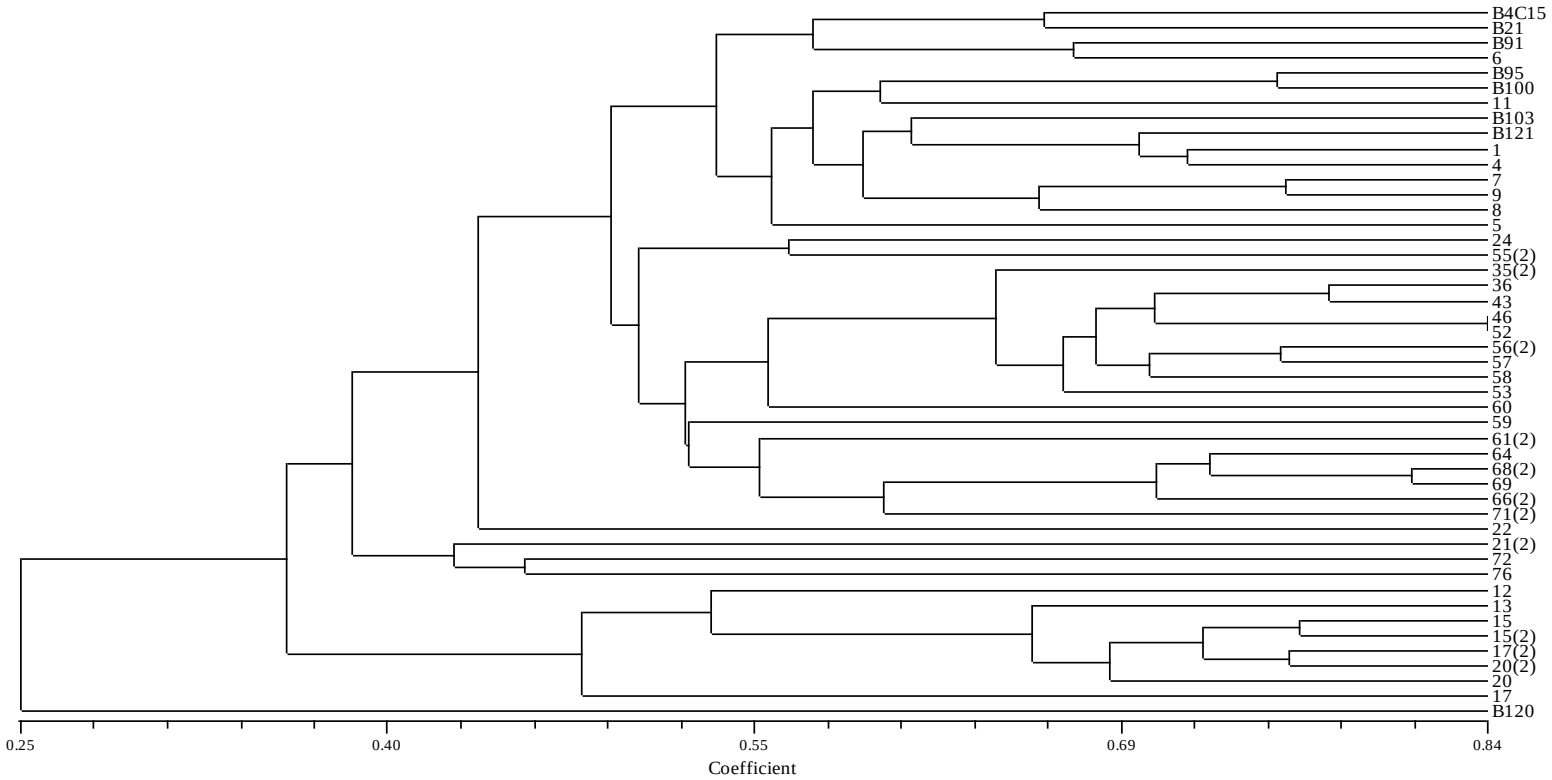


Figure 3.19 Dendogram of individuals based on Nei's (1972) genetic distance and UPGMA done *via* JACKARD method by NTSYS 2.02 and 2.2. Five matrixes made from different primer combinations were used, and at least sixty different polymorphic loci were detected in order to form matrixes, making up 302 loci totally.

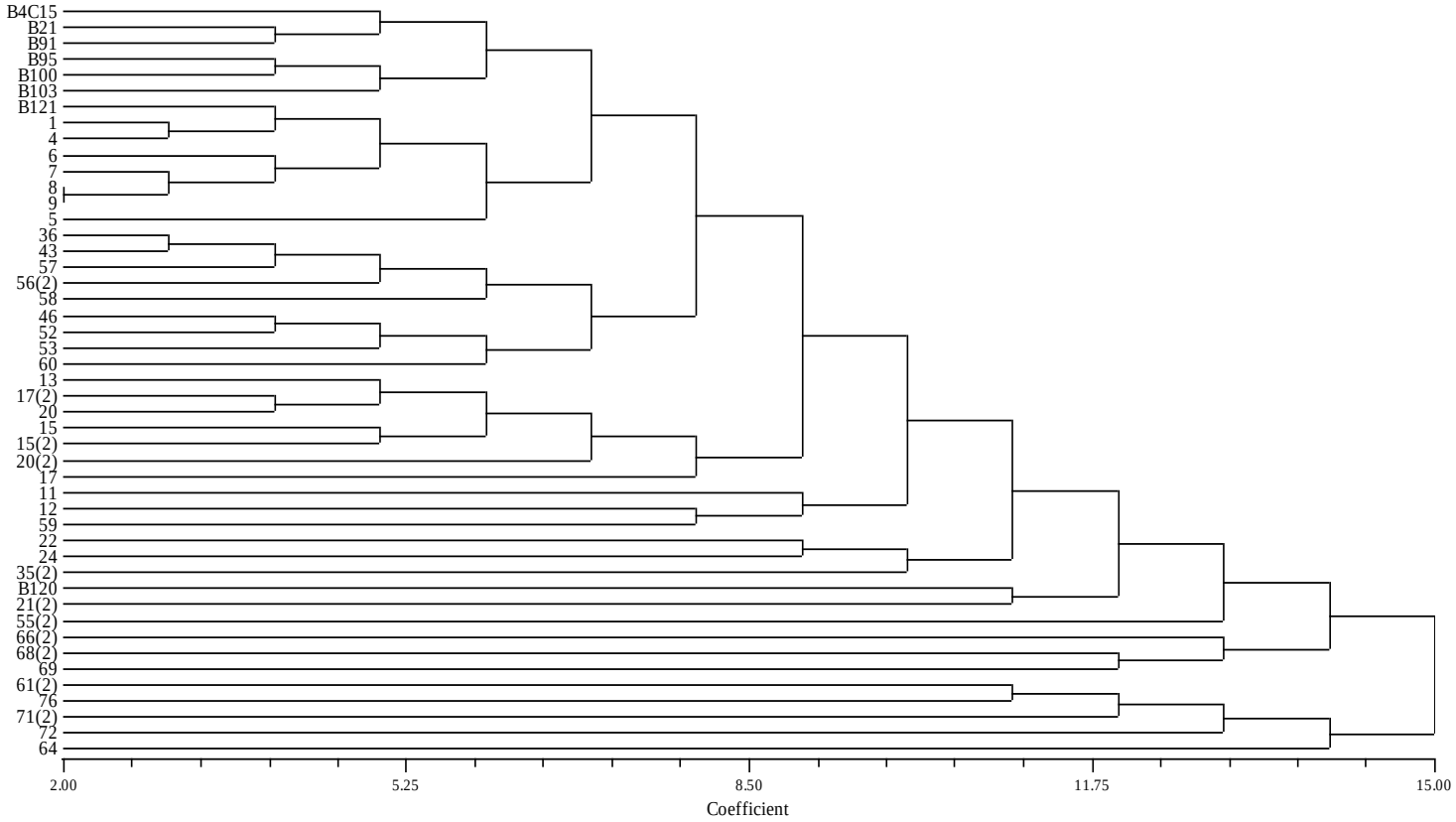


Figure 3.20 Dendogram formed by using Neighbour joining method. Sixteen matrixes made from different primer combinations were used, and at least sixty different polymorphic loci were detected in order to form matrixes, making up 967 loci totally.

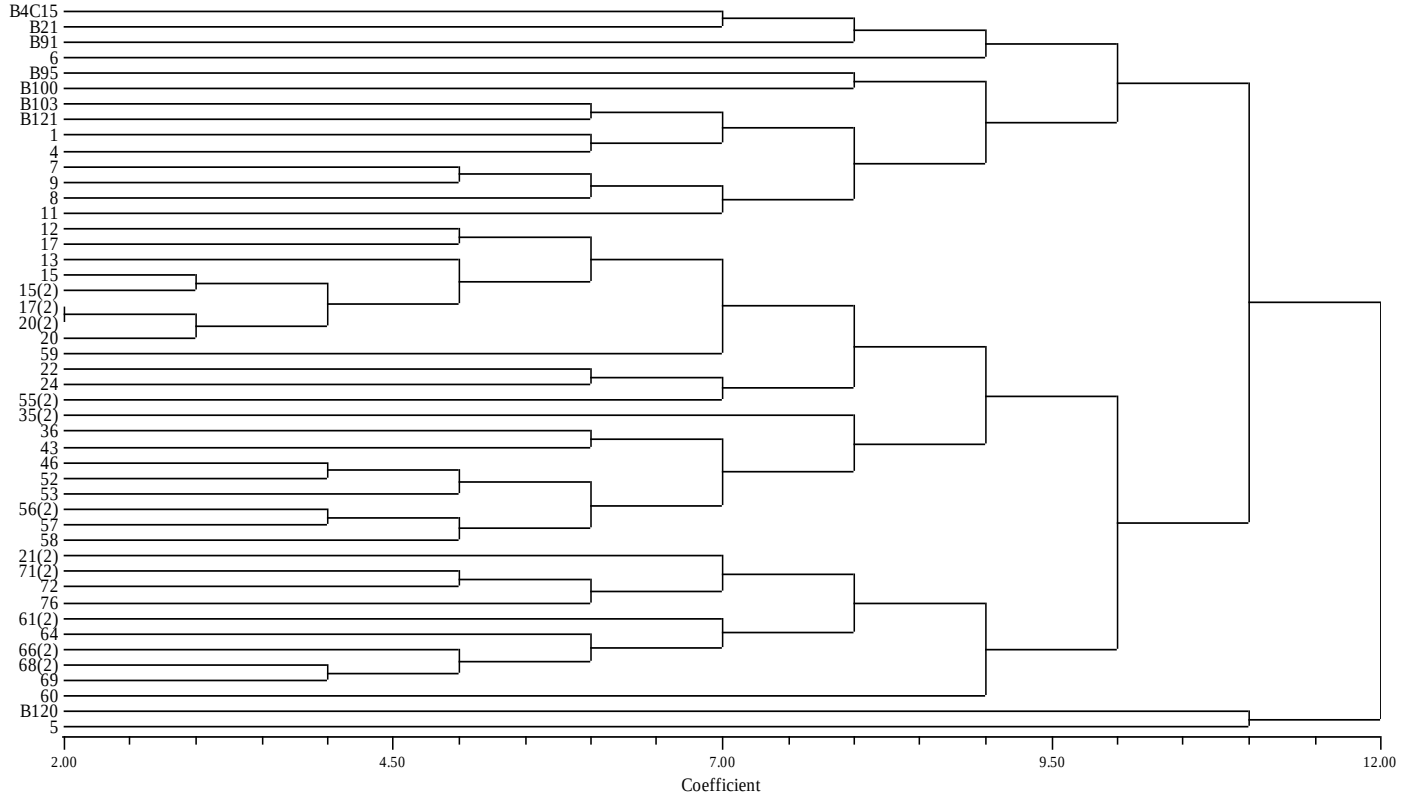


Figure 3.21 Dendogram formed by using Neighbour Joining method with DICE. Five matrixes made from different primer combinations were used, and at least sixty different polymorphic loci were detected in order to form matrixes, making up 302 loci totally.

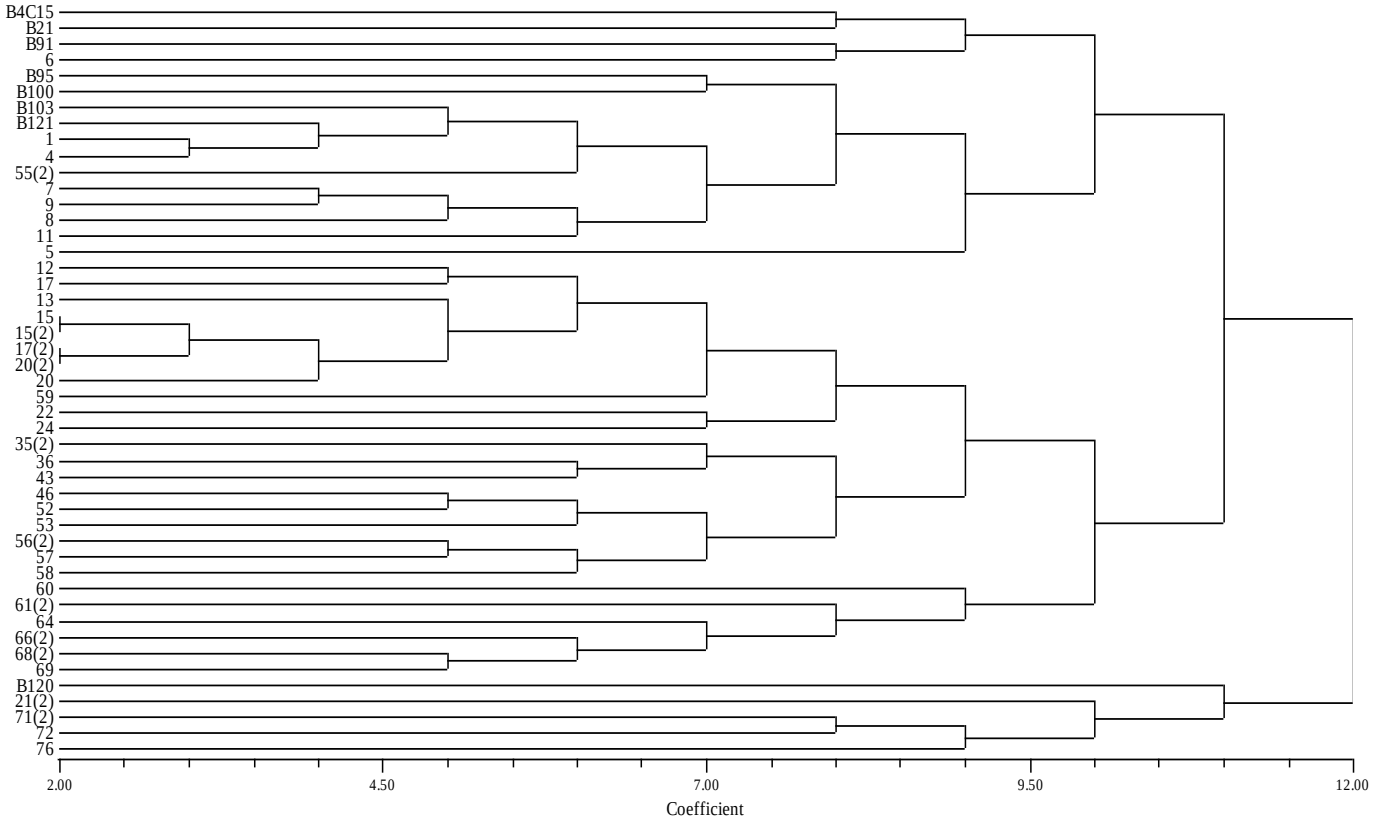


Figure 3.22 Dendogram formed by using Neighbour Joining method with Jaccard. Five matrixes made from different primer combinations were used, and at least sixty different polymorphic loci were detected in order to form matrixes, making up 302 loci.

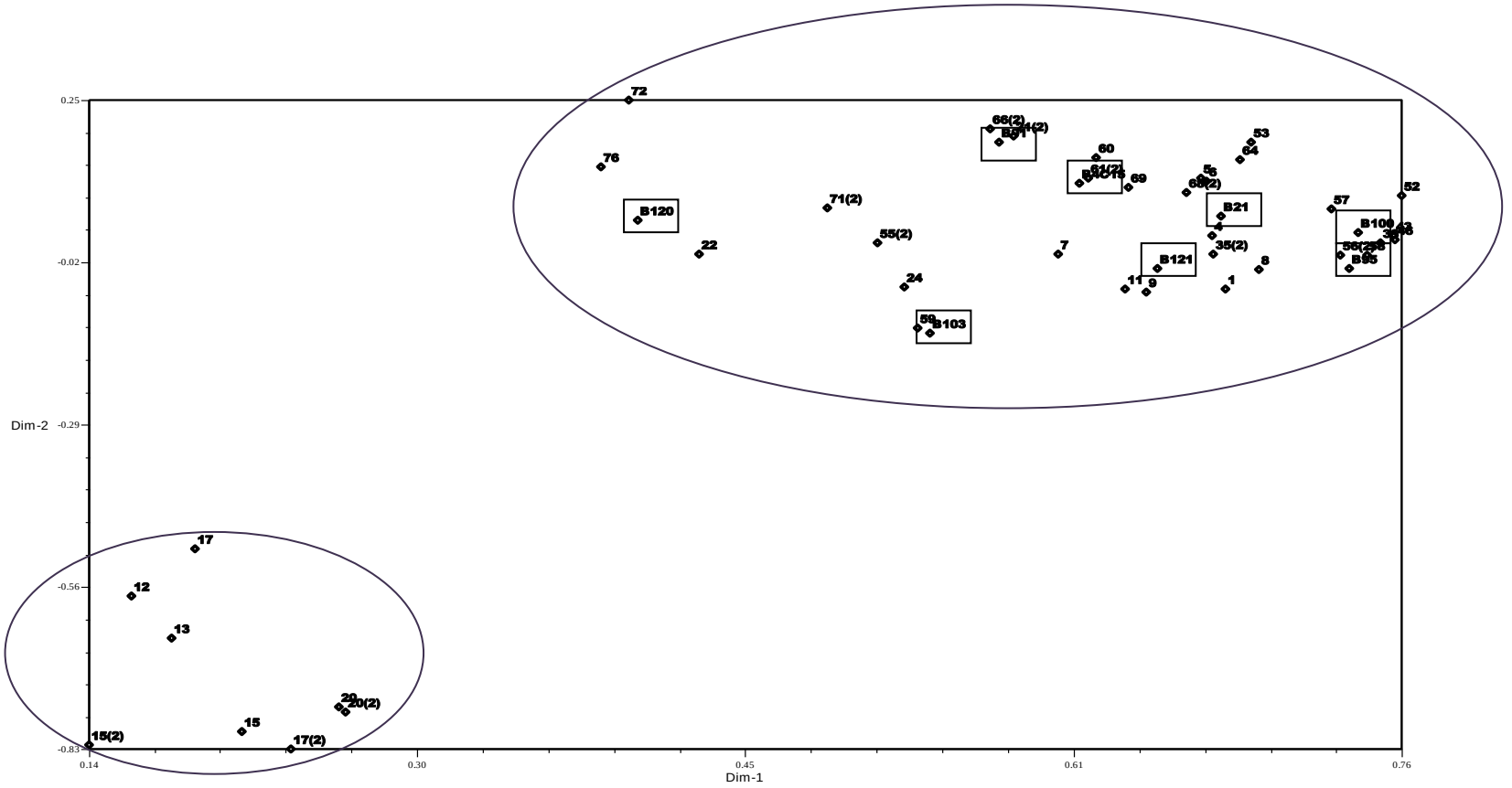


Figure 3.23 Two-dimensional representations of forty-seven samples with five primer combinations. Five matrixes made from different primer combinations were used, and at least sixty different polymorphic loci were detected in order to form matrixes, making up 302 loci. First two coordinate's percent Eigen values are 34.35% and 9.81%. Universal isolates were shown in rectangular boxes.

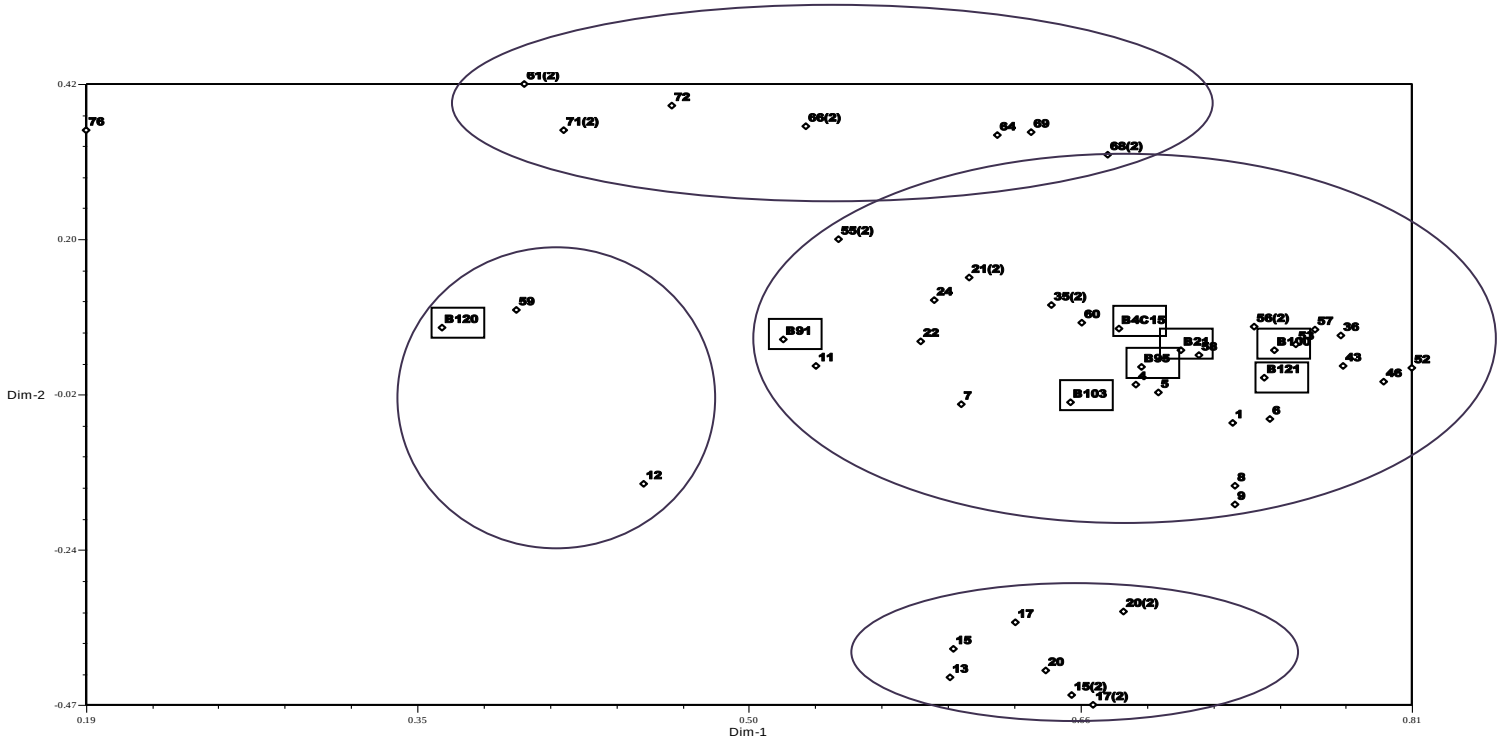


Figure 3.24 Two-dimensional representations of forty-seven samples with sixteen primer combinations. Sixteen matrixes made from different primer combinations were used, and at least sixty different polymorphic loci were detected in order to form matrixes, making up 967 loci. First two coordinate's percent Eigen values are 41.22%, and 5.16%.

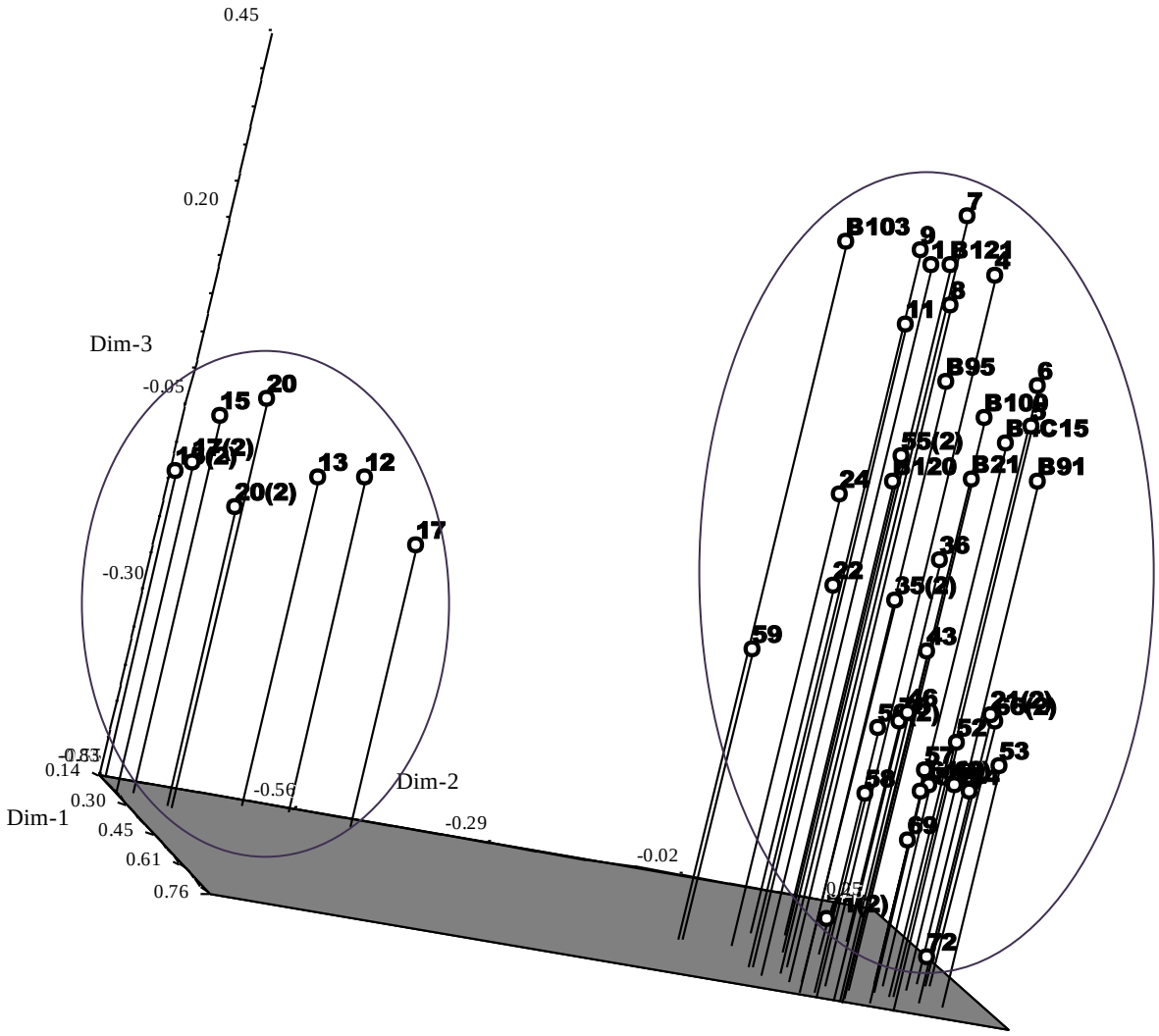


Figure 3.25 Three-dimensional representations of forty-seven samples with five primer combinations. Five matrixes made from different primer combinations were used, and at least sixty different polymorphic loci were detected in order to form matrixes, making up 302 loci.

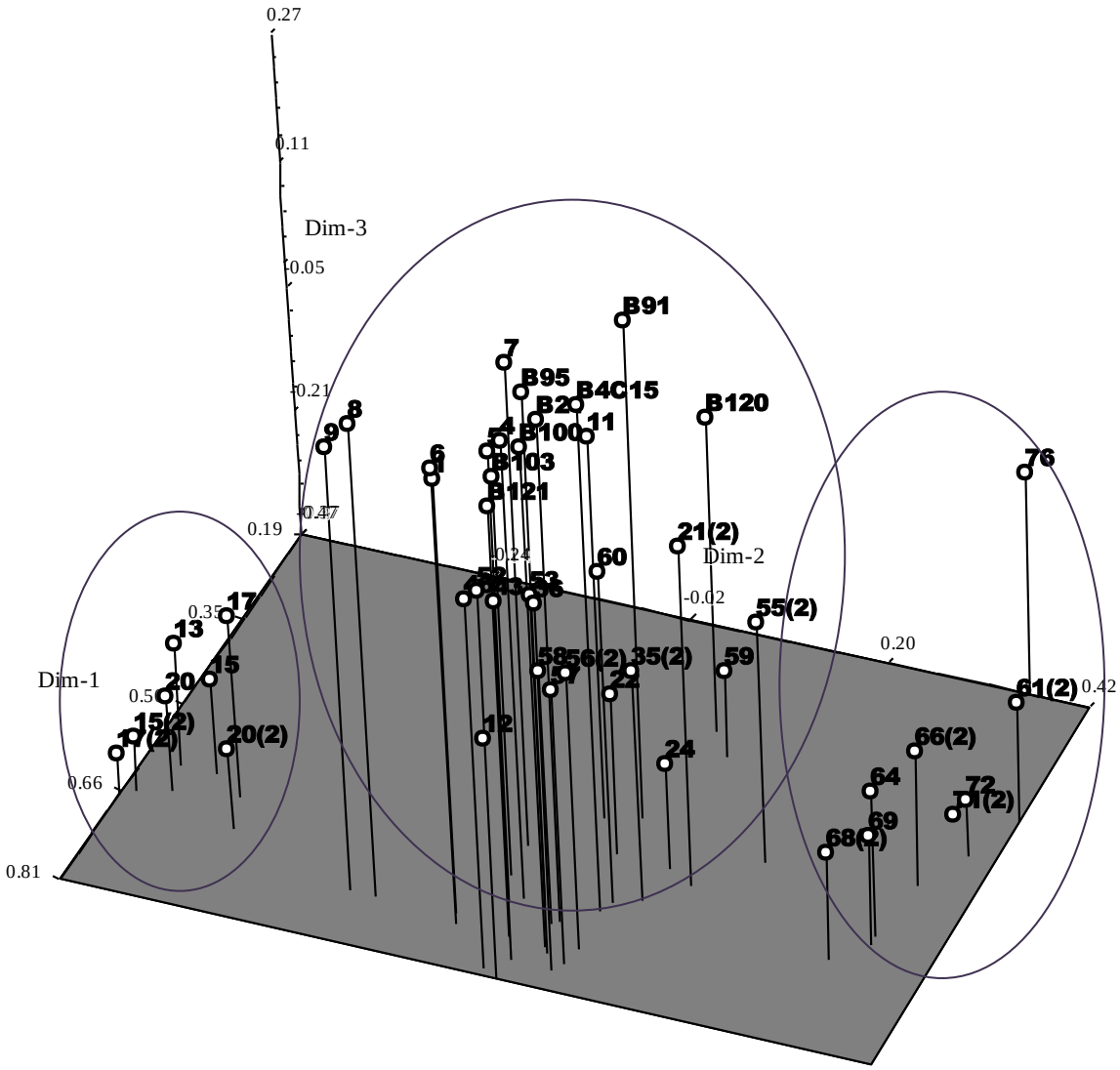


Figure 3.26 Three-dimensional representations of forty-seven samples with sixteen primer combinations. Sixteen matrixes made from different primer combinations were used, and at least sixty different polymorphic loci were detected in order to form matrixes, making up 967 loci.

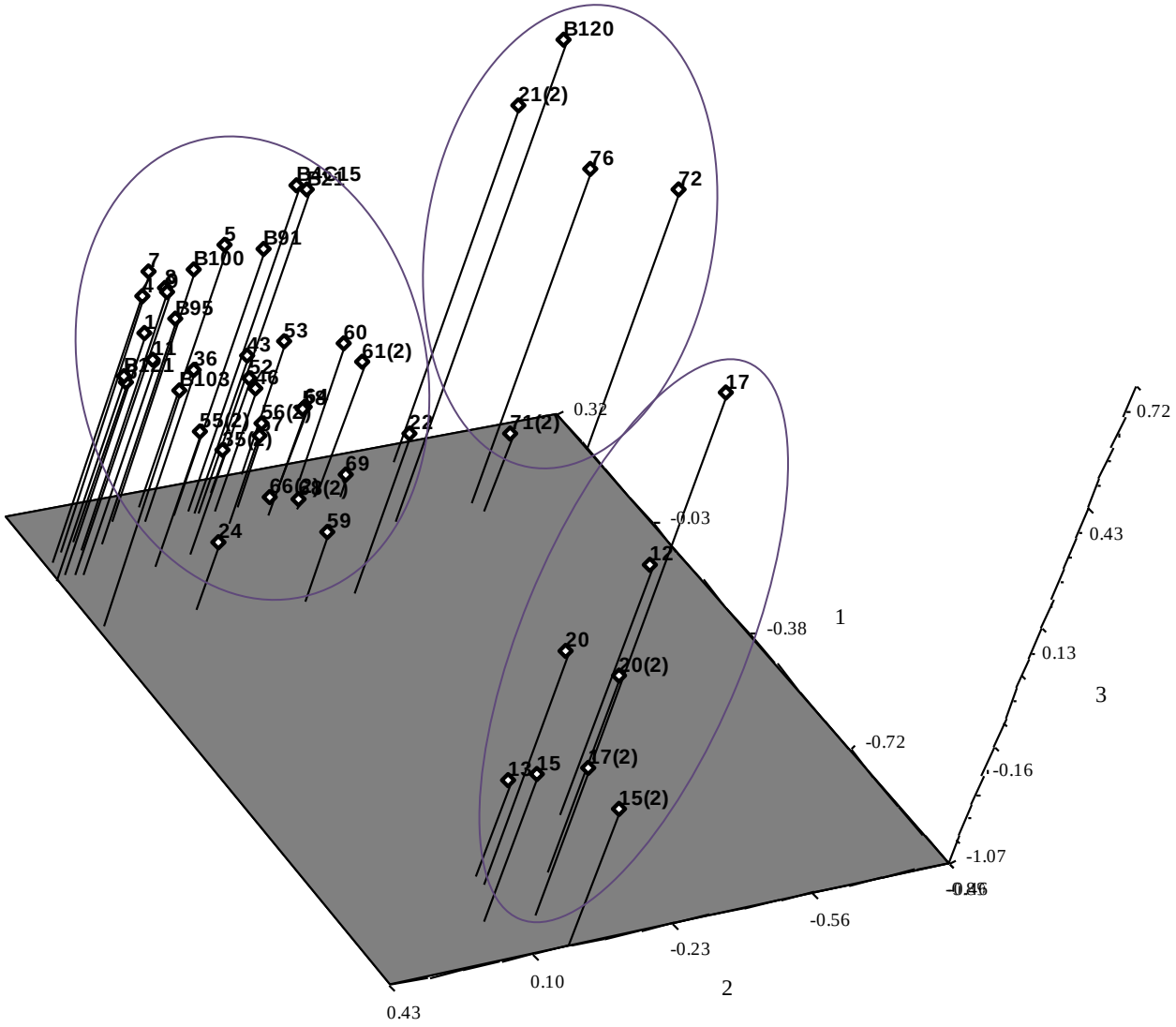


Figure 3.27 Three-dimensional representations of forty-seven samples with five primer combinations by the use of Principle Component Analysis. Five matrixes made from different primer combinations were used, and at least sixty different polymorphic loci were detected in order to form matrixes, making up 302 loci.

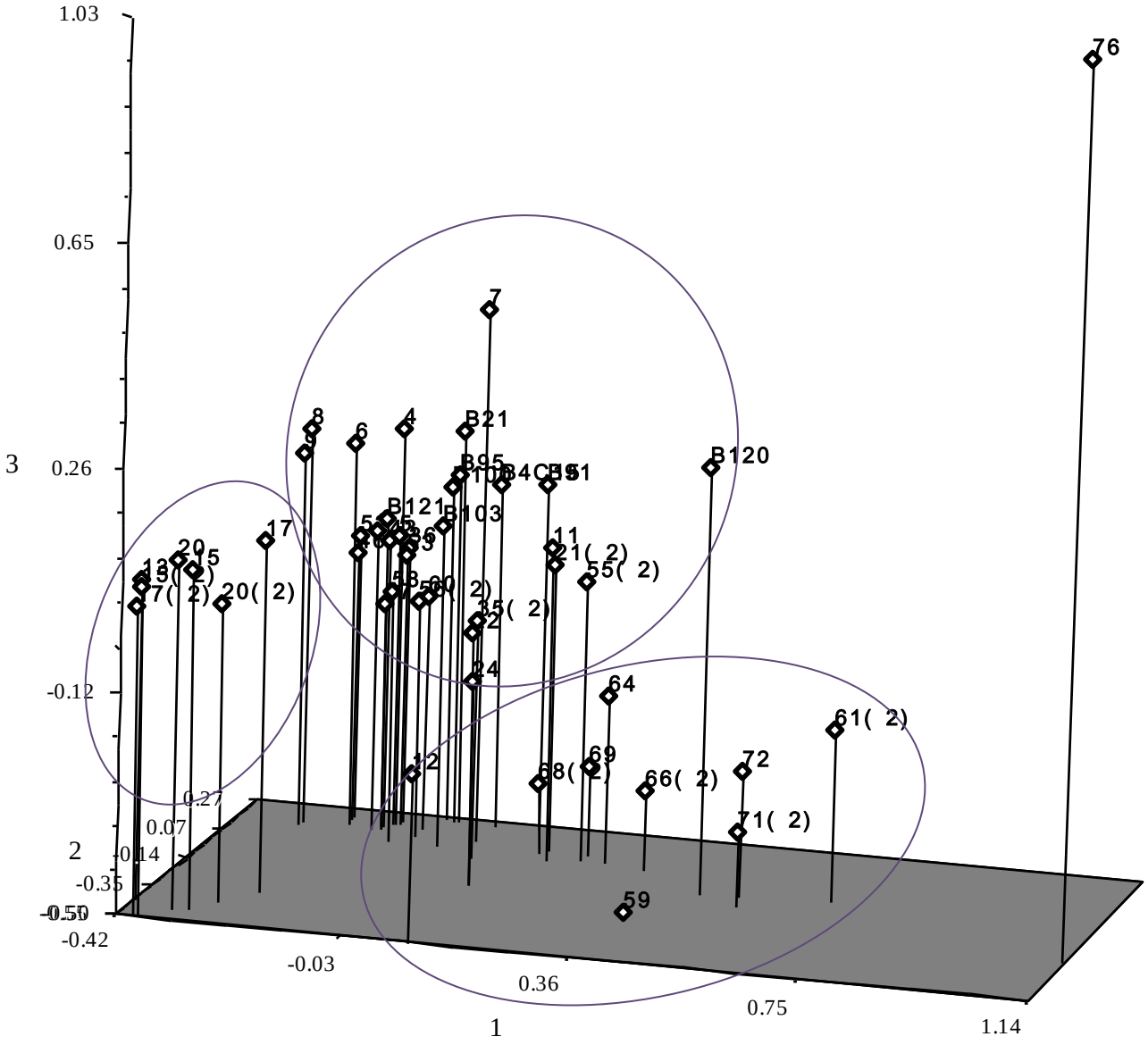


Figure 3.28 Three-dimensional representations of forty-seven samples with sixteen primer combinations by the use of Principle Component Analysis method. Sixteen matrixes made from different primer combinations were used, and at least sixty different polymorphic loci were detected in order to form matrixes, making up 967 loci.

Among sixteen primer pairs studied, five resulted in distinct banding pattern which include a high number of polymorphism. Three separate groups were formed in gel images which have primer combinations M-CTA_E-ACC primer combination read at 700 nm channel, M-CAA_E-AGG primer combination read at 800 nm channel, M-CTC_E-AAC primer combination read at 700 nm channel, M-CTC_E-ACG primer combination read at 800 nm channel, M-CAT_E-ACA primer combination read at 700 nm channel (Figures 3.9, 3.10, 3.13, 3.14, 3.15). Those gel images were analysed independently by the help of computer program NTSYS 2.02 and 2.2, and results were compared with the total sixteen primer combinations. Sixteen primer containing phylogenetic tree by Simple Matching coefficient is not very different than that obtained by DICE and Jaccard methods (Figures 3.17, 3.18, and 3.19). DICE, Jaccard, and Simple Matching similarity matrices are clustered by the application of the UPGMA clustering technique which gives the highest co-phenetic correlation scores ($r>0.9$, means a very good fit; $0.8<r<0.9$, means good fit; $r<0.8$, means a poor fit). Jaccard and DICE coefficients result in very high and comparable correlation values, which were higher than Simple Matching coefficient (Van Droogenbroeck, Breyne et al. 2002). In Figure 3.17 universal isolates were grouped among themselves and they are distant from the Turkish *Bgh* isolates. B91 and B120 is grouped among Turkish isolates, however especially B120 seems to be very distant from the others. Turkish *Bgh* line 76 is the outgroup of the dendogram. It is the most distant relative of all the other isolates. Isolates 46 and 52 are genetically very similar to each other.

In Figure 3.18 which was obtained DICE method, the outgroup becomes B120, and the most distant relative of the isolates are Turkish *Bgh* line 17, the general dendogram divides into three major branches. Isolates 46 and 52 are again very similar to each other. Isolates 68(2) and 69 are close relatives of each other. Universal races are grouped together, but two local races are included in this group. *Bgh* line 6 is similar to B91 and *Bgh* line 11 is similar to B95 and B103.

The dendogram obtained by Jaccard method is very similar to DICE method, explained above. The very same results were obtained (Figure 3.20).

Figures 3.20, 3.21, 3.22 were obtained by using Neighbour Joining (NJ) method. In this method the final tree produced is under the principle of minimum evolution (Nei 1996). The main idea of the method is its finding pairs of **O**perational **T**axonomic **U**nits (OTUs, or neighbours) which minimize the total branch length at each stage of clustering of OTUs beginning with a starlike tree (Nei 1996). NJ method can give meaningful information when used with the character-state data such as nucleotide or amino acid differences, which needs slight modifications from the ordinary procedure (Nei 1996). Because of this reason the values obtained by NJ method are considered carefully. In this method the dendogram obtained is different from the other methods (Figure 3.20). This figure is acquired by using sixteen primer combinations and Simple Matching method. Two general branches were formed and universal races were grouped together without any local *Bgh* isolates, only B120 is grouped outside the universal races' root. Isolates 1 and 4 seemed to be similar to universal isolate B121. *Bgh* isolates 8 and 9 were very similar to each other, and they are grouped with isolate 7. Isolates 36 and 43 were also similar to each other.

Figure 3.21 was obtained by NJ method by using five primer combinations and by DICE coefficient. In this dendogram there were two general branches one of them included the universal race B120 and the other one was the *Bgh* isolate 5. They seemed like distant relatives but they were more similar to each other than the other isolates. Universal races and isolate 6 were grouped together. Isolates 17(2) and 20(2) were very similar to each other and they are in the same root with isolate 20. The same relationship holds for isolate 15 and 15(2).

The last dendogram obtained by NJ method with Jaccard coefficient was slightly different form the DICE coefficient. There were again two general branches, however in this diagram B120, 21(2), 71(2), 72, and 76 were grouped together and

the others formed another group. *Bgh* isolates 15, 15(2) and 17(2), 20(2) were very similar to each other, and these two groups were clusred showing their similarity.

UPGMA and NJ algorithms are supposed to build up a single tree; however they may derive more than one topology from a single matrix depending on the order of data entry. This problem is caused by the ties, and molecular studies including binary or band scoring data (RAPD, RFLP, DNA fingerprinting, and general protein profiles) are prone to formation of ties (Backeljau, DeBruyn et al. 1996). Thus change in isolate similarity values was not very unexpected.

Principle coordinate analysis were performed for both sixteen and five primer containing matrixes and the results were given both in two and three dimensional forms represented in Figures 3.23, 3.24, 3.25, and 3.26. In Figure 3.23 ıt is clearly seen that two groups were formed and Turkish *Bgh* isolates 12, 13, 15, 15(2), 17, 17(2), 20, and 20(2) were very different to the other group and they are unique to Turkey. This graph was formed by using the first two Eigen values which were 34.35% and 9.81%, and the third one was 6.59%, totally making up 50.75% meaning that those three values are explaining the 50.75% of total variation.

Figure 3.24 represents the two dimensional model for sixteen primer combinations. In this graph there are four groups. One of the groups were the same as the above group which contains 13, 15, 15(2), 17, 17(2), 20, and 20(2), only isolate 12 is missing in this group, and the other special group contains 61(2), 64, 66(2), 68(2), 69, 71(2), and 72. B120, 59 and 12 made another group which was consistent with the dendograms obtained. The first two Eigen values which helped to draw this graph were 41.22% and 5.16%, and the third one was 4.16%, totally making up 50.54% meaning that those three values are explaining the 50.54% of total variation. These two representations as percentages were very close to each other, and they are greater than 50% which states that the study was accurate.

Figures 3.25 and 3.26 were the three dimensional representations of Principle Coordinate Analysis. In Figure 3.25 five primer combinations was used whereas in Figure 3.26 sixteen primer combinations were used. The both graph were consistent with the previous two-dimensional graphs.

Figures 3.27 and 3.28 were the three dimensional representations of Principle Component Analysis. In Figure 3.27 five primer combinations were used whereas in Figure 3.28 sixteen primer combinations were used. The both graphs were consistent with Principle Coordinate Analysis. PCA is used in order to decrease the complexity of the data. If researcher is dealing with a large number of variables it becomes difficult to compare the variables. PCA reduces dimentionality by performing a covariance analysis between factors; the method decreases the multiple variables into two dimentions. PCoA is calculated by the distance matrix of Eigen vectors. The basic differences in between these two variables could be written as PCO is equivalent to PCA on covariance matrix of transposed data matrix if distance matrix is Euclidean, PCO is equivalent to PCA on correlation matrix of transposed data matrix if distance matrix is Penrose, and PCO only gives information on units or variables not both. The comparison of the two variables gave nearly same results; the samples were divided into three groups, and the biggest group included the universal isolates.

3.4 Results for Assessment of powdery mildew (*Bgh*) resistance genes in Turkish barley varieties

Thirty-four Turkish barley varieties are studied for identification of R-genes that they contain. The study was performed by “leaf segment test” (Lutz et al. 1992). In order to use powdery mildew resistance genes information in breeding programs, it is essential to test for the presence of those genes in registered cultivars and for the virulence of the pathogen through periodical surveys (Czembor and Bladenopoulos 2001; Czembor and Czembor 1998, 2000a; Czembor and Gacek 1990; Czembor and

Johnston 1999). These experiments generally are conducted based on the gene-for-gene hypothesis, i.e. by inoculating plants with pathogen isolates that present a defined virulence spectrum (Flor 1942, 1955; Moseman 1959) as performed in Table 3.3 (Zeybek et al. 2008)and then the infection spectra is observed makes it possible to determine a 'reaction spectrum' for each interaction, which then makes it possible to identify the resistance phenotype of the tested plant as performed in Figure 3.4 (Czembor and Czembor 1998, 1999, 2001; Dreiseitl and Jørgensen 2000; Zeybek et al. 2008). One of the most important breeding afforts is race specific resistance and it is controlled by R-genes which can be passed from resistant cultivars into susceptible ones those are agriculturally important (Zeybek et al. 2008). Moreover, The Fertile Crescent area, covering the south-eastern part of Turkey, is considered as the centre of origin for barley and wheat (Czembor 1996; Willcox 1995; Zohary 1999), which suggests that barley varieties, landraces and wild relatives from Turkey might represent an interesting source of R-genes for powdery mildew (Jahoor and Fishbeck 1987). Since the best potential sources of new resistance genes for cultivated barley most likely are landraces from the centre of origin (Ceccarelli et al. 1987, 1995) is known, it is very probable to observe R-genes those are not characterized in Turkey barley varieties (Zeybek et al. 2007).

This is the first study identifying R-genes for powdery mildew in commonly grown barley cultivars bred in the National Agricultural Research Institutes of Turkey. Among the tested varieties there are certain R genes which are Mla8, MlLa, Mlg, Ml(CP), Mlh, Mlat, Mla1, Mlh, Mla7, Mlra, and a few uncharacterized genes. The most common R-gene was Mla8; it was found in nine cultivars out of 34 (Zafer-160, Yesilköy-387, Efes-2, Sahin-91, Basgöl, Yesevi-93, Ince-04, Kalaycı-97, and Erginel 90). Several cultivars contained a single (known) R-gene for powdery mildew [namely Orza-96 (Mlra), Serife hanım (Mla7), Bornova-92 (Mla1), Kaya-7794 (Mla(La)), Bilgi 91 (Mlg), Vamık hoca-98 (Ml(Ab)), Sülayman bey-98 (Ml(Ab)), Obruk-86 (Mlh), Özdemir -5 (Mlh), Çıldır 02 (Mlh), and Anodulu-86 (Mlh)], while three cultivars (Tokak-157/37, Beysehir-98, and Konevi-98) contained

both Mlg and Ml(CP). Interestingly, the varieties Gemici-7243, Yea-793.12, and Akhisar-98 were partly resistant to the fungus, but no R-gene could be inferred because their reaction spectra with the test isolates were not conclusive. By contrast, the cultivars Hamidiye-85, Yesevi-93, and Bülbül-89 showed no resistance to any of the isolates tested, as also observed by Lower et al. (1997) (Zeybek et al. 2008).

Table 3.3 Isolates of *Blumeria graminis* f.sp. *hordei* and their infection types on 'Pallas' differential near-isogenic isolates (Kølster *et al*. 1986) and twelve additional cultivars

			Differential test isolates[2]								
No	**Differential isolates**[1]	**R-genes**	**B4(C15)**	**B95(53/01)**	**B100(60/01)**	**B121(26/04)**	**B120(20/04)**	**B97(57/01)**	**B91(98AF066)**	**B21(R86/01)**	**B103(64/01)**
0	Pallas	Mla8	4	4	4	4	4	4	4	4	4
1	P01	Mla1,ML (A12)	0	4	4	4	0	0	0	0	0
2	P02	Mla3,	4	0-1n	0	0	4	4	0-1n	4	0-1n
3	P03	Mla6, Mla14	0	0	0-1n	3n-4	4	4	0	4	4
4	P04B	Mla7, Ml(NO3)	0	4	4	3-4	1-2n	3n	4	1n	4
5	P08B	Mla9	0	0	4	4	4	0	4	0	0
6	P09	Mla10, Ml (Du2)	0	4	3n	0	4	0	4	0	0
7	P10	Mla12, Ml (Em2)	0	4	0-1n	1n	3n-4	4	1n	0-1n	3n-4
8	P11	Mla13, Ml (Ru3)	0	0	0	4	0	4	4	0	4
9	P12	Mla22 Mic)	4	0	0	3n-4	0	4	0	4	0
10	P14	Mlra	4	4	4	0	4	4	4	4	4
11	P16	Mlk	2cn	2cn	4	1-2cn	4	1-2cn	3n-4	1-2cn	3n-4
12	P20	Ml	2n	2n	2n	2-3n	2n	4	1-2n	1-2cn	2n
13	P21	Mlg,Ml (CP)	2-3n	4	4	4	4	4	4	0	4
14	P23	Mlla	1-2n	4	4	4	4	2n	4	4	2n
15	P24	Mlh	4	4	0	0	4	4	4	4	4
16	ISO2R	Mlg	4	4	4	4	4	4	4	1-2n	4
17	SI-1	Sl 1	0	1-2n	3n	0	0	0	0	0	0
18	GUNNAR	Mla3, Ml (Tu2)	1-2n	0	0	0	2n	4	2-3n	4	0
19	SV83380	Mlab	2n	3n	2-3n	3n	4	4	3n-4	2n	4
20	MELTAN	Mla13, Mlı 8lm9),+	0	0	0	4	0	4	0	0	4
21	GOLDİE	Mla 12, U	0	2n	0	4	1n	4	4	0	4
22	STEFFİ	Ml (St)	0	0-1n	0-1n	3n	4	2n	4	0	1n
23	HENNİ		0	1-2n	0-1n	4	4	1n	4	0	1-2n
24	PUNTO	Mla3, Ml (Tu2), Ml (lm9),+	0	0	0	0	1n	4	1-2n	1n	0
25	BENEDİKTE	Mla9, Ml(lm9)	0	0	2n	4	0-1n	0	1n	0	0n
26	SCARLETT		0	0-1n	0	3cn	4	2n	4	0	0-1n
27	CARLSBERG	Mla8	4	4	4	4	4	4	4	4	4
28	Bülbül 89 / control		4	4	4	4	4	4	4	4	4

[1] P01-P24: Pallas differential near-isogenic isolates.
[2] Scale 0-4: 0 = no compatible; 4 = compatible; n = necrosis; c = chlorosis.

Table 3.4 Resistance alleles and infection types of thirty-four Turkish varieties challenged with nine isolates of *Blumaris graminis* f. sp. *hordei*

No	Cultivars	Differential test isolates																		Postulated Alleles
		B4(C15)		B95(53/01)		B100(60/01)		B121(26/04)		B120(20/04)		B97(57/01)		B91(98AF066)		B21(R86/01)		B103(64/01)		
1	Zafer 160	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	Mla8
2	Yeşilköy 387	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	Mla8
3	Gemici 7243	3	3	1	0	2	2	4	4	4	4	2	2	4	4	3	3	3	3	+?
4	Kaya 7794	0	1	4	3	3	2	4	4	3	3	2	2	4	4	2	2	4	4	Mla (La)
5	Tokak 157/37	3	2	4	4	2	2	4	4	4	4	4	4	4	4	0	0	4	4	Mlg, Ml (CP)
6	Cumhuriyet 50	3	3	4	4	2	2	4	4	3	2	4	4	4	4	3	3	4	4	+?
7	Yerçil 147	0	0	2	3	2	2	4	4	4	4	2	2	4	4	0	0	4	4	+?
8	Hamidiye 85	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	None
9	Obruk 86	4	3	4	3	1	0	0	0	4	4	4	4	4	4	4	4	4	4	Mlh
10	Anadolu 86	4	4	4	4	0	0	4	4	2	2	4	4	4	4	4	4	4	3	Mlh +?
11	Bülbül 89	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	None
12	Efes-1	4	3	4	3	2	0	4	4	0	1	2	2	4	4	4	4	3	2	Mla1 +
13	Efes-2	3	4	4	3	3	4	4	4	4	3	4	4	4	4	4	4	4	4	Mla 8
14	Efes-3	3	3	4	3	2	2	4	4	2	2	4	4	4	4	4	4	4	2	Mla +?
15	Şahin 91	4	4	4	4	3	3	4	4	4	4	4	4	4	4	4	4	4	4	Mla8
16	Yea. 793.12	4	4	4	4	4	4	3	3	3	2	2	2	4	4	4	4	4	4	+?
17	Bornova 92	0	0	4	4	4	4	4	4	1	0	0	0	0	1	0	0	1	0	Mla 1+ ?
18	Şerife hanım 98	0	0	3	3	3	2	4	4	1	2	4	4	4	4	2	2	4	3	Mla 7+?
19	Vamık hoca 98	2	3	4	4	2	2	2	2	2	1	2	3	4	4	3	2	4	3	MlAb
20	Akhisar 98	3	2	4	4	3	3	3	2	2	1	4	4	4	4	4	4	3	2	+?
21	Süleymanbey 98	1	2	3	3	4	4	4	4	4	4	4	4	4	4	4	3	4	4	MlAb+?
22	Bilgi 91	4	4	4	4	4	4	4	4	4	4	4	4	4	4	0	1	4	4	Mlg
23	Beyşehir 98	3	2	4	4	4	4	4	4	4	4	4	4	4	4	0	0	4	4	Mlg, Ml (CP)
24	Konevi 98	3	3	4	4	4	4	4	4	4	4	4	4	4	4	0	0	4	4	Mlg, Ml (CP)
25	Başgöl	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	Mla 8
26	Çıldır-02	3	3	4	4	0	0	0	0	4	4	4	4	4	4	4	4	4	4	Mlh
27	Avcı-2002	0	2	4	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	Mla7
28	Yesevi-93	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	None
29	Orza-96	4	4	4	4	0	2	4	4	4	4	4	4	4	4	4	4	4	4	Mlra
30	Aydan hanım	4	4	4	4	4	4	0	0	4	4	4	4	4	4	4	4	4	2	Mlg
31	Özdemir 05	4	4	4	4	0	0	0	0	4	4	4	4	4	4	4	4	4	3	Mlh
32	İnce 04	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	Mla 8
33	Kalaycı 97	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	Mla 8
34	Erginel 90	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	Mla 8

Each cultivar was tested as two replicates with each test isolate.

3.5 Conclusion

In this study, Turkish *Blumeria graminis* f. sp. *hordei* varieties were characterized using Amplified Fragment Length Polymorphism molecular marker technique. Thirty-nine Turkish varieties were compared with eight universal *Bgh* races, and 967 polymorphic loci were detected in sixteen selective primer combinations. Since one of the strogest advantages of AFLP is its reproducibility, all the steps of the experiment was repeated two-weeks after the first application; the same banding pattern was obtained; in our studies the data was analysed on NTSYS, Numerical Taxonomic System, and the genetic relationship among isolates investigated. Principal coordinate analysis, phylogeny, and cluster analysis were conducted. Thirty-nine local isolates together with eight universal races were found to be divided into three major clusteres (Figure 3.17).

This study is crucial in terms of being probably the first one on detecting local pathogen variation, their genetic similarities and distances to the isolates with known virulances (universal isolates). The study is informative in terms of observing *Bgh* diversification in Turkey and the speed of mutation rate of *Bgh* isolates. The samples used in this study are important because they are the isolates originating from a single pathogen species probably by meiosis (crossing-over) or mutations. The AFLP profiles showed unique bands only present in some of the isolates; these fragments may be utilized as “isolate specific markers” once they are further characterized. These analyses can be valuable to assess newly emerging isolates in future studies, also for genetic relationship and evolutionary analysis.

Turkey is the center of origin for both barley and wheat (Czembor 1996; Willcox 1995; Zohary 1999). Thus; in Turkey it is expected that a great range of diversification in Resistance genes in barley and its wild relatives, landraces is present (Ceccarelli et al. 1987, 1995). This is also most likely case for the presence of still uncharacterized powdery mildew resistance genes, possessed by our barley

varieties. There is an arms race in between plants and pathogens, while pathogens are evolving very fast in order to overcome immune responses of plants, plants are trying to resist this invasion by diversifying their R-genes faster than any other genes in its genome (Jones and Dangl 2006; Aguileta, Refregier et al. 2009). Because of pathogens very fast evolution capability, the control mechanisms become functionless through evolutionary adaptation of pathogen populations. Thus disease control attempts should also focus on understanding population processes that causing pathogen to adapt so easily (Wolfe and Mcdermott 1994). Since the *Bgh* isolates used in this study were collected from Çukurova region in Turkey, which is near the center of origin of barley we expect high degree of polymorphism in our samples, due to co-evolutionary processes explained above.

Natural selection of pathogens acts on nucleotide polymorphisms of effectors genes to overcome the host immune responses. Moreover, it acts on the number of repeat regions of effector genes as well (Hogenhout, Van der Hoorn et al. 2009). Effector genes of filamentous pathogens are known to be located in loci, in places where high genome plasticity is seen such as transposon-rich and telomeric regions (Orbach, Farrall et al. 2000; Gout, Fudal et al. 2006). In two effector loci of *Magnaporthe oryzae*, although high degrees of presence or absence mutations observed, nucleotide diversity was low (K. Yoshida and R. Terauchi unpublished data). Unlike its fungi counterparts (Odell, Wolfe et al. 1989; Christiansen and Giese 1990; Rasmussen, Rossen et al. 1993) *Bgh* has repetitive DNA in its genome, and a special repeating element (903 bp) which constitutes more than 5% of the fungi's genetic material, is similar to mammalian SINE (transposon-like short interspersed element) (Rasmussen, Rossen et al. 1993). These type of retrotransposon-like elements are highly conserved and functional; and they may be taking part in virulence activity (Hogenhout, Van der Hoorn et al. 2009). This information is crucial because AFLP is a molecular marker that can easily detect presence or absence mutations. Also, AFLP molecular marker system produces more robust polymorphic products comparing with other molecular marker systems (Jones, Edwards et al. 1997). Due to this characteristics of AFLP, it is found to be the most reliable, and convenient

method for studying genetic relationships of fungal populations (Parrent, Garbelotto et al. 2004; Zeller, Bowden et al. 2004; Nunez, Gallego et al. 2006). Thus, AFLP was a proper choise for studying *Bgh* isolates of Turkey in this study.

Througout the analysis, primer pairs chosen gave consistent results when compared with the AFLP studies conducted on barley. The primer pair M-CTC_E-AGC in 800 nm channel gave no banding pattern, meaning the primer pair was not a good combination for *Bgh* isolates. The same primer pair does not work well for barley AFLP as indicated in the manual provided by LI-COR Biosciences. This is an interesting coincidence and may be important in terms of dynamic interaction between the *Bgh* and barley at the genome level. The same primer pair works properly for plant species like, lettuce, maize, pepper, sugar-beet and tomato.

Powdery mildew population structure varied to a very great extent (Bardin, Nicot et al. 1997; Bardin, Carlier et al. 1999; Urbanietz and Dunemann 2005). For instance apple powdery mildew (*Podosphaera leucotrica*) analysed with AFLP molecular marker, was found to be divided into two clusteres (Urbanietz and Dunemann 2005), and this division is thought to be because of the crossing of sexual and asexual populations (Delye, Laigret et al. 1997). There are other examples indicating the other divisions of powdery mildews such as cucurbit powdery mildew (*Golovinomyces cichoracearum*), this powdery mildew divides into three clusteres which is thought to be reason of host specialization (Bardin, Carlier et al. 1999), and there are also powdery mildews (*Podosphaera xanthii*) not dividing into any groups (Bardin, Nicot et al. 1997; Nunez, Gallego et al. 2006). This situation may be the cause of the differentiation in isolate genetic structure in this study. Although some literature information is present about the “selective incompatibility” between the isolates of different genetic make-up (Delye, Laigret et al. 1997) *in vitro* studies revealed viable ascospores as a result of crossing in sexual and asexual phases (Schneider et al., 1999, 2000; Miazzi et al., 2003). The reason of differentiation could also be cause of the migration capability of powdery mildew spores. It is

known that *Bgh* conidia can disperse long-distances (whole Europe) (Wolfe and Mcdermott 1994; Nunez, Gallego et al. 2006).

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APPENDIX A

Table 1 Matrix for M-CAC/E-AAG primer combination, read at 700 nm channel.....60

Table 2 Matrix for M-CAC/E-ACT primer combination read at 800 nm channel.....61

Table 3 Matrix for M-CAC/E-ACA primer combination read at 700 nm channel……..…….....62

Table 4 Matrix for M-CAC/E-AAG primer combination read at 700 nm channel.....…….....63

Table 5 Matrix for M-CAT/E-AAC primer combination read at 700 nm channel.....64

Table 6 Matrix for M-CAT/E-ACG primer combination read at 800 nm channel …….....65

Table 7 Matrix for M-CAG/E-AAC primer combination read at 700 nm channel.....……..…….....66

Table 8 Matrix for M-CAG/E-AGG primer combination read at 800 nm channel.....67

Table 9 Matrix for M-CTA/E-ACC primer combination read at 700 nm channel …….....68

Table 10 Matrix for M-CAA/E-AGG primer combination read at 800 nm channel.....……...…….....69

Table 11 Matrix for M-CTA/E-AAC primer combination read at 700 nm channel.....70

Table 12 Matrix for M-CTA/E-ACG primer combination read at 800 nm channel ...…….…….....71

Table 13 Matrix for M-CTC/E-AAC primer combination read at 700 nm channel.....…….…….....72

Table 14 Matrix for M-CTC/E-ACG primer combination read at 800 nm channel.....73
Table 15 Matrix for M-CTC/E-ACG primer combination read at 800 nm channel.…….....74
Table 16 Matrix for M-CAT/E-AGG primer combination read at 800 nm channel.....……..…….....75

Appendix Table 1 Matrix for M-CAC/E-AAG primer combination, read at 700 nm channel. The number of polymorphic loci scored was 60. “0” means absence “1” means presence of the allele.

no	Polymorphic loci (1-60)
1	000000000000010000011001111111111111011000100111011111011101
2	001100000000110000011000101111011111110101101110111111001101
3	001111000000110000011000101111011111110001000100111111001000
4	000000000000100001101000101111000110110001000100100010001111
5	001000000000000000011000101111111111110001000111010110011111
6	000000000000100000000000001110011111110001000100100010001001
7	000000000000111000010000111111111111110001101110111111010000
8	000000000000110000011000111111111111110001011111111111011111
9	000000000000000000011000101111111101100110011111111111011011
10	011100000001111100011001111111111111110001001111111111001111
11	001000000000100000011000111111111111110011101111111111111101
12	000000000000100000011000101111111111110001101111111111011111
13	011000000000100000011000111111111111110001001101111111011111
14	000001100000010000010000101110011111110001101111111111011111
15	000000000000000000010000101110011111110001101100111111011111
16	111011100101111011111000000110000110100000000100000010000000
17	001110000000100100011000111110111011000010011111111101010111
18	000000000000000011111000101110011111110001001110111111001111
19	001100000000111000011000101111101111110111111100011111011111
20	000000000000000000010000101110011111111001101100111111011111
21	000000000000000000011000101111001111100010011001111111010001
22	000000000000000000000000101110011111110001101110111111011101
23	000000000000000000000000101000011111110001101100111111011111
24	000000000000100000011000111111111111011000100111111111011111
25	001100000000100000011000111111111100110001101110010111001000
26	000000000000000000010000101111111010000001001111111111111111
27	000000000000000000011000111111111000111101111011111111011111
28	000000000000111000011000101110011010110001111111000111111101
29	000000000000111000011000111111111111110001101111111111011111
30	001100000000100000011000101110100111110001011101111111011111
31	000000000000110000001000111110111111110001111111111111011111
32	001100000000110000011000111111111111110001111111111111011111
33	000010000000100000001000101100011111110001001100001111001000
34	001100000000110010110001111111111001100010111000111110000000
35	000000000000000000001000101111111101100010111110011110111111
36	001100000000110000110001111111111111110011111111111111011111
37	000000000000100000011000101111111111110001101111111111011111
38	011100000000000010000000000000010101111010000000000000000000
39	011100000010100111111111111111111111111101101111111111111111
40	010000000000000000000000000000000000000000000000000000000000
41	011100000000110000110001111111111110000011011110111011011111
42	000000000000011000011111100000011011110011011100110010100011
43	001100000000101000111111011100111111100010011010001110111111
44	000000000000000000011111101111100111100000011111111111011110
45	000100000011000000010000111000110000000011101000000001010010
46	000000000000000000011000000000110100001000011111010101011111
47	111111111111111111110000100100000000000000011110000001000000

Appendix Table 2 Matrix for M-CAC/E-ACT primer combination read at 800 nm channel. The number of polymorphic loci scored was 60. "0" means absence "1" means presence of the allele.

no	Polymorphic loci (1-60)
1	010111110111111001110010000111011100100011101111111111101110
2	011111110111111000010010100111011111100011011111111111101110
3	011111100110011000100001001100111001000110110011111111001110
4	011111100111111000010001111110011001001110111111111111101110
5	011111100111111000110010111111011110100111011111111111111111
6	011111100111111001011010111111011110100111011111111111111111
7	001101110111111001100010101111011110000111011111111111111110
8	011101100111111000111010101111011110100111011111111111111111
9	011111110111111001010010011111011110000111011001111001100110
10	011101100111111001111010111111011110100111011111111111101110
11	001101110111111011100101111110111010111011111111111111111111
12	011111100111111000110010001111011110100111011111111111101110
13	011111110111111000110010011111011110100111011001111101101110
14	011111100111111110110011111111011110100111011111111111101110
15	011111100111111000110000100111011110100111011111111111101110
16	000000000000000001000000011000000010010011010111110110000000
17	011111110111111001010010011111011110000011001111111000101110
18	011111100111111110111001101110111100011101111111111101111
19	011111110110111001111010011111011110000111011111111111111110
20	011111110111111001010101111110111100001101111111101101110
21	011111100111110000111010111111011101001101111111111101111
22	011111100111111000111010111111011110000111001111111111111111
23	011111110101111000111010111111011110000111001111111111111111
24	011111100111111001010101001110111110000111000111111111111111
25	111111100101111001100000000111011110000111011111111111111111
26	011111100101111001111010111011011111101111011111111111111111
27	011111110111111000111010000111011110001111000111111101111111
28	110101100100111000000000000111111000001111000110101100101110
29	011111100111111001111010100111011110101111011111111111111111
30	011111100111111000111010111111011110100111001111111111111111
31	011111110101111001111010111111011110100111011111111111111111
32	011111110111111001111010100111011110100111011111111111111111
33	011111110111111110011010111111011110100111011111111111111111
34	011111101111110000011010111111011110100111011111111111100110
35	011111110111111000111010111111011110100111011111111111111111
36	011111110111111000011011111111011110000111011111111111111111
37	011111110111111011011010011111011110001111011111111111111111
38	000011000000011011001010011111000000000000000000000001101111111
39	011111110111110000010000111111011110101111011111111111111110
40	110011000001100000000000011111000000000000000000000000000110
41	010111111111110000011100001110110000001100001100000001111111
42	011111100101111001011101111111011110000110011111111001111111
43	001111100101110000011101111111011110101111011111111111111111
44	001111100111010000011101111111011110101111011111111111111111
45	010111100111111000001010000111000100000000000000111101111111
46	011110101100111000001110000001111111111111100001000000000110
47	111111100100011000100000000011011110001110000011111111110000

Appendix Table 3 Matrix for M-CAC/E-ACA primer combination read at 700 nm channel. The number of polymorphic loci scored was 60. "0" means absence "1" means presence of the allele.

no	Polymorphic loci (1-60)
1	000000000000101100111100111110001110000010000011100101111111
2	000000000011111100110011111110111110100011000000000000010000
3	000000000000111100110110111110111110000001000001000010111111
4	000000000000111100111110111110111110001001000000010010111111
5	000000000001110000111110111101111000000010000011100000110111
6	000000000000011000111100101110101000000001000001111011111111
7	000000000010001000110000011000000000000000000000000000000101
8	000000000000000010011111111111011110111110100000111001111111
9	000000000000011000011111111111011110110110100000111011111111
10	000000000000011110011111111111101011000010010000011101111111
11	000000000000111000011011111111011111101101100000111011111111
12	000000000000011110011111111111011111101100100000111111111111
13	000000000010110010110001011100000000000000000000000000001011
14	000000000001000100110111111111111111011011011011100111111111
15	000000000000010100110101111111111111011011000010110111111111
16	100000000000000000010000111110110111111100110111101111111111
17	110111000010011100001111111100111000000010000111100000111111
18	000000000000011100111111111111111111111100100101111011111111
19	110001000011111100110010111111001101100001000001110000111110
20	000000000000010100111111111110111111011001010011111111111111
21	110000000000000000100111110101001000000000000001100000100010
22	000000000000000000110111111111111111011001010011111111111111
23	000000000000000100111111111111011111100100100001111111111111
24	100000000000000100111111111110111100011001000011100111110011
25	110011000000011100110011111110011110100000000001000000000000
26	000111000000000100110000000000101001001000110011100111111111
27	111111010000011100110001111111101011000011000011100000111111
28	110000000000000111110000110110100011001001001111100011110101
29	000001000000001110011111111111011111000100000000100000010000
30	000000000000000100111111111111011111100100100000111001011111
31	000000000000000000110111111111011111000101100000111001011111
32	000000000000000100111111111111011111001011000011110111111111
33	000000000000010010011111111111011111100101100001111011111111
34	000001000000000100110000101100100001001100010000010010010000
35	000011000000011100110111111111011111100100100000111001011110
36	000000000000000100110101111110111111011011000011110010111111
37	110011000000100100111111111110011110001001000011111110011111
38	000000000000000000000010111000001111101110000000111000001111
39	000011000000111100111111111110111111001001000011110111111111
40	000000000000000000000000000000000000000000000000000000000000
41	000111111111011100010101111010111001001000000000001010010000
42	000000000000001100110001111010001000001000000000000010011000
43	000000000000000100110000111010101001000000000000010010010000
44	000001100000001100110011111110111111000000000000000000000000
45	000000000000001000000011011111101111011001110011110111111111
46	000001000000000000001000000000000000000000000000000000000000
47	001111111111111100000001000000100001001000111011111111111111

Appendix Table 4 Matrix for M-CAC/E-AAG primer combination read at 700 nm channel. The number of polymorphic loci scored was 60. “0” means absence “1” means presence of the allele.

no	Polymorphic loci (1-60)
1	000000000000000000000000001000000001110000000000000001001110011
2	001000001000001111000010011110010011000000000001001001110011
3	000000000000000100000000011000011001000000010001001001110011
4	000000000000000000110000011110000011000000010001000001110011
5	000000000000000000000000011110000001100000000001000001110001
6	000000000000000000000000000000011001101010000111011111111011
7	000000000001100000000000011110000001110001110111001111111111
8	000000000000000011000000001111001100110001110111111111110011
9	000000000000000010000000010110101001100010000111000101110011
10	000000000001000111000000011000000011110011110101111111111011
11	000000000000100011000000011100000011110011110001011111110011
12	000000000001000111000000011000000011101000000011110111111011
13	011101001000111111111110111011000111111111100011111111111111
14	000000000010000111011000111000000001111001000001111111110011
15	000000000000000111001000011000011011110001000001110101100011
16	000000000000000000000000011100111001110011110101011111111111
17	000000000000000111010000011000000011111010000001010111111111
18	000000000001110111001000011000000001111001000111111111110011
19	000100000000000111001000011111100001110001100001011111111111
20	000000000000000011001000011000000011101011010111111111110011
21	000000000000000010001000011000000001110000000001011111111011
22	000000000000000111000000011000001001111111000111110111111011
23	000000000000000111000000011000011011111011000001111111111111
24	000000000000000011001000011100100011100010000111000101110011
25	000000000000010010001000011111000011100011010001001101110011
26	000000000000010011001000011000000011111111110111011111111111
27	000000001110010111000000011100111011111111110111011111111110
28	000000000000000111000000011100000011111111110111111111111111
29	010000000000000011001000011100000001100001100001111111111011
30	010100000000010111001000011100000011110000000011111111111011
31	000000000010000011000000011000001001100001000001000101110011
32	000000000010000111001000011010001011111001000001110001110011
33	000000000000000111000000011110000001110001000001100101110011
34	000000000010000011000011011100001001110001110111110101110011
35	000100000000000111001000111000011011110011010011001111111011
36	000000000000000011000000011000100001110001000101111111110011
37	000000000000000111000000111000000011111111110111111111101011
38	010100011100000001000100011000100001110000000000111111000000
39	000000000000000111001000111000100001110011100000000000010001
40	011101111000011111100100100101100011100010000000000111110000
41	000000000100000011000000111000001001100000110001111111111011
42	000000000000000110000000011000011001101001011101000111110011
43	000000000000000010000000011000011001101000111101000111111001
44	000011100000000111101111011100111011101001110001101111111111
45	000000100000011111111110000101100111011111110011000001110000
46	000000000000000011100000000000111000100001110111001111111110
47	111111111111011001110110000000000001000011110111111111111111

Appendix Table 5 Matrix for M-CAT/E-AAC primer combination read at 700 nm channel. The number of polymorphic loci scored was 60. “0” means absence “1” means presence of the allele.

no	Polymorphic loci (1-60)
1	000000011110000111000000011111111100001111111111110011111111
2	000000111110000000000011111111111110001111111111111110011111
3	111111000011111111111111111111111000000000000000000000000100
4	000000111110000000000000000011100000011110111111110011111111
5	000000111100010000000001011011110000001111011111110011111111
6	000000111100000000000011011111110000001111000111110000011111
7	110111000000001111111110111111111100100011000111010000011100
8	000000111110000000000001011111111100001111111111111111111111
9	000000111110000000000011000011110000001111111111110000011111
10	000000111110001000000010111111111000001111011111110000011111
11	000000111110000000000000010111100000011110111111100011111111
12	000001111110000000000000111111101100001111111111111111111011
13	000000111110000110000000111011100100001111111111111111111011
14	000000111110000000000000111110101100001111111111111111111111
15	000000111110010000000000111100000000001111101111111111111111
16	000000111110000010000000111011111111011110111111100111111111
17	000000000000000000111010110011111111101111011111111100011111
18	000000111111110000000001011101100000001111011111110010011111
19	000000111100000000010010111111100000000011011111110000011111
20	000000111110010000000000011111101000001111111111111110011111
21	000000111110000011000000001101101100001111111111111111111011
22	000000111110010011100001001111110000001111111111110001111111
23	000001111110011111111110111111111100001111111111111101111111
24	000000111110011110100000111111111000111111111111111111111111
25	000000111110000000000001010111110000001111110111111111111111
26	000000111110010000011111111111101000011111111111111111111111
27	000000000000010000101111111111111111111111111011110110011111
28	000000111000011011011111111111110000001110111111101100111111
29	000010111100011111010010011111011100000111011111110111011111
30	000000111110010000000000000010001000000111111111111011111111
31	000000111110000110000000001111110000001111111111110111111111
32	000000111110010110000000001111111000000111111111110111111111
33	000000111100000000000011111111111000001111111111111100011111
34	000000111010001111111110111111111110001111001001010000001111
35	000000111010010000000011000001111100011111011111111011111111
36	000000111110011100000010111111111000001111010111110000011111
37	011000111110010000000010000001000000011111111111111110011111
38	000000000000000000000010011011111000001111111111110011111100
39	000000111110001111000011111111111100011111111111111111111111
40	000000000000011111111101111111111111110001100011111011011111
41	000000000000010000011101111111111110011101111110110011111111
42	000000111110000000011111011110111111011111101111101100111111
43	000000000010000010111011111111111111110001101111111001111111
44	000000000000000000011111111111011111000110111111100111111111
45	000000000000001100111111111111011111000110001111100100111100
46	000000000000010000111011111111111111101111111111110010011111
47	000000000000011110111111111111111111001111010111110011111111

Appendix Table 6 Matrix for M-CAT/E-ACG primer combination read at 800 nm channel. The number of polymorphic loci scored was 60. “0” means absence “1” means presence of the allele.

no	Polymorphic loci (1-60)
1	111010111001101000001110001111110000010110000110111111100111
2	111011011100111111000111111111110000011111000011111111111111
3	100000000000000000000000000000000000000000000000000000000000
4	101010011101111000000111111111111000111111011111011111111111
5	111111011100111000001111111111111000111111000000111111001111
6	111000000001111000111111111111111000111111000011000111110111 0
7	000000001100000000000011111000000000000110000000001101100001
8	111111111111111001111111111111111001111111000011111111111111
9	111111001100111100000111111111111001111110000110110111111111
10	111111101111110100111111111111111101111110000011111111011110
11	100101001100011000001101111011000011111100000001111111001111
12	101111111101111000011111111111111101111110000111111111111111
13	111111011101110000011111111111111001111110000111111111111111
14	111111111111110100001111111111111111111101111111111111110110
15	111101001110110000001111111111110011111100001111111111100110
16	110001111111111000111111111111110011111100001101111111010 10
17	110101101111111100000010001111111001111100000110011111110000
18	100111001100111100000011111111111100111110110110110111111111
19	110111001111111000000011111111110000111110001001111110110010
20	100011001100011000000011111111110000111110000011111111111111
21	100101101100110000000111111111100011111100000111111111110010
22	100111101100110000000111111111100001111100011111111111111111
23	111111111111011000001111111111110001111110000011111011010010
24	101111001100001100000011111111111000111111000001011111111111
25	111111001101000000001111111111111001111110000010111000110010
26	101011000000100000011111111111111000100110000010111001100000
27	110001101110000000011111110111100011111100000110010111111111
28	110001101100111000000110111111111000010110000011111011011001
29	111101111000111000001110111111111000111110000010111111111111
30	111101111000111111011111111111111001111110000110111111011111
31	111111001000111111011111111111111001111110000110111111011111
32	110011011000111000011111111011111001111110000110111111111111
33	100010001000111000011111111111110000111110111110111111110010
34	100110001000111111011111111111111111111100000101110001111 10
35	111011111001111000011111111111111001111110000111111111111111
36	100001001000111000100111111111111000111111000011011111100111 1
37	111111111110111001010111111111111000111111000011111111001010
38	111110000011111000000000000101110001111111000000000000110000
39	100111001010111000000001110111111000000110000111111111111111
40	111101111010000000000111111111111000100111000110111111110010
41	110001001000110000101000110111111000111111000011011101111111
42	111100001000001111000111110111111000111111000110001111111111
43	110101000000110000000111111111111000111111000110111111111110
44	111100000011111111011111111111111011111111000110111111111110
45	110100001000001111010010110111111000011110100011011110011110
46	110000001000001111011011111111111000111111000111111111111110
47	111010101000001000000111111111111011111111000110111011110010

Appendix Table 7 Matrix for M-CAG/E-AAC primer combination read at 700 nm channel. The number of polymorphic loci scored was 60. “0” means absence “1” means presence of the allele.

no	Polymorphic loci (1-60)
1	101110101101111110101000111111000000000000001000001110000000
2	101111111111001110111000111111000010000000000000001010000000
3	101111111100111100111000110111001000000000010000001110000000
4	100100100001110000000000000011000001000010000000001010000010
5	100111110101110100000010110011000000000000000000001010000000
6	100100011101111110011011110111010010001100111110001110000010
7	000010100011110110001000000100011001111111111111001111111111
8	101111111100111111101111111111000000000000000000000010000000
9	000011111100111110011000110100001111001100000000001110000010
10	001000111101111010010000000000010010111001110000001110110010
11	000011111111111110011111111111000011111111111110001110000011
12	101111011100111101111111111110011111011100000000000010000000
13	000100100011000000000000000000000000000000000000000000000000
14	101111111100111111111111111111001001001100011001101110000010
15	101111111101111111111001000110001001011100111000001010110011
16	101111101111001111111111111111010111111010011100011110011011
17	001011111100001111111111111111011001111111110010001001111111
18	101111111100111111111001001000011011111111111110011110111110
19	001011111100110001110000000011000110000000000000001110000010
20	001011111111110001110000000100001000000000001000001110100010
21	000011111100111111111000111111000111000000001100001110000000
22	101111111100111111111010000111001011111111111111011110000111
23	101111111111111111111000000011000001010100111111111010001111
24	101111111100001111111111111111011000000110111111001110000010
25	001010110000000011011110000011111111100000000000011000010010
26	011000111000000111111000110110000000111010011000011111101111
27	011011110000000000011010000011000000111110000011011001111111
28	101111111100110010011000000111001001011100000011101110000010
29	101111111100110111011000110111000111000000000000001110000000
30	101111111111110010011000110111000111000000110011001010000110
31	101111111111111111011001110111011001110110111111001110110011
32	101111111111111111011001110110001001010110111111001110110010
33	100111111101001111111001111111001001011100001100001010010010
34	000011110011111011111000000000111111000000000000011000000011
35	100111111100111111111111111111000001001111111111011010001111
36	001011111100110101111010000111000110001000001000001110000001
37	001010110000000100011010000011001000010011000000001000110110
38	001010110001001110101000000100111001111111101011111001111111
39	101111111111111111111000001111001011011100111111001110110010
40	001011111100111111100001111010000000000000000000001000000010
41	000010111000110001110000000011011001011000000000001000100011
42	011011110001000110001000000000111001111111111100001001111011
43	000011111100110111111000000111001011111101001111001111000010
44	000011000100110111101000000111000011111101001111001110000000
45	000011110100110001100010000111000000000000000011001111100010
46	011011110100110001100000000011000000011000000000001000000000
47	111100011100010000000000110000000000000000000000000000100010

Appendix Table 8 Matrix for M-CAG/E-AGG primer combination read at 800 nm channel. The number of polymorphic loci scored was 60. “0” means absence “1” means presence of the allele.

no	Polymorphic loci (1-60)
1	000001000100000000000000000000000000000000000001111001110000000
2	000000001110110000000000000000000000000000000001111000110000000
3	000000000110000000000000000000000000000000000001111011110000000
4	000000000010000010000000000000000000000000000001111111110000000
5	000000000010000001000000000000000000000000000001111111110000000
6	000000000000110000000000000000010000000000000001111111110000000
7	000000000111000000000001110111101111110111111111100001100000000
8	000000000010000000000000010000000000000000000001111111111110000
9	000000000000000000000000000000000000010000000011111111110000001
10	000000000001000000000000000000000000000000000001111011110000000
11	000000000011000000000000000000000000000000000001111111111111100
12	000000000011000000001110000000111010000000001111111111111100
13	111110110000000000000000000000000000000000000000000000000000000
14	000000000000010000000000000000000000000000000001111111111110000
15	000000000000000000000000000000000000000000000001111111111100000
16	000000000010010000000000000000000010000000000111101111001000
17	000000000000000000000000000000000000000000000001111111111111110
18	000000000001000000000000000000000000000000000001111111111110100
19	000000000011000011000000000000000000000000000001111111110000000
20	000000000001000000000000000000000000000000000001111111110000000
21	000000000011000000000000000000000000000000000001111011110000000
22	000000000011000000000000000000000000000000000001111111110000000
23	000000000011000000000000000000000000000000000001111111110000000
24	000000000001000000000000000000000000000000000001111111110000000
25	000000000000000000000000000000000000000000000001111111110000000
26	000000000000010000000000100000000000000000000001111111101000000
27	000000000001010000000000000000000000000000000001111111111100010
28	000000000001000000000000000000000000000000000001111111110000000
29	000000000001000000000000000000000000000000000001111111110000000
30	000000000001000000000000000000000000000000000001111111111100000
31	000000000001000000000000000000000000000000000001111111111100000
32	000000000000000000000000000000000000000000000001111111110000000
33	000000000001000000000001000000000000000000000001111111110000000
34	000000000001000011000000000100000000000000000001111111100000000
35	000000000001010000000000000000000000000000000001111111111011000
36	000000000001000000000000000000000000000000000001111111111110000
37	000000000001000000000001000000000000000000000001111111111100000
38	000000000000000000000000100100000000000000000001111111111111101
39	000000000000000000000000000000000000000000000001111111111110000
40	000000000000000000000000000000000000000000000001111001100000000
41	000000000000000000000001000000000000000000000001111101110000000
42	000100000000000000000011010111100000110011011111110111111110
43	000000000000000000000000000000000000000000000001111111111111100
44	000000000000000000000000000000000000000000000001111111111110000
45	000000000000000000000000000000000000000000000001111111111110000
46	000000000000000000000000000000000000000000000001111111111110000
47	111111111110101111111111111111111111111111111100000000001000000

Appendix Table 9 Matrix for M-CTA/E-ACC primer combination read at 700 nm channel. The number of polymorphic loci detected was 62. “0” means absence “1” means presence of the allele.

no	Polymorphic loci (1-62)
1	11010000100000000011100000111111000011111111111111100000011111
2	00000000000000000011000000011011000010111100111011100000011111
3	11000000000000000011111100011111001011111111111111100000011111
4	11000000000000000011100000011111000011111111111111100000011111
5	11110000000000000011100000011110000011111111111111111000011111
6	11010000000000000011101111111000000010111100111011100000011111
7	11010000100000000011100000011011000000000000011011100000011111
8	11111000010000000111000000111110000111111101110111100010011111
9	11011000010000000111000000111110000111111101111111001000111111
10	11011000010000000111000000111111010111111111111111111110011111
11	01011000010000000110000000111110000111111101110111001000111111
12	01011000010000000011000000111111010111111111111111110100011111
13	00000000010000000010000000001011100000011000000001000011011110
14	00000000010000000001010000011011000011111110011011100001011110
15	00000000000000000010000000011011100000111100000001000000011110
16	11101000010000000011110000011011101011111111011111110000011110
17	10211111111110011100001111110100001110000000111110001100001001
18	00000011111010011100110001111100000111111101011111010100000 1001
19	00011111111111001110110001111100000111111100010111000100001010 1
20	10111111111110111010010111110000011100000001011100010000010 01
21	00011000000000100010000011111000001110000000101110001000000001
22	00011111111111000001110001111000001111111000111110001000001001
23	00000000000000000010010011111000001111111000111110001000000011
24	10111111111110000001111101111000001111000000111111001000000011
25	11111100000000000010100000011000000011111111111111111110010111
26	10111111111111111111111000011100100111111110111111110000011110
27	11111100011110000000111000011101101111111111111111111111000011111
28	01111100001110000000111000011100001111111111111111111100011111
29	01111100001000000000100000001101101001111111111011111100011111
30	01111100001000000000111000011101101111111111011111111100011111
31	01111100001000000000100000011001100111111111111011100000011111
32	01111100001000000000100000011101100011111110111111111101011111
33	00011100001000001100100000011101100101111110111011111111011111
34	01111100001000001100111000011100001111111110111011111111011111
35	00111110001000000000111000011101101111111110111011111110011111
36	00111100001000001100111000011101101111111110111111111110011111
37	11111100001000000000111000011101100110111110111111111100011111
38	11111111111100000000100000011100000111110110111111111100011111
39	00111000000000000000100000011101100001111110011011100110011111
40	11111101011100000000110000011100001111111110111111111100011111
41	11111100000000000100110000011101001111111110111111111010011111
42	11111100010000001000110000011101100011101110111011101100011111
43	01111100000001000100110000000101101111101110111111110100011111
44	01111100000000000000111000000101110100101110111100111100111111
45	01111110111010001100111000110101111111100010111100111100011111
46	01111100001000000000011000100100000100100010111110111100011111
47	01111110001010000010011100110100000110111111111110111110001111

Appendix Table 10 Matrix for M-CAA/E-AGG primer combination read at 800 nm channel. The number of polymorphic loci scored was 60. "0" means absence "1" means presence of the allele.

no	Polymorphic loci (1-60)
1	000000110000010000000011011011110001111110000101111111010111
2	000000000000110000000010010000100001100110000101111111110111
3	000000000000100000000100110010100011101110000011101100110111
4	000000000001100000001100100000110101000110000011001101110110
5	000000000001100000001100100000101110001100000011011101010110
6	000000000001100000001100100000100000001100000011101110110111
7	000000100011000000000000000000000111100100100000001100000000
8	000000000001000000001001100000101000001110000011111100110111
9	000000000001000000001111100000110111001000000111101100110101
10	000000000001100000001101100011111111001110000110111001110111
11	000000000001000000001001100101001000000111000110111001100111
12	000010000001000000001101101001110111001110000110111000110111
13	000000000001100000001101100010111111001110000011111100010111
14	000000000001100000001100100000111100000110000111111100000111
15	000010000001100000001101100100101001000111000111101100010111
16	000000000001000000001100001000000110000110000000001100010110
17	000000000000011111110000000000110011000011111000001100101110
18	001111111100111111110000010000011000001010100110001111011111
19	011011101100111110110000010000011110001010100110001111001111
20	111111011100111110110000010000011000001010100110001111001111
21	000000000110001111110000011000101000000111101110001111111111
22	010111100110111111110000010000010000001110100110000110001111
23	011111101100111110110000010000011000001010100100001110101011
24	010001101100011111100000010000011000001010100110010110001111
25	000000000001000000000001100000001000001111011111100101010111
26	110000100001000001110001110101110110001110000000010001111110
27	001111110001100000011100110011111111001111101001111111111110
28	000000111001100001001101100000000111100111000001011110100011
29	000000000001100000001100100000110111001110001100111100010111
30	000000000001100000001100100011111111001111001101111100010111
31	000000000001000000001001000000010110011100000111111010101111
32	000000000001100000001100100100100111001110001011111101010111
33	000000000001100000001101101011010000011110111111111101010111
34	000000000001100000001111110010100111001110001001111010101110
35	000100000001100000001001100001110101000110000111101100000111
36	000010000001000000001101100001111101000110010100111101000111
37	000000000000100000001100100000001000000110100100101111110111
38	000000110000100000000001001000100001011100000100011011100110
39	000000000000000000000000100110011000101000100011000101111111
40	000000010000000000000000000011010000011010000101011000101110
41	000000000000000000000000100010000000011101010001111110111011
42	000000000000000000000000000100010000110010101111101101110111
43	000000000000000000000000100000010000111011101111101111111111
44	000000000000000000000000111000000000011111101111101111111111
45	000000000000000000000000111000000000011111100011101111111111
46	000000000000000000000000000100000000111111000111111111101111
47	000000100000000000000000000111001110011111100011010111111101110

Appendix Table 11 Matrix for M-CTA/E-AAC primer combination read at 700 nm channel. The number of polymorphic loci scored was 60. “0” means absence “1” means presence of the allele.

no	Polymorphic loci (1-60)
1	000000000000000000000100110001111111101001010011011110111111
2	111100000000000111111101111011111111011011100110000100111111
3	011100000000100010110101110011111111000010110100000101111110
4	010000000000000010101001101011100111011011110110001000111111
5	000000000000000000001101110011111111000011000110001100111111
6	000000000000000000011001100011110110000011010110001111111110
7	010000000000000000000001000000000010000011010010000000011111
8	000000000000000010101000101010000010000011010111001101111011
9	000000000000000010101101011011111111111011110111111111111111
10	111111111111111111110011111111111111111111110110011100111011
11	000000000000000000001001100000000110100010010110011000111110
12	000000000000000011101011101111111111010110100101011001111110
13	010100000000000010111101111011111111101111010111101110111111
14	010000001111100010101101111011111111010110101111011001111111
15	011100000000000010001101110011111111101011010111101100111111
16	000100000000000000001000000000010000001001010111101100111111
17	000000000000000000001000000000110000000000010000000011000001
18	001000000000000000101101110011111111101110101111010001111111
19	010010000000000010111101111011111111100011010011101100111110
20	010000000000000010111101111011111111101011010011101100111111
21	010100000000000010111101111011111111100011010011101100111111
22	000000000000000010111101111011111111100011010011001100111111
23	010000000001110001111011100111111111000110100110010001111111
24	000100000000000010111101101011111111101011010111100000111111
25	010000000000000000111011100011011111000010101000001000111111
26	000000000000000000011101100000000001100101010111100000111111
27	000000000000000010111111101001110011000110001111010001110010
28	000000000000000010111101101001100111101011000000000100011011
29	000000000000000000101101100011111111100001000110100100111111
30	000000000000000010001101100000000111100001000000100100111111
31	000100000000000110111101101011101111010110101100111001111110
32	000100000000000010101101100011111111101011010110111001111111
33	000000000000000010110101100011111111101011010110101100111110
34	000100000000000010111101110000001111101011010100000000011111
35	000000000000000000101100110011001111010010101000011001111110
36	000100000000000000001101110011111111011001001001001100111111
37	000000000000000010111101111011111111101011110111111100111111
38	000000000000100000111000000001111000101010100011101001001000
39	011100000011000110111101111111111111001011100110001000111110
40	000100011000000111101101100010011110010000100000000000011111
41	000100000000000010111111110011111110101010100000001100111011
42	000000000000000000110000000011111110011111100100011111111111
43	000100000000000000101100000011011111011000100100100000111110
44	000100000000000000101101000000001010100000000100100000111100
45	000100000000000001110000000000101111010010000100011111101011
46	000100000000000100001111110001101010000010000001000000101011
47	000100000000000111101111111011111111100011010000000000111111

Appendix Table 12 Matrix for M-CTA/E-ACG primer combination read at 800 nm channel. The number of polymorphic loci scored was 60. "0" means absence "1" means presence of the allele.

no	Polymorphic loci (1-60)
1	000000010000100100001111100000000001111000001110000100111111
2	000000000010000000001111100000000001110011111100100111111111
3	000000000010100000001111100001000001110111011001000011111111
4	000000000000000111111111100000000011100000011100000001111111
5	000000010000000000000011111000000011110000011100100001111111
6	000000010000000100000111100000000001110000011101101111111111
7	000011101001111110001111100000000101110001101101000000000000
8	000000100000011100000111100000000001100000011100111101111111
9	000000000000000000000011110000010000110000011111111111111111
10	000000000000000110111111100000000001110011110111000000000000
11	000000000000000000000000000000000001110000101110110111111111
12	000000000010000000001111000110000011110101011001000111111111
13	000000000000000000000011110001100000111101111110100111111111
14	000000000010000000001111000111000111110111111011001111111111
15	000000000111000010111111100111000001111011101100100011111111
16	000000000000000000000000000000000001110001011101000011111111
17	000000000000001110000000000000000000110000000000000000000110001
18	000000000010011000000111100011100001111000101111100111111111
19	000000000000000000000011100001100000011011101111100001111111
20	000000000111000100001111100001000001111011101100100001111111
21	000000000000000000000111100000000011110001101111101101111111
22	000001000000000000001111100000000111110011101100100001111111
23	000000100010000100000111100001000001111111101100100100110111
24	000000000000100000000111100000000011110011111100000000011111
25	111110010000100000000111100000000001110000000000001110000001
26	000000000000000000000000000000000001100111100000000001111111
27	000000000000000000000111100000000000000011100000000010100001
28	101011100000001100000111100000000000110111100000100011101110
29	000000000000000000000011100000000000000000000111100010111111
30	000000000000000000000011100000000111100011111100100101111111
31	000000000000000000000111100001000001110011011100100101111111
32	000000000000000000000111100000000001110011011100100101111111
33	000000000000000000001011100000000001100011100100100101111111
34	000000110000100100000110100000000000110010011100000000010001
35	000000000000000000000111100000000000100011100100100000111011
36	000000000000000000000011100000000000011011111111000101111111
37	100000000000000100000111100001000000111111111100111100011100
38	000000000000000000000000000000011100000000110110011111111111
39	000000000000100100001111100001000111111000110111100001111010
40	000110000011000100000000000001000000111100000000100000000001
41	000000000000000000000110100001000001111000001100111100111111
42	000000000000100000000111100000000000000010100000000001110000
43	000000000010000000000111000000000000011000000111000001111111
44	000000000000000000000011000000000000010000001111000101111111
45	000000000000110000001111100000000100001111100000000000000000
46	000100000010000100000111100000000000110000000100011100111111
47	000000000011000000000000000000000000110011000010110000000001

Appendix Table 13 Matrix for M-CTC/E-AAC primer combination read at 700 nm channel. The number of polymorphic loci scored was 60. "0" means absence "1" means presence of the allele.

no	Polymorphic loci (1-60)
1	011110000011111100000000111000000111001001000000011000110000
2	011111000010001100000000111000000101000000000000011100110000
3	111111000110001101111000111000000100000001000000011000110000
4	010100000000001100000000111000000101000000000000011000110000
5	110110000110001100000000111000000110001000000000011000110000
6	010110100011001111111101111111101111011011110000011011111000
7	000000000000000000000000000000000000000000000000000000000000
8	000011000111001111011100111111101111111101110000011111111000
9	010000010111001101001100111111101111001101110000011111110000
10	101011010111001101001100111111101111001101110000011111111000
11	011111000010001101000100111100000101000101000000011000110000
12	011100000011001101010000111100000111000000000000011110110000
13	011011010011001101000110111111001111000101110000011111111000
14	001000000010001101000000111100000111000000000000011110110000
15	001010010011001101000100111111001111000001110000011111111000
16	001000000011001101000000111000000111000100011110011010110000
17	000000100000000000111100000011100000000011111000001111111000 1
18	000001000000000000100000100011111000000111100011011111100000
19	000000100000000000111000010001111100001111111001001111111000
20	000000100000000000111001100011111100011011110011111111110001
21	000001110000000000111000000111000000011111110011111111111001
22	000000100000000000111000000111111000000111100011011111111000
23	000000000000000000111000000001011100000011110000001111111001
24	000000100000000000111000000011000000001011110010011111011001
25	111111000110000100000000011000000000010000000001000000100000
26	010110000000001101000001111010011111100000000000110000010000
27	010000011000001011100001111111101111110010000000110000110000
28	010110100111001111001001110101100111000011000000110011000000
29	010110100111001101000000110100000110001111000000110011111000
30	001111000011000101000011110100000100011110000000010001110000
31	110000000010000111000011110000000100011100000001110001001000
32	110000000010000111000011110000000100011000000001110001001000
33	111100000111000111000011110000000100001100000001110001110000
34	000010111111111101000000011010000101010101010001111111111101
35	000110001100000111000001110001000100011100000001110011111000
36	110101001100001101000011111001000101101100000001110011111000
37	110100001100000101000011010010000100001000000001110011111000
38	100001000000000001000001001111101111110110000001111111110000
39	110100001100000101000011110000000100000100000011110000001000
40	111111000000000111000111110000000000001000000000000000001000
41	111111111111101111000111110100000100000000100011110000111000
42	111110001011001110011111111011001111001011000111100001011111
43	111000011001001111000111110100101111100000100011110000111100
44	111100001001000111000111110000100111100000000011110000111000
45	111100000000000101000111000000000010100000000011110000111111
46	111111010000001101000111110000000100000000000011110000101111
47	111111000011001101010000000000000100000000000000000000101000

Appendix Table 14 Matrix for M-CTC/E-ACG primer combination read at 800 nm channel. The number of polymorphic loci scored was 60. "0" means absence "1" means presence of the allele.

no	Polymorphic loci (1-60)
1	00000011000101010000100110011011111111001000000000000000001
2	00000001000101011000000110011011111111001000000000000000001
3	00000001000001010000000110011011111111001000000000000000001
4	01111011000111111110000110011011111111111111110000000011111
5	00100011000111111100000110011011111111111111110000000001001
6	01111111000111111101111100111111111111111111000000000011111
7	00000011000001010000000000000111000000000000000000000000001
8	00001011000111111110000110011111111111111111000000000011111
9	00101011000111111100101100111111111111111111100000000001101
10	00001011000111111100000100011111111111111001000000000001101
11	00101011000111111100000100011011111111001100000000000011101
12	10001111000111111100000110011011111111111110110000000001111
13	00001111000111111100000111011011111111111000000000000001111
14	01001111000111111100000110011011111111111000000000000011111
15	10001111000111111100000111011011111111111000000000000011111
16	00111111101111111110111111111111111111111111000000000000111
17	00001111111111111100000100000100011111111101111111100000101
18	11111111111111111101001111111111111111111101111100011100010
19	11111111111111111100001100001111111111111111111111111000011
20	11111111111111111101000100111111111111111111111111111110011
21	11101000000000000000000000101111110110000001100111100000001
22	11111111111111111110000010111111111111111111111111111110010
23	00001111111111111110000010011111111111111111111111111110011
24	11001111111111111100000010001111111111111111111111111110011
25	00000000000000000000000110000001100000000000000000000000001
26	11111100000000100000011110000011111111101101000000000111100
27	11011101100111111110111111011011111111111110000000000111111
28	00001101100011111100001110111111111111011101100000000000001
29	00001100100011111100000111011011111111101010110000000000001
30	00001100100011111000000111001011111111101010110000000000001
31	00001100100011111000000111001011111111101110110000000001111
32	00001000100011111100000111001011111111101010110000000000001
33	00001000100001111100000110001011111011010000000000000000001
34	11111111101111111111111111011111111111111111000000000111111
35	00101100100011111100000111001011111111101000110000000000001
36	00101100100011111000000111001011111111101000110000000000101
37	00101100100011111100011111001011111111111000000000000000001
38	00101111111111111100111110000011111111111000000100000111111
39	00110000000001111100000110011011111111001000000000000111001
40	00001111000011111100000111001011111111101100000000000001100
41	01101111001111111100000110011011111111101110000000000111111
42	01111111001111111110000111011011111111111110000000000111111
43	00111111001111111110000111011011111111111110000000000111111
44	01111111001111111110000110011011111111111110000000000111111
45	01110011001111111000000110001011111110001000000000000000001
46	00000000000000000000000000000010000000000000000000000000000
47	00000100000000000001111000011010000111001000001100000000111111

Appendix Table 15 Matrix for M-CAT/E-ACA primer combination read at 700 nm channel. The number of polymorphic loci scored was 60. "0" means absence "1" means presence of the allele.

no	Polymorphic loci (1-60)
1	111111111111110000000000111000000000000100000000000101110000
2	111000011111110000000011111100000111110100000000000111111010
3	111101111111110000001111111101111111111111111011111111111111
4	111101111110100101000001110000011011101000000000000011000001
5	111001111110000000000000000000010011101000000000000101000011
6	101011111110100011000000000000000000010000100000000111000010
7	111111110111111000000000000000001011000100000000000000000000
8	111000100111110000000000111100000000111111111000001111111011
9	111111011011110010100000111100001001110100000000000000001111
10	111011111100000000000000011100000000000100000000000101111011
11	111111111111000000000000000000001001111100011111100000000000
12	111111111111111100001110111011111111111111111011111111111111
13	111000000111110000000000011100001000110100000000000011011010
14	111000001111111100000011011100001000110100000001000100111111
15	111111111111110000000011011100001000110100000000000000000011
16	111000001111110000000011111000001000110100000000000011111111
17	000111111111010000000001111000000011100000000000000000011111
18	111000011111110000000111111101111011111111111011111101111111
19	111010111111110000000111111100001000010111111010000111110011
20	111111111111100000000011111100001000010100011000000011010011
21	111000011011100000000011111100001000110100000000000000001011
22	111111111111100000000000111100001001110100010000000111000011
23	111101111111110011000000111100001000110100010000000000000011
24	111001111111110000000000011100001001110100000000000011000010
25	111000001111110000000011111100001001110100000000000000010011
26	000010011011110000000011111100001000110100000000000000010001
27	000011100100000000000000011000001000110100000000000011110011
28	101111111111111100001110111000001011111100000000000111110010
29	111111111110110000001110011100001000110100000000000011110011
30	111111111111010000000010011100001000110100000000000001010010
31	111111111111110000000111111100001111110100001000000011110010
32	111111111111110000000111111100001111110100001000000011110111
33	111000001111100000001111111100001111110100001000000011010010
34	111000000101010000000000000000000010110001000000000100000001
35	111000001111110000000010111000001101110100000000000011110001
36	111111111111110000000111111110001111110100000000000011110010
37	111111111111110000000100111000001111110100000000000001010010
38	111111111111111111111111110000011111101000000000000011000000
39	111111111011110000000111111100011111110100000101101011110011
40	111000010100000000000010111000001111110100100000000100000110
41	111000011111110000000000111000001111110100100000000000001100
42	111000011111111100111011111101111111110100110101100001000101
43	111101111111110000000101111100111111111010000000000000000010
44	111011011111110000000000111000111111111010000000000000000000
45	111000000110110100000000111001111111110100000000000101010101
46	101101111111110100000011111101111111110100100000000000001100
47	111000000000010000000000111000000000000100000000000000000010

Appendix Table 16 Matrix for M-CAT/E-AGG primer combination read at 800 nm channel. The number of polymorphic loci detected was 65. “0” means absence “1” means presence of the allele.

no	Polymorphic loci (1-65)
1	11100001101001000000000000011100000000011000111111000000111000000
2	01000001110001000000000001111111000000111100011110000000011000001
3	01000000100000000000000001111111000001111000111100000000110000001
4	11110010000000000000000000010110000000111000100000000000000000000
5	11110000000000000000000000000010000001011000100000000000110000001
6	11110000000001000000000000000110000000111000001100000000100100000
7	10000000000110000000000001111100000010011000101100000000000000000
8	01111000000000000000000001111111000000101100011110000000011000000
9	00000101000001000000000001111111000000111100111111000000000100011
10	00000000110110000000000001111111000000111100011110000000011000100
11	00000001000001000010000011111110001111111000111111000000001001001
12	00000001010001001000000001111110000001111000111100010000110011000
13	00000001000001000000000011111110000001111100111100001000111111111
14	00000001110001000000000011111111010001111100111100001100111011111
15	00000001110000000001010001111111000001111100111100001000111011111
16	10110001110000000000000001111110000001111100101110000000000000000
17	10110000000001000000000000000010000011111100111111000111000000000
18	00000001110001001010000001111110000011111110111001110001110111111
19	11010001110111000010000011111110000001111111111100011000110000000
20	00000001110100001010000011111110100001111000111100000000110000000
21	00000001110001001010000001111110000001111000111100001000110001111
22	00000001110001001110000001111110000001111010100000001100000001111
23	01000001110001111010000011111110000000111100111000001000110001111
24	00000000000001000010000001111110000001111000100111000000110100000
25	00110011111100100100000001011001010000101100011111000000110000000
26	00001000000001000000000001011100000011110001001100011100110001111
27	00000000000001100010000001111110000001111000111111000000000000000
28	00010000000001000000010001111110000001111100111100000000110100000
29	00000011110001000010000001111110001101111100111100000000110100000
30	00000001110001000000000001111110000001111100101000000000110000000
31	00010000110001000010000001111111010001111101111100001000110000111
32	00010000110001000000000001111111010001111001111100001000110000111
33	00000001110000000000000001110110000000111000111100011000110000000
34	11001110000000000000000000000000000000000000000010000000000000000
35	00001110000001001000000000010000000100000001000000000101000000
36	00000000110111001000000001111111000110111101011111000000111000000
37	00010000000000110111000011111110001100111010100000000000010000000
38	00110000000001100000100000000000000001011010111111000000100000000
39	00000010001000000000000001111111000000011111111110011100011110000
40	11010000000001000000000000000000000001010000000000000000100000000
41	00110000000001000000000001111111000001010101011100111001111100000
42	00010000000000100000000000010010000001011000111100000011110100000
43	00001000000000000001000110111111000000111100011110000000011000000
44	00001000000000000000001101111110000001111000111100000000110000000
45	00010110001000000000001110000000000000000000000000000000000000000
46	00000000000001001000000001111100000001011001111100111000110100000
47	11111100000001000000000000000000000000010000000000000000110000000

APPENDIX B

Similarity indices for sixteen primer combinations with 967 polymorphic loci, NTSYS program version 2.1, Simple Matching module.

1.0000000
0.8097208 1.0000000
0.7456050 0.7704240 1.0000000
0.7859359 0.7693899 0.7052740 1.0000000
0.8055843 0.7869700 0.7228542 0.8603930 1.0000000
0.7538780 0.7145812 0.6794209 0.7838676 0.7993795 1.0000000
0.6680455 0.6349535 0.6514995 0.6359876 0.6411582 0.6204757 1.0000000
0.7600827 0.7600827 0.6856256 0.7631851 0.7952430 0.7869700 0.5997932
1.0000000
0.7518097 0.7497415 0.6794209 0.7817994 0.7952430 0.7786970 0.6122027
0.8076525 1.0000000
0.7362978 0.7342296 0.6721820 0.7352637 0.7507756 0.7321613 0.6318511
0.7817994 0.7859359 1.0000000
0.7487073 0.7383661 0.6721820 0.7518097 0.7859359 0.7466391 0.6732161
0.7755946 0.7797311 0.7476732 1.0000000
0.7466391 0.7714581 0.7197518 0.7518097 0.7714581 0.7197518 0.5925543
0.8066184 0.7900724 0.7683557 0.7766287 1.0000000
0.7011375 0.7569804 0.6246122 0.7166494 0.7300931 0.6887280 0.6049638
0.7445708 0.7528438 0.7435367 0.7042399 0.7373320 1.0000000
0.7342296 0.7797311 0.6804550 0.7394002 0.7549121 0.7176836 0.5801448
0.7880041 0.7673216 0.7600827 0.7662875 0.8262668 0.7724922 1.0000000
0.7311272 0.7683557 0.6876939 0.7487073 0.7497415 0.7414685 0.6101344
0.7704240 0.7745605 0.7735264 0.7631851 0.7921406 0.7921406 0.8541882
1.0000000
0.6835574 0.6814891 0.6235781 0.7156153 0.7042399 0.7125129 0.5997932
0.7249224 0.7104447 0.6742503 0.7135471 0.6970010 0.6556360 0.7280248
0.7021717 1.0000000
0.6297828 0.6173733 0.5780765 0.6246122 0.6401241 0.6566701 0.6122027
0.6566701 0.6628749 0.6390900 0.6597725 0.6452947 0.5873837 0.6246122
0.6318511 0.6421923 1.0000000
0.6390900 0.6701138 0.6287487 0.6587384 0.6597725 0.6577042 0.5429162
0.6949328 0.7052740 0.6711479 0.6835574 0.7435367 0.6401241 0.7476732
0.7342296 0.6390900 0.6577042 1.0000000
0.6577042 0.6949328 0.6370217 0.6732161 0.7032058 0.6742503 0.5760083
0.6990693 0.7052740 0.6959669 0.6566701 0.7001034 0.6794209 0.6938987
0.6928645 0.6349535 0.6763185 0.7704240 1.0000000
0.6783868 0.6990693 0.6266805 0.6959669 0.6990693 0.7073423 0.5822130
0.7156153 0.7238883 0.7063082 0.6773526 0.7207859 0.6773526 0.7228542
0.7549121 0.6287487 0.6866598 0.8014478 0.8241986 1.0000000
0.7021717 0.7331954 0.6504654 0.6742503 0.7083764 0.6876939 0.6184074
0.7104447 0.7228542 0.6866598 0.7032058 0.7052740 0.7052740 0.7280248
0.7373320 0.6442606 0.6773526 0.7238883 0.7673216 0.7797311 1.0000000
0.6659772 0.6783868 0.6184074 0.6918304 0.7073423 0.7135471 0.5946225
0.7156153 0.7259566 0.6856256 0.7145812 0.7311272 0.6483971 0.7373320
0.7487073 0.6535677 0.6887280 0.8200620 0.7911065 0.8469493 0.7880041
1.0000000
0.6794209 0.6980352 0.6421923 0.6845915 0.6959669 0.6980352 0.6059979
0.7145812 0.6980352 0.6990693 0.6907963 0.7135471 0.6763185 0.7487073
0.7642192 0.6401241 0.6897622 0.7735264 0.7735264 0.8190279 0.7704240
0.8562565 1.0000000
0.7135471 0.7114788 0.6370217 0.7001034 0.7280248 0.7114788 0.6194416
0.7404343 0.7362978 0.6938987 0.7063082 0.7249224 0.6732161 0.7269907
0.7218201 0.6556360 0.7238883 0.7600827 0.7600827 0.8304033 0.7776629
0.8407446 0.8004137 1.0000000
0.7280248 0.7466391 0.6701138 0.7125129 0.7507756 0.6680455 0.6608066
0.7094105 0.7032058 0.6876939 0.7063082 0.6876939 0.6773526 0.6897622
0.6949328 0.6473630 0.6308170 0.6215098 0.6608066 0.6442606 0.7176836
0.6297828 0.6452947 0.6835574 1.0000000
0.6783868 0.6597725 0.6163392 0.6690796 0.6970010 0.6804550 0.6277146
0.7011375 0.6928645 0.6711479 0.7083764 0.6835574 0.6649431 0.7042399
0.6970010 0.6742503 0.6556360 0.6546019 0.6546019 0.6876939 0.6825233

0.6938987 0.6659772 0.7021717 0.6897622 1.0000000
0.6577042 0.6659772 0.6142709 0.6711479 0.6887280 0.6845915 0.6091003
0.7011375 0.6845915 0.7001034 0.6814891 0.6918304 0.6773526 0.6773526
0.6866598 0.6494312 0.6721820 0.6442606 0.6794209 0.6918304 0.6349535
0.6918304 0.6804550 0.6959669 0.6732161 0.7435367 1.0000000
0.6907963 0.6970010 0.6783868 0.6918304 0.7176836 0.7011375 0.6359876
0.7197518 0.7135471 0.7187177 0.6938987 0.7331954 0.6711479 0.7042399
0.7052740 0.6783868 0.6411582 0.6587384 0.6856256 0.6959669 0.6494312
0.7001034 0.6866598 0.7063082 0.6959669 0.7083764 0.7435367 1.0000000
0.7755946 0.7693899 0.7094105 0.7435367 0.8004137 0.7342296 0.6401241
0.7776629 0.7735264 0.7621510 0.7559462 0.7931748 0.7394002 0.7600827
0.7590486 0.6907963 0.6514995 0.6897622 0.7311272 0.7145812 0.7466391
0.7125129 0.7156153 0.7497415 0.7518097 0.7104447 0.7228542 0.7890383
1.0000000
0.7569804 0.7611169 0.6804550 0.7600827 0.8004137 0.7300931 0.6173733
0.7776629 0.7652534 0.7559462 0.7766287 0.7849018 0.7311272 0.7745605
0.7776629 0.6970010 0.6639090 0.6938987 0.7083764 0.7207859 0.7383661
0.7352637 0.7342296 0.7435367 0.7373320 0.7269907 0.7269907 0.7683557
0.8593588 1.0000000
0.7518097 0.7745605 0.7001034 0.7569804 0.7869700 0.7497415 0.6349535
0.7786970 0.7724922 0.7507756 0.7817994 0.7838676 0.7259566 0.7900724
0.7931748 0.7001034 0.6463289 0.7218201 0.7073423 0.7321613 0.7331954
0.7528438 0.7414685 0.7673216 0.7321613 0.7135471 0.7094105 0.7549121
0.8210962 0.8459152 1.0000000
0.7631851 0.7755946 0.7114788 0.7580145 0.7983454 0.7342296 0.6277146
0.7817994 0.7817994 0.7621510 0.7724922 0.7931748 0.7269907 0.7973113
0.8045502 0.7011375 0.6494312 0.7249224 0.7021717 0.7394002 0.7259566
0.7435367 0.7321613 0.7621510 0.7394002 0.7249224 0.7187177 0.7662875
0.8407446 0.8552223 0.9265770 1.0000000
0.7476732 0.7828335 0.7166494 0.7466391 0.7683557 0.7311272 0.6184074
0.7600827 0.7559462 0.7425026 0.7549121 0.7735264 0.7032058 0.7817994
0.7745605 0.6959669 0.6359876 0.7094105 0.7011375 0.7259566 0.7331954
0.7156153 0.7104447 0.7197518 0.7466391 0.7094105 0.6907963 0.7383661
0.7962771 0.8128232 0.8324716 0.8521200 1.0000000
0.6670114 0.6690796 0.6297828 0.7011375 0.7104447 0.6980352 0.6204757
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Similarity indices for five primer combinations with 302 polymorphic loci, NTSYS program version 2.1, DICE module.

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0.6478873 0.6713781 0.6953405 0.6877193 0.7000000 0.6732673 0.6881720
0.7090301 0.7410072 0.6887417 0.7084871 0.6920152 0.8327645 0.8138801
0.8940397 1.0000000
0.5945946 0.5761317 0.6000000 0.5747126 0.5859375 0.4982206 0.3834197
0.5753425 0.5473684 0.5644599 0.5680934 0.6118421 0.5076923 0.5603113
0.4827586 0.5608856 0.4943820 0.5750799 0.5389610 0.5372168 0.4720000
0.5503356 0.4837545 0.5583039 0.5789474 0.5714286 0.5906040 0.6048110
0.5808824 0.6199262 0.6142322 0.6446886 0.6641791 0.6048110 0.6741573
0.6759582 0.6842105 0.6275862 0.6409266 0.6613546 0.7330961 0.7213115
0.7379310 0.7985612 1.0000000
0.5172414 0.5555556 0.5928854 0.4615385 0.4803493 0.4173228 0.3734940
0.4603774 0.4651163 0.4692308 0.5217391 0.5270758 0.4120172 0.4521739
0.4444444 0.4098361 0.3916667 0.4335664 0.3772242 0.4113475 0.4753363
0.3985240 0.3840000 0.4140625 0.6169154 0.4878049 0.4428044 0.5530303
0.5306122 0.5819672 0.5833333 0.6178862 0.5975104 0.4545455 0.5583333
0.6153846 0.6192469 0.5247148 0.6034483 0.6071429 0.6771654 0.6258993
0.6615970 0.6693227 0.7112971 1.0000000
0.5887446 0.5209302 0.5238095 0.4978541 0.5175439 0.5059289 0.3151515
0.5151515 0.4747082 0.5559846 0.5414847 0.5072464 0.4568966 0.4890830
0.4463519 0.4938272 0.4016736 0.4140351 0.4214286 0.4128114 0.4144144
0.4296296 0.4417671 0.4549020 0.5800000 0.5224490 0.5555556 0.5551331
0.5327869 0.5843621 0.5271967 0.5142857 0.5416667 0.5627376 0.5355649
0.5559846 0.5798319 0.5190840 0.5194805 0.6098655 0.6245059 0.5198556
0.5572519 0.5920000 0.5798319 0.6255924 1.0000000

Similarity indices for five primer combinations with 302 polymorphic loci, NTSYS program version 2.1, JACCARD module.

1.0000000
0.6619718 1.0000000
0.5780347 0.6369427 1.0000000
0.5582822 0.5657895 0.5536723 1.0000000
0.5660377 0.5328947 0.5428571 0.7552448 1.0000000
0.4810811 0.4494382 0.4532020 0.5953757 0.5755814 1.0000000
0.3381295 0.2878788 0.2857143 0.3146853 0.3260870 0.2235294 1.0000000
0.5079365 0.5112360 0.5532995 0.5683060 0.5580110 0.6417112 0.2443182
1.0000000
0.5274725 0.4886364 0.5101010 0.6666667 0.6176471 0.6129032 0.3086420
0.6902174 1.0000000
0.5642458 0.4915254 0.5202020 0.5580110 0.6198830 0.5729167 0.2814371
0.7103825 0.7191011 1.0000000
0.5337423 0.5194805 0.5139665 0.5949367 0.5833333 0.5111111 0.3333333
0.5722222 0.5771429 0.5977011 1.0000000
0.5388601 0.5271739 0.6736842 0.6162162 0.5806452 0.4976526 0.3050847
0.6336634 0.6395939 0.6414141 0.6032609 1.0000000
0.4457143 0.5095541 0.4421053 0.5088757 0.4880952 0.5277778 0.2384106
0.6342857 0.6034483 0.6432749 0.4940476 0.5360825 1.0000000
0.4880952 0.5918367 0.5139665 0.6050955 0.5632911 0.5111111 0.2777778
0.5988701 0.5862069 0.5795455 0.5696203 0.6388889 0.6513158 1.0000000
0.4682081 0.5159236 0.4550265 0.5515152 0.5029940 0.5681818 0.2617450
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1.0000000
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0.5375723 1.0000000
0.2682927 0.2642487 0.2949309 0.3232323 0.2979798 0.3623188 0.1757576
0.3440367 0.4019608 0.3714286 0.2900000 0.3555556 0.2920792 0.2964824
0.3165829 0.3333333 1.0000000
0.3362445 0.3615023 0.4342105 0.3811659 0.3466667 0.3781513 0.1594203
0.4364407 0.4067797 0.3858921 0.3333333 0.4747899 0.3524229 0.4074074
0.3628319 0.4324324 0.4672897 1.0000000
0.3437500 0.3380282 0.3702128 0.3835616 0.3669725 0.4355556 0.1928934
0.4585153 0.4279476 0.4181034 0.3230088 0.4356846 0.3665158 0.3842593
0.3835616 0.4292237 0.5297030 0.7317073 1.0000000
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0.4135021 0.4017094 0.3807531 0.3100437 0.3715415 0.3289474 0.3452915
0.3755656 0.3771930 0.5656566 0.6635514 0.7638191 1.0000000
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0.3333333 0.3517588 0.3415842 0.2819149 0.2914798 0.2978723 0.2887701
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1.0000000
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0.7597765 0.6800000 1.0000000
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0.4375000 0.4527363 0.4271845 0.4042553 0.4532710 0.3622449 0.4270270
0.3886010 0.4479167 0.3365854 0.3675214 0.4062500 0.3920705 0.3045685
0.3863636 0.2968037 0.3679245 0.3823529 1.0000000
0.4405941 0.3819095 0.4181818 0.5340314 0.5078534 0.5268293 0.2228261

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1.0000000
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0.6748466 0.6894410 1.0000000
0.4696133 0.5060241 0.5513514 0.5672515 0.5939394 0.4400000 0.3071895
0.5177665 0.5449735 0.5312500 0.4748603 0.5948718 0.4670330 0.5085714
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0.5615764 0.5346535 0.5522388 0.4687500 0.4819820 0.4843750 0.4842105
0.4818653 0.5257732 0.3456221 0.4024896 0.4110169 0.3916667 0.2971698
0.4043478 0.3727273 0.3936652 0.3529412 0.4326923 0.5603865 0.5265700
0.5230769 0.5179487 0.4600000 0.4752475 0.4797980 1.0000000
0.4444444 0.4787879 0.4712042 0.5321637 0.5389222 0.4461538 0.2356688
0.5260417 0.5132275 0.5238095 0.4659091 0.5250000 0.4745763 0.5176471
0.4719101 0.5111111 0.3333333 0.4080717 0.4174312 0.3901345 0.3422460
0.4103774 0.3694581 0.3990148 0.4774194 0.4652406 0.5177665 0.6222222
0.6851852 0.7000000 0.6441718 0.6913580 0.6708075 0.5368421 1.0000000
0.4814815 0.5000000 0.5050000 0.5326087 0.5303867 0.4380952 0.2662722
0.5120773 0.5300000 0.5634518 0.5027027 0.5853659 0.4789474 0.5027027
0.4920635 0.5051546 0.3714286 0.4152542 0.4304348 0.4042553 0.3482587
0.3991228 0.3732719 0.4009217 0.4733728 0.5000000 0.5336538 0.6507937
0.6839080 0.7076023 0.6941176 0.7093023 0.6900585 0.5145631 0.7560976
1.0000000
0.4632768 0.4817073 0.4973262 0.5352941 0.5515152 0.4946809 0.2866667
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0.4056604 0.3989899 0.4150000 0.4709677 0.4368421 0.5360825 0.6166667
0.6385542 0.6625767 0.6792453 0.6956522 0.6441718 0.5077720 0.6792453
0.7289157 1.0000000
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0.4393939 0.4676617 0.4195122 0.4463519 0.4561404 0.4669604 0.3236715
0.4770642 0.4265403 0.4549763 0.3548387 0.4850000 0.5333333 0.5517241
0.4800000 0.4824121 0.5235602 0.5076142 0.4747475 0.4928910 0.5000000
0.5472637 0.5675676 1.0000000
0.4566474 0.4936709 0.5082873 0.4941176 0.4561404 0.3700000 0.2916667
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0.4350282 0.4193548 0.2621359 0.3721973 0.3318584 0.3303965 0.3135135
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0.5317919 0.4831461 0.5853659 0.6024096 0.5722892 0.3786408 0.5568862
0.5909091 0.5792683 0.4816754 1.0000000
0.4104046 0.4250000 0.4480874 0.4470588 0.4876543 0.3926702 0.2361111

0.4502618 0.4285714 0.4702703 0.4756098 0.4670051 0.3764045 0.4319527
0.3743017 0.4222222 0.3125000 0.3484163 0.3257919 0.2951542 0.2566845
0.3349057 0.2970297 0.3535354 0.4105960 0.4175824 0.4587629 0.5081967
0.4685714 0.5329341 0.5272727 0.5542169 0.5059524 0.4226804 0.5180723
0.5111111 0.5398773 0.4864865 0.4610778 1.0000000
0.4891304 0.5000000 0.4824121 0.5333333 0.5224719 0.4874372 0.2530120
0.5427136 0.5384615 0.5175879 0.5632184 0.5336538 0.4627660 0.5027624
0.4838710 0.4895833 0.3301887 0.3898305 0.3628692 0.3613445 0.3250000
0.3911111 0.3644860 0.3925234 0.4464286 0.4472362 0.4834123 0.5300000
0.5026178 0.5294118 0.5494505 0.5401070 0.5464481 0.5532995 0.5666667
0.5647668 0.5786517 0.5326633 0.5747126 0.6419753 1.0000000
0.4326923 0.4536082 0.4837209 0.5228426 0.4750000 0.4953271 0.2473118
0.5467290 0.5355450 0.4885845 0.5179487 0.5733945 0.4444444 0.5102041
0.4778325 0.5121951 0.3304348 0.4308943 0.3769841 0.3864542 0.3076923
0.3925620 0.3562232 0.3702128 0.3906250 0.4511628 0.4911504 0.5000000
0.4880383 0.4761905 0.5148515 0.5145631 0.5123153 0.5068493 0.5000000
0.5233645 0.4950980 0.5592417 0.5684211 0.5343915 0.6931217 1.0000000
0.4587629 0.4590164 0.4545455 0.5489130 0.4973262 0.4805825 0.2471264
0.5339806 0.5450000 0.5024155 0.5026738 0.5471698 0.4639175 0.4867725
0.5240642 0.5051020 0.3534884 0.4100418 0.3949580 0.3991597 0.3173077
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0.5179487 0.5284974 0.5729730 0.5631579 0.5368421 0.5144231 0.5156250
0.5948718 0.5760870 0.5621891 0.5810056 0.5449438 0.7428571 0.7407407
1.0000000
0.4491979 0.4325843 0.4174757 0.5337079 0.4888889 0.4797980 0.2424242
0.4901961 0.4924623 0.4585366 0.4944444 0.5047619 0.4315789 0.4540541
0.4598930 0.4663212 0.3160377 0.3829787 0.3675214 0.3896104 0.2906404
0.4090909 0.3827751 0.3915094 0.4035088 0.4467005 0.5196078 0.5000000
0.4791667 0.5053191 0.5329670 0.5240642 0.5384615 0.5074627 0.5245902
0.5492228 0.5885714 0.5252525 0.5485714 0.5290698 0.7134503 0.6861702
0.8083832 1.0000000
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0.4038462 0.3768116 0.3932039 0.3967391 0.4407583 0.3402062 0.3891892
0.3181818 0.3897436 0.3283582 0.4035874 0.3688889 0.3672566 0.3089005
0.3796296 0.3190476 0.3872549 0.4074074 0.4000000 0.4190476 0.4334975
0.4093264 0.4491979 0.4432432 0.4756757 0.4972067 0.4334975 0.5084746
0.5105263 0.5200000 0.4572864 0.4715909 0.4940476 0.5786517 0.5641026
0.5846995 0.6646707 1.0000000
0.3488372 0.3846154 0.4213483 0.3000000 0.3160920 0.2636816 0.2296296
0.2990196 0.3030303 0.3065327 0.3529412 0.3578431 0.2594595 0.2921348
0.2857143 0.2577320 0.2435233 0.2767857 0.2324561 0.2589286 0.3117647
0.2488479 0.2376238 0.2610837 0.4460432 0.3225806 0.2843602 0.3821990
0.3611111 0.4104046 0.4117647 0.4470588 0.4260355 0.2941176 0.3872832
0.4444444 0.4484848 0.3556701 0.4320988 0.4358974 0.5119048 0.4554974
0.4943182 0.5029940 0.5519481 1.0000000
0.4171779 0.3522013 0.3548387 0.3314286 0.3491124 0.3386243 0.1870504
0.3469388 0.3112245 0.3850267 0.3712575 0.3398058 0.2960894 0.3236994
0.2872928 0.3278689 0.2513089 0.2610619 0.2669683 0.2600897 0.2613636
0.2735849 0.2835052 0.2944162 0.4084507 0.3535912 0.3846154 0.3842105
0.3631285 0.4127907 0.3579545 0.3461538 0.3714286 0.3915344 0.3657143
0.3850267 0.4082840 0.3505155 0.3508772 0.4387097 0.4540230 0.3512195
0.3862434 0.4204545 0.4082840 0.4551724 1.0000000

Similarity indices for five primer combinations with 302 polymorphic loci, NTSYS program version 2.1, Simple Matching module.

1.0000000
0.8410596 1.0000000
0.7582781 0.8112583 1.0000000
0.7615894 0.7814570 0.7384106 1.0000000
0.7715232 0.7649007 0.7350993 0.8841060 1.0000000
0.6821192 0.6754967 0.6324503 0.7682119 0.7582781 1.0000000
0.6953642 0.6887417 0.6192053 0.6754967 0.6920530 0.5629139 1.0000000
0.6920530 0.7119205 0.7086093 0.7384106 0.7350993 0.7781457 0.5596026
1.0000000
0.7152318 0.7019868 0.6788079 0.8145695 0.7847682 0.7615894 0.6291391
0.8112583 1.0000000
0.7417219 0.7019868 0.6854305 0.7350993 0.7847682 0.7284768 0.6026490
0.8245033 0.8344371 1.0000000
0.7483444 0.7549669 0.7119205 0.7880795 0.7847682 0.7086093 0.6953642
0.7450331 0.7549669 0.7682119 1.0000000
0.7052980 0.7119205 0.7947020 0.7649007 0.7417219 0.6456954 0.5927152
0.7549669 0.7649007 0.7649007 0.7582781 1.0000000
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0.7880795 0.7715232 0.7980132 0.7185430 0.7019868 1.0000000
0.7152318 0.8013245 0.7119205 0.7947020 0.7715232 0.7086093 0.6556291
0.7649007 0.7615894 0.7549669 0.7748344 0.7847682 0.8245033 1.0000000
0.6953642 0.7483444 0.6589404 0.7549669 0.7251656 0.7483444 0.6357616
0.7582781 0.7814570 0.7748344 0.7682119 0.7185430 0.8841060 0.8344371
1.0000000
0.6953642 0.7218543 0.6788079 0.7947020 0.7649007 0.7019868 0.6225166
0.7384106 0.7615894 0.7284768 0.6887417 0.7450331 0.7450331 0.8079470
0.7350993 1.0000000
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0.5264901 0.5960265 0.5629139 0.5298013 0.5198675 0.5264901 0.5364238
0.5496689 0.5496689 1.0000000
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0.5231788 0.5827815 0.6225166 1.0000000
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0.5529801 0.5860927 0.6854305 0.8178808 1.0000000
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0.5629139 0.5860927 0.5463576 0.5331126 0.5364238 0.5298013 0.5662252
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1.0000000
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