MOLECULAR MAPPING OF N GENE CONFERRING RESISTANCE TO ROOT-KNOT NEMATODES IN PEPPER

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ABSTRACT

MOLECULAR MAPPING OF N GENE CONFERRING RESISTANCE TO ROOT-KNOT NEMATODES IN PEPPER

Pepper (C. *annuum*) is one of the most important agricultural crops worldwide and Turkey ranks third among all countries in pepper production. Pepper species have economical and also pharmaceutical importance so, it is vital to develop different methods to increase pepper yields. The root-knot nematode (*Meloidogyne* species) is one of the most important biotic factors that affect pepper growth and development in Turkey. The dominantly inherited N gene which was mapped on chromosome P9, 7 cM from Me1 and 2 cM from Me3, confers resistance to pepper species against *Meloidogyne* species. The aim of this work was to develop a marker tightly linked to the N gene which can be used in marker-assisted selection. A total of 132 SSR Hpms primers, 230 EST-SSR markers and 45 chromosome 9 specific primers were used to a construct linkage map and find an N linked marker. Hpms SSR markers gave 19% polymorphism by capillary electrophoresis, EST-SSR markers showed 5.2% polymorphism by agarose gel electrophoresis while the chromosome 9 specific markers, yielded 20% polymorphism by fragment analyzer. When all 407 analyzed markers are considered, only 11.3% polymorphism was observed and these results were expected because we used an intraspecific population. The, polymorphic markers were mapped in a “Carolina Wonder” X “AZN-1” F2 population and analyzed with JoinMap software. Three markers were linked with the N gene. These markers are ScarPM6a (3.6 cM), ScarPM6b (10.2 cM) and ScarN (22.6 cM) which are located with same segregation group with the N gene. These markers will allow development of a marker tightly linked to the N gene which can be used in marker-assisted selection to increase the efficiency and effectiveness of pepper breeding for nematode resistance.

ÖZET

BİBER'DE KÖK-URU NEMATODUNA DAYANIKLILIK SAĞLAYAN N GENİNİN HARİTALANMASI

Biber (C. *annuum*) tarım ürünleri arasında dünya çapında büyük bir öneme sahiptir ve Türkiye biber üretimi bakımından dünya sıralamsında üçüncü sırada gelmektedir. Biber türünün ekonomik değerinin yanında, sağlıklı besin içerikleri bakımından da büyük öneme sahiptir, bu yüzden biber üretiminin arttırılması ve geliştirilmesi büyük önem arz etmektedir. Türkiye de kök uru nematodları (*Meloidogyne* species) biber büyümesini ve gelişmesini etkileyen en önemli biyotik faktörlerden birisidir. Kök uru nematoduna karşı dirençlilik sağlayan dominant olarak kalıtılan N geni, biber türünün 9. kromozomunda; Me1 genine 7 cM ve Me3 genine 2 cM uzaklıkta olduğu araştırmalar sonucunda tespit edilmiştir. Bu projenin amacı; N genine yakın bir konumda bulunacak ve MAS'de (Marker Assisted Selection) kullanışlı olabilecek bir makör geliştirmektir. Bu amaca ulaşabilmek için 132 SSR Hpms primeri, 230 EST-SSR markörü ve 45 kromozom 9 spesifik primeri belirlenmiştir ve bu markörler bir linkage haritası oluşturmada kullanılmıştr. SSR Hpms markörleri kapillar analizleri sonucunda 19% polimorfizim, EST-SSR markörleri 5.2% ve 9. kromozom spesifik markörler 20% polimorfizim göstermiştir. Toplama bakıldığında, kullanılan 407 adet markörün yanlızca 11.3%'i polimorfizm göstermiştir ki bu sonuçlar beklentilerin çok altındadır. Sonuç olarak, bulunan polimorfik markörler JoinMap programı ile analiz edilmiştir ve 3 adet N geni ile bağlantılı markör bulunmuştur. Bulunan markörlerden ScarPM6a N genine 3.6 cM mesafe ile en yakın olanıdır. ScarPM6bN genine 10.2 cM yakınlıkta ve ScarN N genine 22.6 cM yakınlıkta bulunmuştur. Analiz edilen bu markörler ilerde N genine sıkı bir şekilde bağlı bir markör bulmada ve kök uru nematoduna karşı yeni biber türleri geliştirme de MAS'de uygulama alanlarında kullanılabilir.

To My Mother Ayda Pervin ARSLAN

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CHAPTER 1

INTRODUCTION

1.1. Pepper (*Capsicum sp.*)

Pepper (*Capsicum sp.*) originated in South America and nearly 30 species in the pepper genus were domesticated and cultivated in this region nearly 6000 years ago (Andrews 1995). *Capsicum* is a member of the tribe Solanae in the family Solanaceae (Hunziker 2001). In the fifteenth century, vegetable pepper spread rapidly around the world and both pungent and nunpungent types became an important constituent of worldwide diets. In addition to its nutritional importance and spicy taste which results from capsaicinoid compounds, pepper has been widely used in medicinal applications (Bosland and Votava 2000). *C. annuum* is dominant in agriculture worldwide with *C. chinense* and *C. frutescens* among the 30 *Capsicum* species which are cultivated. Many types of cultivated *C. annuum* species such as New Mexico Chile, ancho, Anaheim and banana pepper are grown throughout the world but especially in Latin America. All of the wild parents of the domesticated species have been found and determined except for the wild form of *C. pubescens* (Pickersgill 1997).

Generally *Capsicum* species have 12 chromosomes and a diploid genome but, *C. ciliatum* is an exception and has 13 pairs of chromosomes. Genome size was estimated with flow cytometry analysis at 7.65 pg/nucleus for *C. annuum* and at 9.72 pg/nucleus for *C. pubescens*, while total genome length is nearly 3000 Mbp (Arumuganathan and Earle 1991). Polyploidy is not seen widely in the genus but it is known that some tetraploid *Capsicum* species exist (Lippert et al. 1966). Different types of chromosomal rearrangements can occur within and between species. Reciprocal translocation between chromosomes 1 and 8 in *C. annuum* and *C. chinense* is the most analyzed chromosomal rearrangement between pepper species (Livingstone et al. 1999).

Capsicum is one of the most produced vegetables crops worldwide and ranks third compared to other vegetables. Approximately 30 million tons of *Capsicum* is produced per year and Turkey, with 1.9 million tons annual production, ranks third after China with 15 million tons per year and Mexico with 2.3 million tons per year

(FAO 2010, Figure 1.1.). According to antioxidant content, pepper ranks first among other vegetables and is very rich in vitamin C content (Palevitch and Craker 1995).

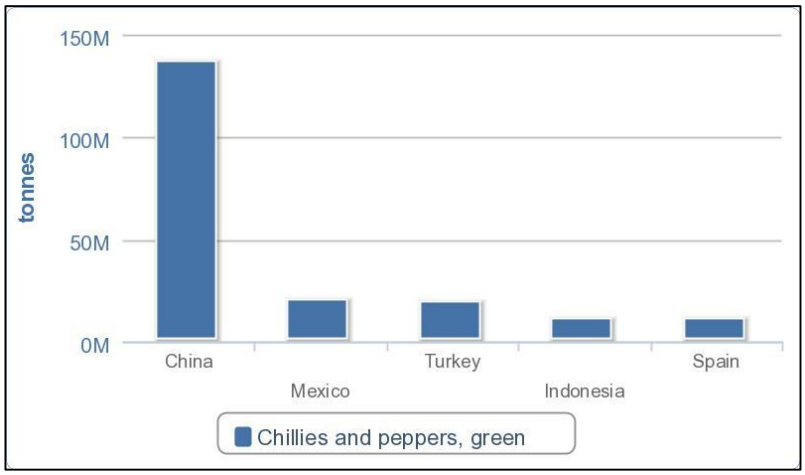

Figure 1.1. World production of pepper.
(Source:FAOSTAT 2000 - 2010)

1.2. Defense Systems in Pepper

Capsicum species have developed protective mechanisms throughout evolution. One example is the biosynthesis of capsaicinoids which are related to many other alkaloids produced in the epidermal cell of the fruit interior. These alkaloids produce a hot or pungent sensation when pepper is consumed and are important for plant survival by keeping away herbivorous mammals. Although capsaicinoids are sensed by mammals, birds cannot taste the capsaicinoids and the bright colors of pepper fruit attract birds' attention (Tewksbury and Nabhan 2001). This attraction allows seed dispersal. Presence or absence of pungency is used to classify *Capsicum* species as chile (or chilli) peppers for pungent types and sweet peppers for nunpungent types. In addition to their importance in fruit color, carotenoids and anthocyanin pigments also have important roles in nutritional content. Mature peppers may have green, red, yellow, orange or purple color, while immature fruits are green. This color spectrum is the result of accumulation of different types of carotenoid and anthocyanin pigments in fruit chromoplasts and vacuoles, respectively.

In addition to herbivory, there are many other biotic (viruses, bacteria and nematodes) and abiotic factors (high wind, extreme temperature, flood and drought) that negatively affect crop plant production. Among biotic factors, root knot nematodes are

the most important limiting factors for many plant species. Root-knot nematodes (RKN), Meloidogyne spp., are obligate, sedentary endoparasites of plants and are localized in plant roots. RKN prevent uptake of water and nutrition by plants, and feed themselves by attaching to plant root cells (Abad 2003).

1.3. Species of Root-Knot Nematode

There are several species of root-knot nematodes which differ according to their preferred hosts, environments and pathogenicity. ***Meloidogyne hapla*** is different from other nematodes in its ability to live in freezing temperatures and withstand cold winters. *M. hapla* is less pathogenic than other nematodes and it produce smaller galls on host plants. It attaches behind growing root tips and causes unbalanced root branching and prevents root growth. Common hosts of *M. hapla* are African violet, asters, barberry, beans, sugar beet, blueberry, broccoli, cabbage, carrot, cauliflower, cherry, eggplant, potato, tomato and pepper (Opperman 2008). ***M. incognita*** cannot withstand cold weather and is generally seen on plants in the greenhouse which are isolated from freezing conditions. *M. incognita* generally lives in hot and sandy soils and it severely damages plants by infecting them in the summer season, while the nematode population usually dies in the first winter weather. *M. incognita* affects plants much more severely than *M. hapla* and generally causes death by forming large root galls on infected plants. *M. incognita* infects many crops including asparagus, beans, cabbage, carrot, clovers, corn, cotton, cucumber, eggplant, grape, lettuce, okra, onion, peach, pepper, potato, radish, soybeans, spinach, potato, tobacco, tomato, and watermelon (Abdal 2008). ***M. arenaria*** and ***M. javanica*** generally live in greenhouses and are very rarely seen in the field because these nematodes cannot tolerate cold temperature environments. *M. arenaria* and *M. javanica* generally prefer the same hosts such as beans, beet, cabbage, carnation, carrot, corn, cucurbits, eggplant, grape, impatiens, peach, potato, radish, snapdragon, soybean, tomato, and zinnia. Many peach rootstock and tomato cultivars that are resistant to *M. incognita* are also resistant to *M. arenaria* and *M. javanica* (Zijlstra 2000). ***M. megatyla*** and ***M. naasi*** are not very common but affect small grains and grasses. *M. naasi* is generally found in isolated locations in northern Eurepean countries, the USA and former Soviet Union. When *M. naasi* infects a plant, it causes stunting, chlorosis and slow decline (Eisenback 1979).

This nematode species forms small root galls like *M. hapla* and infection causes severe yield decreases in grains. *M. naasi* can endure cold winters and low temperatures like *M. hapla*. Unlike other root-knot nematode species, *M. naasi* infects the grass family such as barley, bent grasses, bluegrass, rice, and wheat as well as soybean and sugar beet (Babadoost 2002).

1.4. Root-Knot Parasitism and Feeding Site Development

Root-knot nematodes (RKN), Meloidogyne spp., are obligate, sedentary endoparasites. To complete their life cycle, they must infect plants by invading their roots and transform the host's root cells into highly metabolic feeding sides. Only second stage juveniles (J2s) can infect and penetrate plant roots, and once the nematode invades the root, it migrates through the root until it finds a cell favorable for producing a feeding site. RKNs move intercellularly and find the zone of cell division where vascular parenchymal cells are present. RKNs have proteins and other metabolites which are called secretions and these metabolites are used for host cell differentiation. Nematodes inject secretions into plant root cells with their stylets which are specialized organs that extend from their heads (Caillaud 2008). When the secretions are injected, the cells turn into highly metabolically active, expanded 'giant' cells (Figure 1.2.). The infected cell passes through numerous rounds of mitosis, and cytokinesis is initiated but not completed. This mechanism results in an extensively reduplicated, large nucleus and localized swelling in roots which is called a root-knot or gall (Niebel 1993). By feeding from giant cells, nematodes reach maturity and release several hundred eggs into a gelatin-like matrix. *Meloidogyne* species reproduce in three different ways. *M. incognita* and *M. javanica* use obligate meiotic or mitotic parthenogenesis. *M. megatyla* and restricted host range nematodes have classical sexual reproduction. Other nematodes have facultative parthenogenesis: when sperm exists amphimixis occurs and when there is no sperm available, meiotic parthenogenesis takes place (Figure 1.2.).

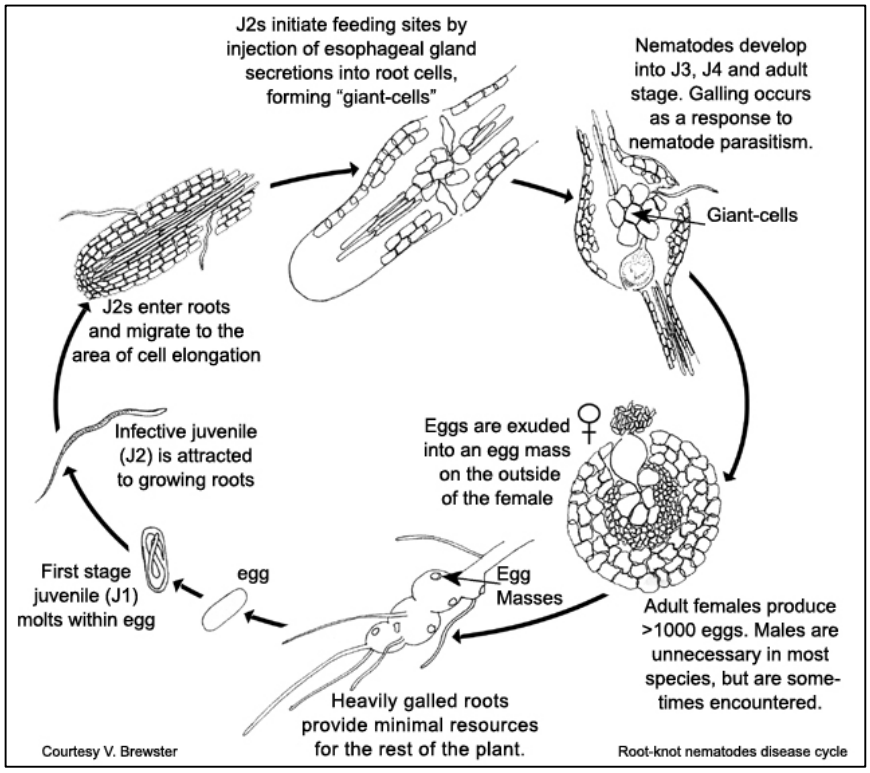

Figure 1.2. Life cycle of Root-knot nematode (Source: Mitkowski 2003).

1.5. Root-Knot Nematode Control Methods

Root-knot nematodes can infect plant roots and prevent water and nutrient transport through vascular tissues and decrease plant development and growth. Thus, they are very harmful for many plants and nematodes have a negative economic impact with 100 billion dollars lost annually in agricultural crops (Sasser 1987). Many methods have been developed to control RKNs. Some of these methods are difficult to maintain and some are very expensive. Therefore, it is important to select a suitable control method to eliminate nematodes from a particular area.

Crop rotation is an excellent control method for RKNs in which resistant and nonhost crops are rotationally planted for two or three years. For each Meloidogyne species, resistant rotation crops have been suggested and it is crucial to use crops that are free of weeds so that rotation is not neutralized (Babadoost 2002). In order to suppress development of RKNs, large amounts of organic matter that contain slightly decayed plant material can be incorporated into the soil. Organic matter induces increases in bacteria, fungi, and other soil microorganism populations which are antagonistic to nematodes however, this method may not be reliable. RKN reproduction

is reduced at low temperature thus, for crops which grow at low temperature, planting as early as possible can prevent infection and early season damage. In the greenhouse, freezing the soil can kill nematode eggs and larvae and solarization (high temperature exposure) can greatly reduce the number of RKNs in the soil. Using chemicals, nematicides, against RKNs is also very effective and a practical control method where economically important crops are planted and crop rotation with resistant varieties is not available. For instance methyl bromide is a very effective chemical for limiting the number of nematodes but, this chemical has harmful effects to other organisms and is being phased-out of use in 160 countries because it depletes the ozone layer (Madhava 2000). Thus, the best method to get rid of RKNs is using resistant plant varieties. However, a limitation for this control method is that one resistant variety is often effective against only one or two Meloidogyne species: thus, if there is more than one nematode species in the field, the resistant plants will not grow successfully (Babadoost 2002).

1.6. Plant Resistance Genes

The best method to protect plants from nematode infection is using the plant's resistance mechanism to control parasitism and this method is relatively cheap and chemical free (Williamson and Kumar 2006). The resistance mechanism prevents nematode development and growth on the root by expressing particular plant genes. This mechanism cannot prevent parasite invasion, it is effective only after the parasite invades the plant and then it protects plants by preventing formation of nematode feeding sites (Trudgill 1991). Many resistance mechanisms are complex traits and regulated by polygenes. All of the genes involved in such mechanisms have not yet been identified. On the other hand, some resistance mechanisms are regulated by single dominant genes (*R* genes) which are expressed in the host's genome after nematode infection. The R gene interacts with a parasite avirulence (*Avr*) gene to initiate the defense response pathway in the host. This gene for gene interaction is crucial in the resistance mechanism because a nematode invasion recognition complex is formed by the plant as a result of these interactions. Pathogen avirulence effector recognition by the R gene product can occur by direct or indirect interactions. When indirect interaction occurs, this is called the guard hypothesis (van der Biezen and Jones 1998).

After recognition, the defense response pathway is initiated and generally results in a hypersensitive response (HR). The hypersensitive response (HR) is used to prevent the spread of infection by pathogens because HR is characterized by rapid cell death in the local region surrounding an infection (Cabrera Poch 2006). If the plant is susceptible to nematode infection, it means that the plant does not have a R gene for interaction with the Avr product of the nematode and therefore cannot initiate the resistance pathway. This resistance mechanism is very useful in agriculture in many ways but, sometimes this method has limitations. For example, there is a barley cultivar with resistance genes for *M. naasi* but these genes do not provide resistance against another nematode species. Thus, if the soil contains different types of nematode species, the natural resistance mechanism cannot be used for agriculture in this area (Cook 2004).

1.7. Nematode Resistance Genes in Pepper (*Capsicum sp.)*

In pepper (*Capsicum sp.*), several dominant genes related to nematode resistance have been identified and it is thought that they have different gene for gene interaction properties (Hare 1956, Hendy 1985, Djian-Caporalino 1999). Six different heat stable RKN resistance genes were found and were named the "Me genes" in different pepper cultivars (PM687, PM217and PM702). It was shown that Me1, Me3 and Me7 can be effective against a wide range of nematode species and that pepper lines containing these genes show different responses against different nematode species (Pegard 2005). Molecular markers have been developed in order to use these genes in marker-assisted selection (MAS) (Djian-Caporalino 2001). By using a bulked segregant analysis-amplified fragment length polymorphism mapping method, these six nematode resistance genes were found to be clustered in a 28 cM area on chromosome P9 of an intraspecific pepper map (Djian-Caporalino 2007). Moreover, a different RKN resistance gene was identified in "Mississippi Nemaheart" (Hare 1957) and transferred to "Yolo Wonder B" and "Keystone Resistant Giant" (Fery 1998) to developed two resistant pepper lines "Carolina Wonder" and "Charleston Bell." By using an allelism test, the N and Me3 genes were identified as distinct genes for resistance (Thies 2000) and the N gene was mapped on chromosome P9, 7 cM from Me1 and 2 cM from Me3 (Djian-Caporalino 2012). Other research showed that there are at least nine RKN resistance genes (N, Me1, Me2, Me3, Me4, Me5, Me7, Mech1 and Mech2) and nearly

all of them are clustered on chromosome P9 of the intraspecific pepper map (Wang 2009) (Table 1.1).

Table 1.1. Pepper (*C. annuum*) accessions which have RKN resistance genes, pepper species, nematode species and their references.

Accession	**Root-Knot Nematode**	**Gene**	**References**
Charleston Cayenne	*M. incognita, race 1,2,3 and 4*	N	Zamora et al. 1994 Thies et al. 1997
Charleston Cayenne	*M. arenaria race 1 and 2*	N	Noe 1992
Charleston Belle	*M. arenaria race 1 and 2, M. javanica*	N	Thies et al. 2000
Carolina Wonder	*M. arenaria race 1 and 2, M. javanica*	N	Thies et al. 2000
PA-353	*M. incognita, race 3*	N	Fery and Thies 1997
PA-398	*M. incognita, race 3*	N	Fery and Thies 1997
PA-426	*M. incognita, race 3*	N	Fery and Thies 1997
PI 322719	*M. javanica, M. incognita, M. arenaria*	Me3, Me4	Djian-Caporalino 2007
PI 201234	*M. javanica, M. incognita, M. arenaria, M chitwoodi*	Me1, Mech2	Djian-Caporalino 2007
CM344	*M. javanica, M. incognita, M. arenaria, M chitwoodi*	Me7, Mech1	Djian-Caporalino 2007
Yolo Wonder	*M. arenaria*	Me5	Djian-Caporalino 2007

1.8. Linkage Mapping

Linkage analysis is used to find the arrangement of genes on specific chromosomes of species. Examining the coinheritance frequency of two traits (or a marker and a trait) can indicate whether the traits are on the same chromosome or not. This information can then be used to calculate the genetic distance separating the linked loci. Pairwise distance and the orders of three or more genes are determined and used to

construct a genetic-linkage map. By looking at the probability of crossing over in a particular region, the distance between the gene pairs can be estimated. Once the recombination fractions for many pairs of genes are determined, linkage maps of the chromosome can be constructed. First, using recombination fractions, gene pairs are separated into linkage groups which are sets of gene pairs linked to at least one member of the set on the same chromosome. Recombination fraction can be used to order the loci of the gene pairs, because recombination increases proportionally to the distance between the two linked genes. Then each recombination fraction is converted to the genetic distance and the loci are drawn on a line where the distance between any two loci is proportional to the genetic distance between these two loci (Walker 1992). A linkage map of pepper chromosome 9 is shown in Figure 1.3.

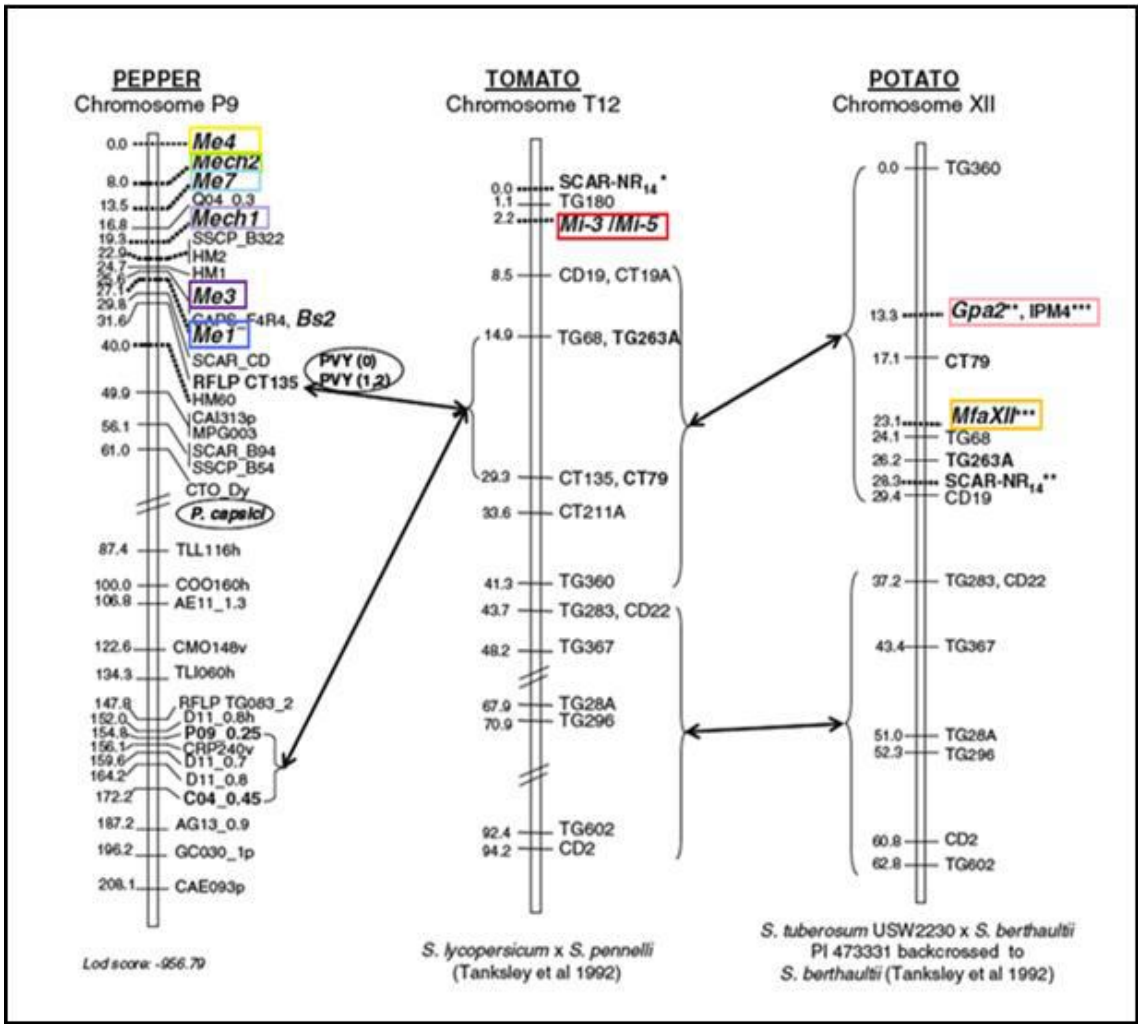

Figure 1.3. Comparative linkage mapping of nematode-R loci of pepper, tomato and potato (Source: Djian-Caporalino 2007).

1.9. Marker-assisted selection (MAS)

Over the past century, plant breeding has developed and made remarkable progress in increasing crop yields. With changes in agricultural practices, the need for developing genotypes which have specific agronomic characteristics arose. Furthermore organisms in the environment constantly change such that microorganisms and pests continually evolve and escape from host resistance. Consumer demands, preferences and requirements are also changing. Thus plant breeders have to overcome endless hurdles to develop new crop varieties (Evans 1997). The human population is increasing very quickly and this causes a need for increased crop production but, scientists report that the rate of crop yield increase is recently declining (Pingali 1999). Plant breeders must focus on certain factors such as current yield trends, pressure on the environment, traits relating to yield stability and sustainability and these factors include traits such as durable disease resistance, abiotic stress tolerance and nutrient and water-use efficiency (Mackill 1999). It is possible to improve plant yield to some degree using conventional breeding but, it is certain that new technologies such as biotechnology are crucial to continually maximize yield improvement. Molecular genetics and genomics offer great opportunities for plant breeding with the help of DNA marker technology. By using genetic linkage analysis and DNA marker technologies, allelic variation in the genes related to certain traits can be detected and these technologies can greatly increase efficiency and precision in plant breeding. Molecular breeding is a recently developed method which involves the use of DNA markers in plant breeding, otherwise known as marker-assisted selection (MAS) (Collard 2008).

There are many different types of markers used for marker-assisted selection such as SSR, COSII, SNP and SCAR markers. SSR (Simple Sequence Repeat) are tandem repeats shorter than 6 bp and, these repeats are mainly composed of dinucleotides $(AC)_n$, $(AG)_n$, $(AT)_n$; trinucleotides $(TCT)_n$ and tetranucleotides $(TATG)_n$ (Jones et al. 2009). COSII (Conserved Ortholog Set II) markers are PCR-based markers developed from single-copy conserved orthologous genes in Asterid species. Each COSII gene matches only one single-copy Arabidopsis gene (Fulton et al. 2002). SNP (Single nucleotide polymorphism) markers are DNA sequence variations occurring when a single nucleotide in the genome differs between members of a biological species. SNPs usually occur in non-coding regions more frequently than in coding regions (Barreiro et al. 2008). SCAR (Sequence Characterized Amplification Region) markers are derived from RAPD markers

and have the advantages of RAPD markers and have the additional benefits of increased specificity and reproducibility (Paran 1993).

1.10. Aim of the Study

Pepper (C. *annuum*) is one of the most valuable agricultural crops worldwide and Turkey ranks third among all countries in pepper production. Pepper species have economical and also pharmaceutical importance so, it is essential to develop methods to increase pepper production in the field and greenhouse. There are many biotic and abiotic factors that severely affect pepper growth and, one of the most important biotic factors that affect pepper growth and development is root-knot nematode (*Meloidogyne* species). Some pepper species developed resistance gene mechanisms to protect themselves from nematodes. One of the resistance gene mechanisms includes the dominantly inherited N gene which was mapped on chromosome P9, 7 cM from Me1 and 2 cM from Me3 (Djian-Caporalino 2012). The N gene has broad spectrum resistance against many nematode species thus, it is crucial to develop markers which are tightly linked to the N gene for use in marker-assisted selection. The aim of this work was to develop a marker tightly linked to the N gene which can be used in molecular breeding. To achieve this, F1 plants and an F2 population were developed by crossing "Carolina Wonder" (1013N), a resistant inbred line which carries the N gene, and "AZN-1," a susceptible inbred Turkish pepper line. Phenotypes of the progeny were monitored by nematode test and genotypes were analyzed with SSR, COSII, SNP and SCAR markers. By saturating the area on chromosome P9 where the N gene is localized, we developed N gene-linked markers for use in breeding of pepper.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

In this study “Carolina Wonder” (1013N) was used as a resistant line and the Turkish cultivar (“AZN-1”) was used as a susceptible line. “Carolina Wonder” was developed at the U.S. Vegetable Laboratory, Charleston, SC. It was created by backcross breeding to transfer the dominant N gene for root-knot nematode resistance from “Mississippi Nemaheart” into “Yolo Wonder B” (Fery 1997). “Carolina Wonder” (1013N) was crossed with Turkish susceptible cultivar “AZN-1” to generate 20 F1 hybrids. Highly resistant F1 individuals were selected and self-pollinated to generate a segregating F2 population of 250 individuals which was used for nematode tests and genotyping. Plants were grown in growth chambers at Multi Tarım, Antalya at 24°C during 18 hour day light, 22 °C at night with nearly 65% humidity.

2.2. Methods

2.2.1 Nematode Testing

The susceptible tomato variety “Tueza F1” was inoculated with second stage juveniles (J2) of *M. incognita* (race 2) in the growth chamber at Assist. Prof. Dr. Mehmet Ali Söğüt’s laboratory at Süleyman Demirel University, Isparta, for multiplication of *M. incognita* eggs. Eight weeks after inoculation, *M. incognita* eggs were collected from tomato roots. At the four-leaf stage, 32 individuals of each parent and 20 F1 generation individuals were inoculated with approximately 4000 *M. incognita* eggs and second stage juvenile (J2) embryos.Similarly, 256 F2 individuals of the *C. annuum* population were inoculated with approximately 4000 eggs and second

stage juveniles of *M. incognita* (race 2) in 250 ml pots. The plants were grown in a growth chamber at 24°C during the day (18 hour day length) and 22°C at night with 65% humidity. Eight weeks after treatment, egg masses (EM) and rate of gall formation were calculated. Root systems were rated according to number of egg masses and gall formation. According to egg masses, plant roots which had 20 or fewer egg masses were considered resistant and those with more than 20 egg masses were considered susceptible. According to root galling index scale, roots with two or fewer galling index scale were considered resistant and roots with three or more galling index scale were considered susceptible (Table 3.3).

Table 3.3. Root galling index scale (Source: Harman and Sasser 1987).

Root gallindex	**Root status**
0	No galls
1	1 to 10 galls
2	11 to 25 galls
3	26 to 50 galls
4	51 to 75 galls
5	>75 galls

2.2.2. DNA Extraction

Genomic DNA of *C. annuum* individuals was isolated for genotypic analysis using molecular markers. Promega Wizard® Genomic DNA Purification Kit was used to purify DNA from fresh leaf tissue according to the manufacturer's protocol. After isolation, the quantities of each sample DNA were analyzed using Nanodrop ND-1000 spectrophotometer. All DNA samples were dissolved in distilled H2O and stored at -20 °C.

2.2.3. Molecular Marker Analysis

2.2.3.1. SSR Analysis

Parental pepper DNAs were surveyed with 132 SSR (Hpms) primers (Yi 2006) (Table 2.1). Polymerase chain reaction (PCR) was carried out in 25 µL volume containing 2.5 µl 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, pH: 8.3), 1 µl MgCl2, 0.5 µl dNTP (0.2 mM), 1.5 µl forward and 1.5 µl reverse primers (10 pmol), 0.25 µl Taq polymerase (0.25 U), 15.75 µl sterile distilled water, and 2 µl DNA (~50 ng/µl).

DNA samples were amplified according to the PCR protocol: one step of 5 min at 94°C, 35 cycles with 30 sec at 94°C, 45 seconds at 55 °C annealing temperature, 1 min at 72°C and a final extension step of 5 min at 72°C in BIO-RAD Thermal Cycler™. Amplified DNA samples were separated by 2 or 3% agarose gels in 1X TAE buffer (0.25 M Tris base, 12.75 M EDTA adjusted to 1 L with distilled water and pH 8.3 with acetic acid). Samples were run at 100 V for 2 hours and visualized under UV light after ethidium bromide staining. QIAxcel® Novel 12-channel capillary electrophoresis and Fragment Analyzer™ Automated CE System were used for high resolution separation of amplified DNA samples.

Table 2.1 Simple sequence repeat (SSR) primers used in parental survey.

SSR PRIMERS					
HpmsE001	HpmsE027	HpmsE053	HpmsE089	HpmsE122	HpmsE148
HpmsE002	HpmsE028	HpmsE054	HpmsE091	HpmsE123	HpmsE149
HpmsE003	HpmsE029	HpmsE055	HpmsE093	HpmsE124	
HpmsE004	HpmsE030	HpmsE056	HpmsE094	HpmsE125	
HpmsE005	HpmsE031	HpmsE057	HpmsE095	HpmsE126	
HpmsE006	HpmsE032	HpmsE058	HpmsE096	HpmsE127	
HpmsE007	HpmsE033	HpmsE064	HpmsE097	HpmsE128	
HpmsE008	HpmsE034	HpmsE065	HpmsE098	HpmsE129	
HpmsE009	HpmsE035	HpmsE066	HpmsE099	HpmsE130	
HpmsE010	HpmsE036	HpmsE067	HpmsE100	HpmsE131	
HpmsE011	HpmsE037	HpmsE068	HpmsE101	HpmsE132	
HpmsE012	HpmsE038	HpmsE069	HpmsE102	HpmsE133	
HpmsE013	HpmsE039	HpmsE070	HpmsE103	HpmsE134	
HpmsE014	HpmsE040	HpmsE071	HpmsE104	HpmsE135	
HpmsE015	HpmsE041	HpmsE072	HpmsE107	HpmsE136	
HpmsE016	HpmsE042	HpmsE073	HpmsE108	HpmsE137	
HpmsE017	HpmsE043	HpmsE074	HpmsE110	HpmsE138	
HpmsE018	HpmsE044	HpmsE078	HpmsE111	HpmsE139	
HpmsE019	HpmsE045	HpmsE080	HpmsE112	HpmsE140	
HpmsE020	HpmsE046	HpmsE081	HpmsE113	HpmsE141	
HpmsE021	HpmsE047	HpmsE082	HpmsE115	HpmsE142	
HpmsE022	HpmsE048	HpmsE083	HpmsE116	HpmsE143	
HpmsE023	HpmsE049	HpmsE084	HpmsE118	HpmsE144	
HpmsE024	HpmsE050	HpmsE086	HpmsE119	HpmsE145	
HpmsE025	HpmsE051	HpmsE087	HpmsE120	HpmsE146	
HpmsE026	HpmsE052	HpmsE088	HpmsE121	HpmsE147	

2.2.3.2. EST-SSR Analysis

In this project, non-redundant expressed sequence tags (ESTs) which were developed by Yi et al. (2006) were used as a source of SSR markers. Gibum Yi designed the primers using parameters of product size 100–350 bp, primer length 20–24 bp, and melting temperature 60–68 °C (Yi et al. 2006).

A total of 230 EST-derived SSR markers (Table 2.2) were used to survey parental DNAs. To amplify fragments, the Hpms primer PCR conditions were used. Electrophoresis with 3% agarose gels was used to separate large fragment differences

while a fragment analyser was used to separate fragments with small polymorphism differences (3-4 bp).

Table 2.2 Expressed sequence tags (ESTs) based SSR primers.

PRIMERS								
4CL	CA516044	CP10061	EPMS369	EPMS416	EPMS497	GP20095	GPMS161	GPMS1
AA840689	CA516334	CP10081	EPMS372	EPMS417	EPMS501	GP20117	GPMS162	GPMS200
AA840692	CA516439	CP10131	EPMS373	EPMS418	EPMS507	GPMS100	GPMS163	GPMS201
AA840739	CA517699	CT232	EPMS374	EPMS419	EPMS514	GPMS101	GPMS164	GPMS202
Actin SR	CA519548	CT253	EPMS376	EPMS420	EPMS538	GPMS103	GPMS165	GPMS203
A-39662	CA523558	CT59	EPMS377	EPMS421	EPMS539	GPMS104	GPMS166	GPMS205
A-39662	CA523715	CT94	EPMS378	EPMS424	EPMS540	GPMS107	GPMS169	GPMS29
asu11	CA523880	E492334	EPMS382	EPMS426	EPMS542	GPMS109	GPMS171	GPMS37
asu2	CA524065	EPMS303	EPMS386	EPMS427	EPMS543	GPMS111	GPMS176	GPMS3
asu5	CA525274	EPMS305	EPMS387	EPMS428	EPMS546	GPMS112	GPMS178	GPMS4
asu7	CA525390	EPMS309	EPMS390	EPMS429	EPMS547	GPMS113	GPMS181	GPMS6
asu9	CA526211	EPMS310	EPMS391	EPMS430	EPMS549	GPMS117	GPMS183	GPMS8
BD76366	CA847460	EPMS316	EPMS395	EPMS438	EPMS554	GPMS119	GPMS185	GPMS93
BM59622	CA847580	EPMS327	EPMS396	EPMS439	GP1017	GPMS140	GPMS186	GSP
BM61028	CACCEL1i	EPMS330	EPMS397	EPMS440	GP1049	GPMS141	GPMS187	Hba181H07SP6
BM61461	CAN130829	EPMS331	EPMS399	EPMS441	GP1078	GPMS142	GPMS188	hp2
BM61910	CaSn-R	EPMS335	EPMS402	EPMS443	GP1102	GPMS147	GPMS189	Hpms1-117
BM62655	CaSn-SR	EPMS340	EPMS404	EPMS446	GP1127	GPMS150	GPMS191	Hpms1-143
BM64867	CB164833	EPMS342	EPMS409	EPMS448	GP20031	GPMS151	GPMS193	Hpms1-165
BM67271	CB164897	EPMS343	EPMS410	EPMS449	GP20036	GPMS153	GPMS194	Hpms2-41
CA514272	cLPT5E7	EPMS345	EPMS411	EPMS451	GP20056	GPMS154	GPMS195	Idh-1
CA514621	CM10	EPMS349	EPMS412	EPMS472	GP20062	GPMS155	GPMS196	MboI77E18SP6
CA515055	CP10020	EPMS350	EPMS413	EPMS480	GP20064	GPMS156	GPMS197	ovate
CA515275	CP10023	EPMS353	EPMS414	EPMS490	GP20068	GPMS157	GPMS198	P1-P2
CA515649	CP10060	EPMS366	EPMS415	EPMS492	GP20087	GPMS159	GPMS199	Pgm-2
SCAR	Skdh-1	T0408	T0463	TG132	TG517	U217183	U221402	U223436

2.2.3.3. Pepper Chromosome 9 COSII, SSR, SCAR and SNP marker Analysis

Five nematode resistance genes have been assigned to pepper chromosome P9 by integrated mapping (Wang 2009). Thus, it is supposed that a cluster of genes resistant to Meloidogyne spp. is present on pepper chromosome P9 and it was also shown that the N gene is located on pepper chromosome 9 (Djian-Caporalino 2012). Therefore, different types of markers on chromosome P9 were analyzed. A total of 45 COSII, SSR, SCAR and SNP markers on chromosome P9 (Table 2.3) were applied to parent DNA for polymorphism analysis. Agarose gel electrophoresis and fragment analyser methods were used to separate amplified PCR products.

Table 2.3 P9 chromosome COSII, SSR, SCAR and SNP markers

PRIMERS		
Scar	SSCP B54	HpmsE117
P1-P2	C2at5g06130	CL011634-0160
ActinFSFR	C2at2g29210	CL000081-0555
CaSnFS-FR	Scar HM6	CL001943-1222
Hpms1-3	Scar PM6b	CL011732-0346
ScarCD	Scar HM60	CL010825-0217
CAPS F4R4	Scar Pm6a	CL011597-0298
C2at2g37240	SSCP PM5	CL010426-0078
SSCP-B322	GPMS160	CL010328-0429
C2at3g09920	HpmsE094	CL012112-0218
C2at3g09925	HpmsE0102	CL010608-0194
Hpms 2-41	HpmsE082	CL012920-0252
Scar B94	At5g58410	CL003067-0303
GPMS171	HpmsE025	CL012747-0303
Hpms1-117	HpmsE007	CL005231-0326

2.2.4. Data Analysis

Chi-square goodness of fit test was used to analyze the segregation data. To construct a linkage map of molecular markers, JoinMap software (Kyazma B.V. 1996) with a minimum LOD score of 3.0 was used. JoinMap software uses the Kosambi mapping function to estimate distances between markers in centiMorgans (cM). Recombination frequencies and their standard errors were calculated using maximum likelihood method.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Nematode Testing and Phenotype Analysis

First 32 individuals of each parent ("Carolina Wonder" (1013N), Turkish cultivar ("AZN-1") and the F1 generation generated from these two lines were tested with *M. incognita* (race 2) nematodes (Table 3.1). Results showed that the Turkish cultivar ("AZN-1") is completely susceptible to the root knot nematode, however both "Carolina Wonder" (1013N) and "Carolina Wonder" (1013) X "AZN-1" F1 hybrids are resistant to the root knot nematode. Analysis showed that the results are consistent with each other and standard errors are low. Susceptible plants have many galls and egg masses on their roots and resistant plants have no galls and egg masses on their roots (Table 3.2.).

Table 3.1. Nematode testing of parents and F1 generation.

Materials	Number of Plants	Description
AZN-1	32 Plants	susceptible pepper line (Turkish pepper)
Carolina Wonder (1013N)	32 Plants	root knot nematode resistant plants (N gene)
Carolina Wonder (1013) X AZN-1	32 Plants	F1 generation plants

The F2 population generated from the F1 hybrids was tested with *M. incognita* (race 2). The scale of root galling is shown in Table 3.3. and phenotype analysis was performed according to this scale. A scale value of 0 to 1 is considered as resistant while values of 2 to 5 are considered as susceptible. The average gall number in the F2 population was nearly 50 and the range was from 0 to 780 galls (Table 3.4.). According

to the gall formation test results, 75.4% of the F2 population was resistant to *M. incognita* (race 2) (root knot index between 0 to 1) and 24.6% of the F2 population was susceptible to *M. incognita* (race 2) (root knot index greater than 2) (Figure 3.1.). The results showed that the F2 generation segregated for the nematode resistance N gene. The resistance allele is inherited dominantly and should segregate according to the 3:1 Mendelian segregation ratio. This was confirmed in our results with a Chi-square goodness of fit test ($p = 0.88$).

Table 3.2. Nematode test results according to Gall index and Gall number for egg and larvae inoculations with standard errors and consistency (a,c and d).

Pepper	**Egg inoculation**		**Larvae inoculation (j2)**	
	Gall index	**Gall number**	**Gall index**	**Gall number**
AZN1	5.0±0.4 d	258±25.6 c	5.0±0.4 c	78.6±5.4 c
Caroline Wonder (1013N)	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
AZN 1 X Caroline Wonder (1013N) (F1)	0.4±0.3 a	0.8±0.6 a	0.14±0.2 a	0.3±0.3 a

Table 3.4. Gall number analysis in F2 population.

Gall number	
Mean	50,09
Standard Error	7,47
Standard Deviation	119,50
Range	780
Minimum	0
Maximum	780
Sum	12824
Count	256

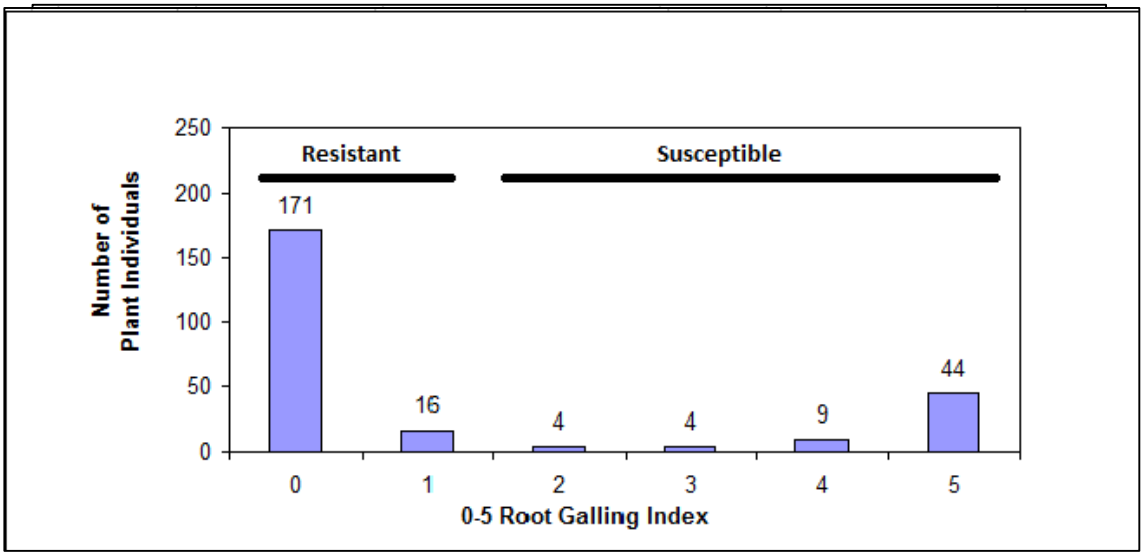

Figure 3.1 Distribution of Root Galling Index in F2 plants tested with *M. incognita* (race2).

3.2. DNA Extraction

Genomic DNA of *C. annuum* individuals was isolated and samples were separated by agarose gels (Figure 3.2). Quantities of each sample DNA were analyzed using Nanodrop ND-1000 spectrophotometer (Table 3.5). All DNA samples were dissolved in dH2O and stored at -20 °C.

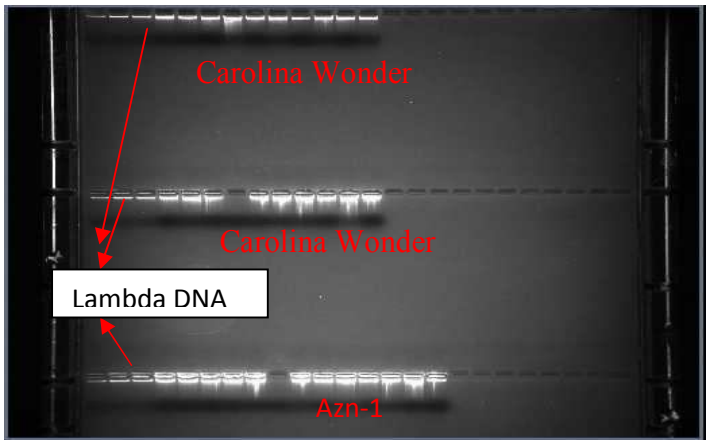

Figure 3.2 Agarose gel electrophoresis of *C. annuum* Parental DNA (First three bands are lambda DNA; 50 ng, 100 ng,150 ng).

Table 3.5 Nanodrop ND-1000 spectrophotometer results of Parental DNAs.

Sample	ng/ul	A260	A280	260/280	260/230	Sample	ng/ul	A260	A280	260/280	260/230
cw-1	270.58	5.412	2.856	1.89	2.11	cb-7	761.74	15.235	8.132	1.87	1.61
cw-2	256.70	5.134	2.772	1.85	2.33	cb-8	813.53	16.271	8.386	1.94	1.76
cw-3	364.54	7.291	4.003	1.82	2.06	cb-9	673.04	13.461	7.216	1.87	1.94
cw-4	819.40	16.388	8.942	1.83	2.16	cb-10	580.99	11.620	6.127	1.90	1.87
cw-5	251.11	5.022	2.670	1.88	1.92	a1-1	355.79	7.116	3.766	1.89	1.87
cw-6	321.85	6.437	3.543	1.82	1.72	a1-2	868.27	17.365	8.963	1.94	2.33
cw-6	104.67	2.093	1.139	1.84	1.60	a1-3	809.74	16.195	8.537	1.90	2.02
cw-8	1320.18	26.404	14.109	1.87	1.87	a1-4	651.79	13.036	7.153	1.82	1.77
cw-9	214.02	4.280	2.278	1.88	2.15	a1-5	529.44	10.589	5.852	1.81	1.82
cw-10	299.89	5.998	3.280	1.83	2.13	a1-6	26.86	0.537	0.240	2.23	1.15
cb-1	469.69	9.394	5.051	1.86	2.01	a1-7	1433.52	28.670	15.450	1.86	1.88
cb-2	487.43	9.749	5.591	1.74	1.14	a1-8	374.12	7.482	3.956	1.89	2.13
cb-3	976.38	19.528	10.516	1.86	1.97	a1-9	615.85	12.317	6.490	1.90	1.86
cb-4	538.94	10.779	5.759	1.87	1.31	a1-10	475.34	9.507	4.950	1.92	1.84
cb-5	596.83	11.937	6.409	1.86	1.62	a1-11	637.55	12.751	6.680	1.91	1.61
cb-6	540.36	10.807	5.871	1.84	2.07	a1-12	1075.80	21.516	11.941	1.80	1.72
						a1-13	933.38	18.668	9.948	1.88	2.02

3.3. SSR Analysis

Parental pepper DNAs were surveyed with 132 SSR (Hpms) primers (Yi 2006). Amplified DNA samples were separated by 3% agarose gels in 1X TAE buffer (Figure 3.3) and QIAxcel® Novel 12-channel capillary electrophoresis (Figure 3.4).

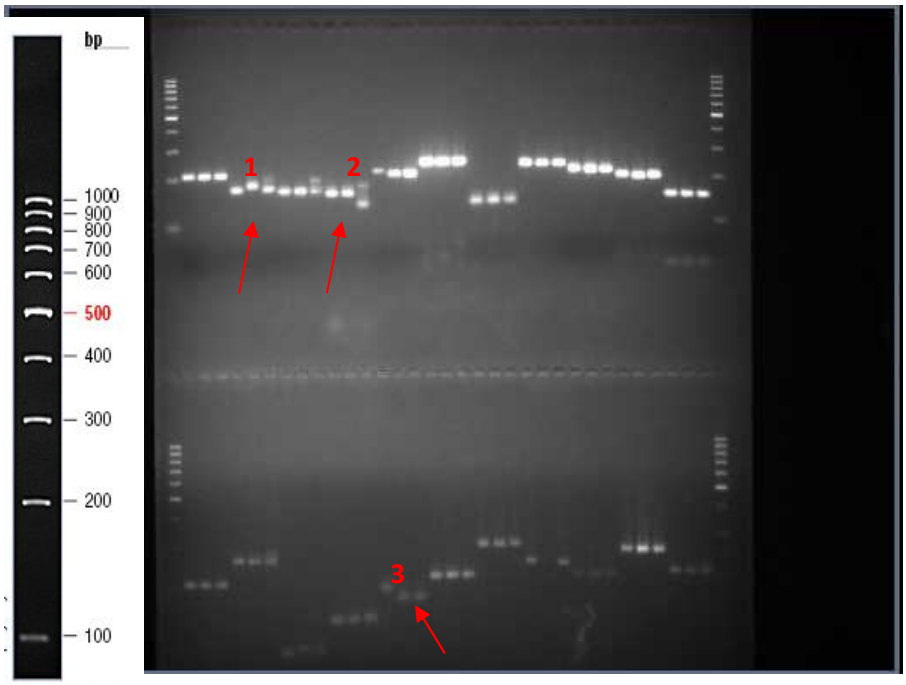

Figure 3.3 Sample agarose gel electrophoresis for some polymorphic bands (**1**: HpmsE002 2: Hpms E004 3: Hpms E016).

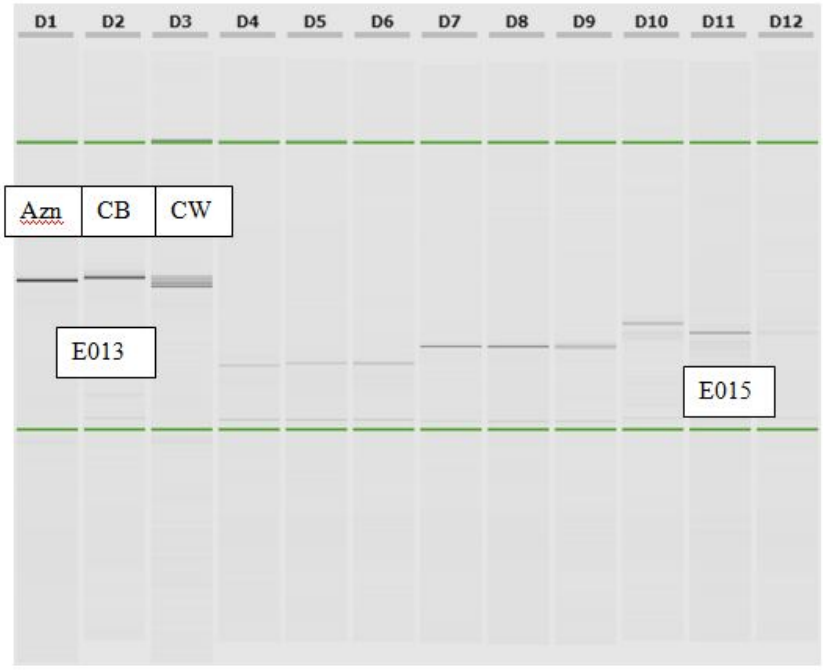

Figure 3.4 QIAxcel® Novel 12-channel capillary electrophoresis analysis, with polymorphic bands labeled (Azn: Turkish cultivar, CW: *Carolina Wonder* and CB: *Charleston Bell*).

With agarose gel electrophoresis analysis, 7.6% (10 polymorphic markers) of all tested marker showed polymorphism (Table 3.6) and in QIAxcel® Novel 12-channel capillary electrophoresis analysis, 25 polymorphic markers were observed which is 19% of 132 SSR markers (Table 3.7). In both systems, the same markers were analyzed, but gave different results, because capillary electrophoresis is more sensitive and has higher resolution than agarose gel electrophoresis. Capillary electrophoresis can differentiate fragments up to 3 bp but agarose electrophoresis separates 7 to 10 bp succesfully. Some of the agarose gel result did not agree with capillary results and this may have resulted from PCR conditions (Table 3.6 and 3.7).

Table 3.6 Polymorphic markers observed in agarose gel electrophoresis.

HPMS
E002
E003
E004
E014
E016
E034
E119
E133
E139
E149

Table 3.7 Polymorphic markers observed in QIAxcel® capillary electrophoresis.

CAPILLARY ELECTROPHORESIS	
E002	E067
E004	E071
E005	E088
E009	E095
E013	E119
E015	E120
E016	E126
E017	E133
E036	E139
E050	E141
E053	E145
E055	E147

3.4. EST-SSR Analysis

A total of 230 EST-derived SSR markers were used to survey parental DNAs. Some of the polymorphic markers are shown in Figure 3.5. After survey analysis in agarose gel electrophoresis, 12 polymorphic markers were identified which is 5.2% of all tested markers as shown in Table 3.8.

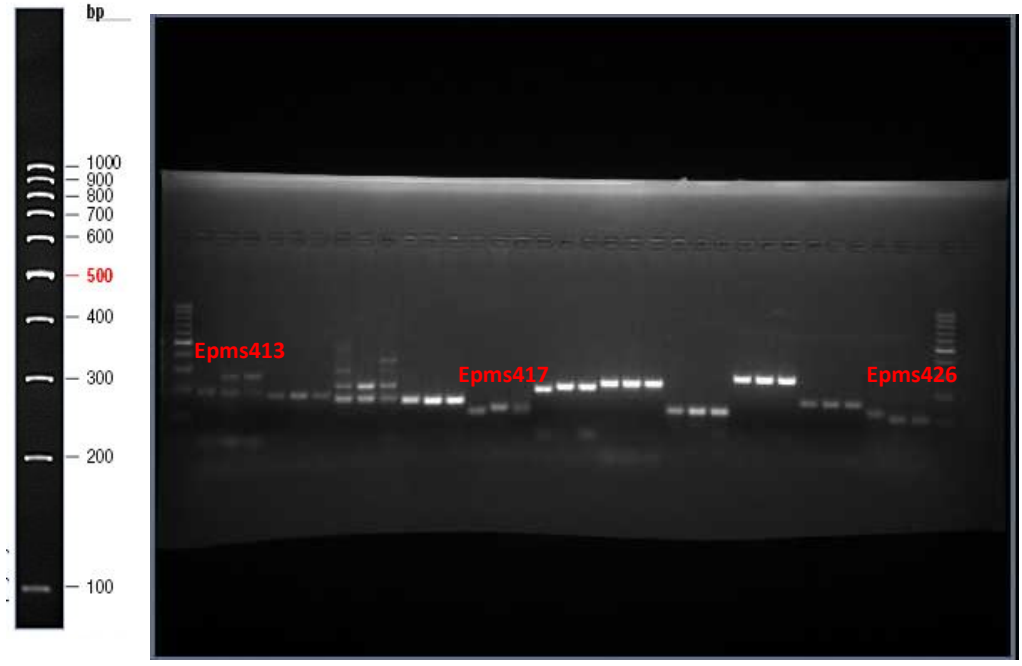

Figure 3.5 Several polymorphic EST-SSR markers visualized on 3% agarose gel (Epms413, Epms417 and Epms426)

Table 3.8 Polymorphic markers found in parental survey of EST-SSR markers.

POLYMORPHIC PRIMERS	
CT232	Epms417
Gpms171	Epms418
Gpms185	Epms426
Gpms191	Epms443
Epms310	Epms472
Epms413	Epms480

3.5. Pepper Chromosome 9 COSII, SSR, SCAR and SNP marker Analysis

A total of 45 COSII, SSR, SCAR and SNP markers on chromosome P9 (Table 2.3) were applied to parent DNA for polymorphism analysis. A Fragment Analyzer™ Automated CE System was used to separate amplified PCR products of these chromosome 9 specific markers (Figure 3.6). Polymorphic markers are listed in Table 3.9.

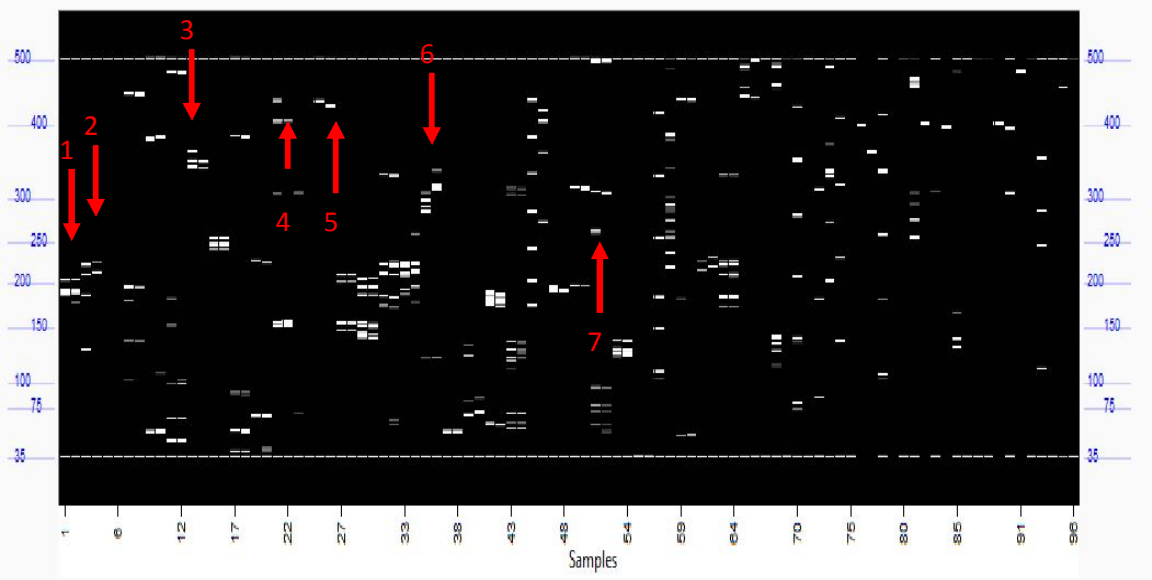

Figure 3.6 Fragment Analyzer™ Automated CE System parental survey analysis of chromosome 9 specific markers. Polymorphic markers are indicated by arrows. (1-Hpms117, 2-Hpms1-3, 3-SSCPB54, 4- At5g06130, 5- CapsF4R4, 6- SSCPB322 and 7- Epms472).

Table 3.9 Polymorphic markers of chromosome 9 specific primers.

POLYMORPHIC MARKERS	
ScarN	Hpms117
ScarPM6a	Hpms1-3
ScarPM6b	SSCPB54
At5g06130	CapsF4R4
SSCPB322	Epms472
SSCPPM5	CaSnFR

In general interspecific populations in the Solanaceae family show high polymorphic genome structures, while intraspecific populations have lower polymorphism (Foolad et al. 2012). Our population is an intraspecific pepper population thus, we expected lowpolymorpism results when markers were tested in parental survey. When the SSR markers were tested, 7.6% of 132 markers shows polymorphism in agarose gel electrophoresis and 19% in capillary electrophoresis. The 230 EST-SSRs surveyed gave 12 polymorphisms which accounted for 5.2% of all EST-SSR markers. The 45 chromosome 9 specific marker surveyed gave 9 polymorphic makers which is 20% of all markers. Overall 11.3% polymorphism was obtained which is according to our expectations for an intraspecific population.

3.6. Polymorphic Marker Segregation in F2 Population

Polymorphic markers were applied to the F2 population to confirm 3:1 segregation and also to correlate the population nematode test phenotypes and marker genotypes. The expected 3:1 marker data segregation in the F2 population was confirmed with a Chi-square goodness of fit test and the p values were 0.48 for ScarN marker, 0.81 for ScarPM6a marker and 0.48 for ScarPM6b marker. By using Kyazma JoinMap software, marker genotype results and individual phenotype test results were associated to each other. The aim of this analysis was to find linkage between markers and the N gene phenotype. When the polymorphic markers were applied to the F2 population, three markers were found to be linked to the N gene. ScarPM6a marker were found to be closest, 3.6 cM from the N gene. ScarPM6b was 10.2 cM and ScarN was found 22.6 cM from the N gene (Figure 3.10). Fragment Analyzer results for

ScarPM6a (Figure 3.7), agarose gel elecrophoresis analysis (Figure 3.8) and Fragment analyzer results (Figure 3.9) for the ScarN marker are shown in Figure 3.6, 3.7 and 3.8. Previous analysis showed that ScarPM6a is the closest marker to N gene at a distance of 3.9 cM (Ariane 2012) while we found the same marker to be 3.6 cM from the N gene. Thus, very similar results were obtained in both studies.

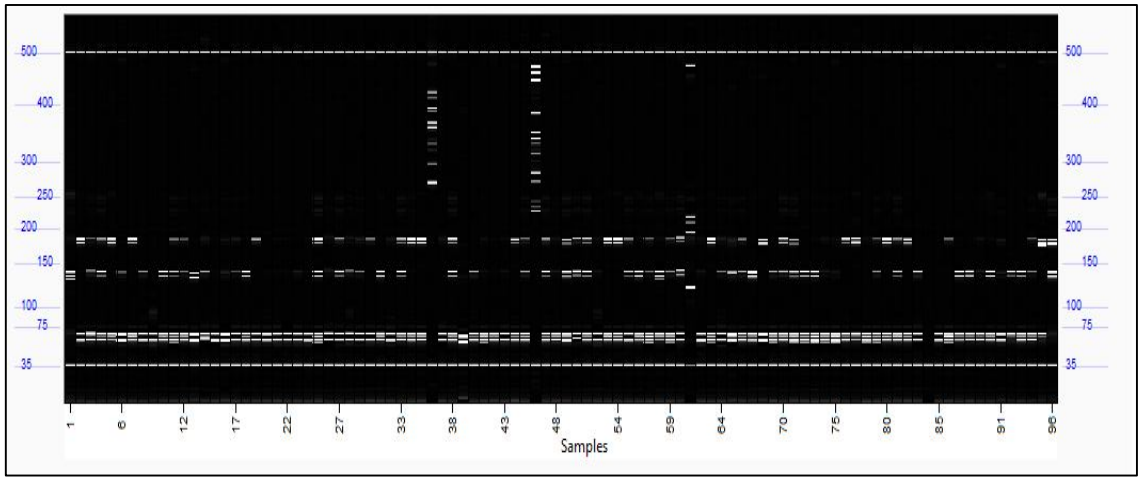

Figure 3.7 Fragment analyzer result of ScarPM6a marker on F2 population.

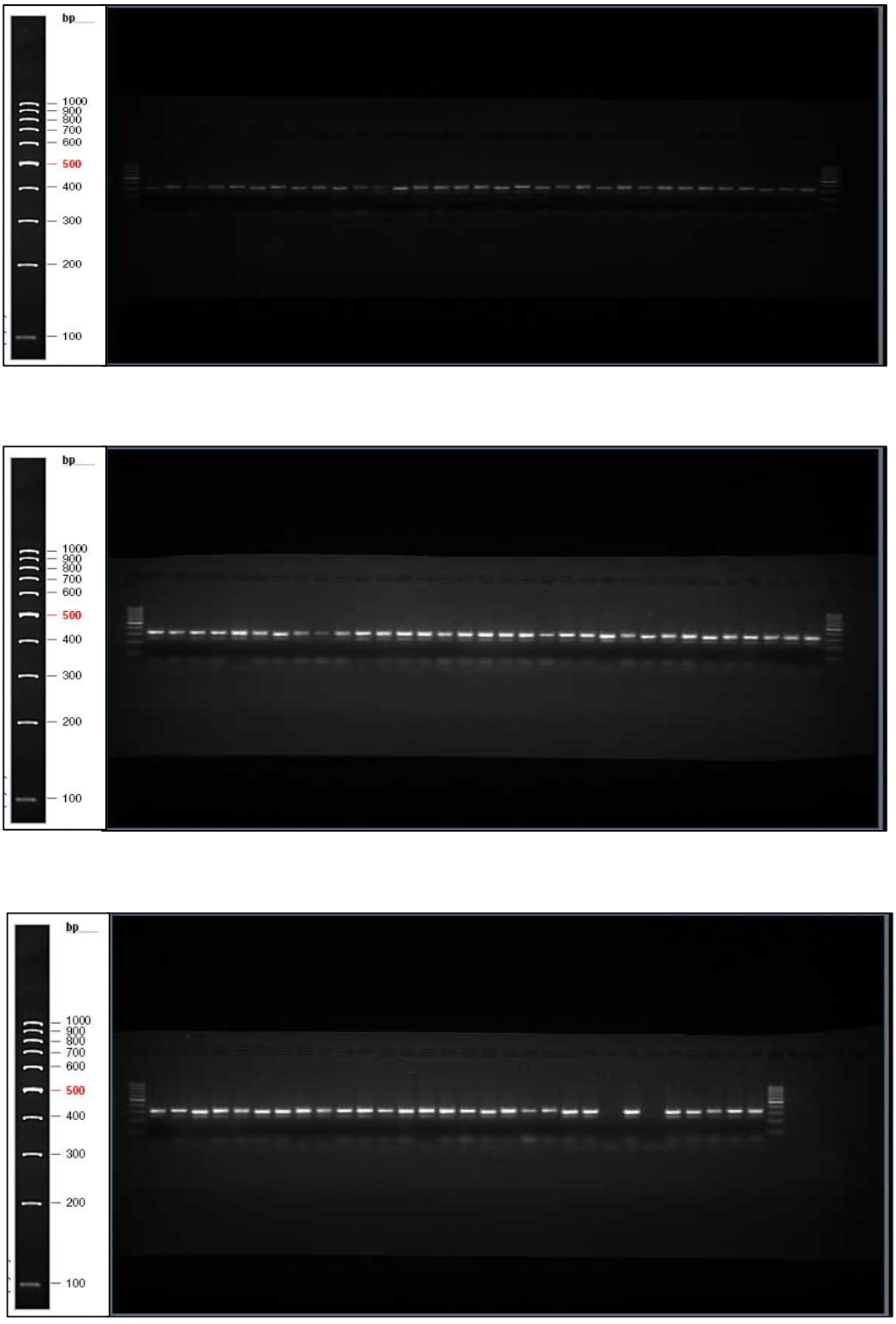

Figure 3.8 Agarose gel electrophoresis results of ScarN marker on F2 Population.

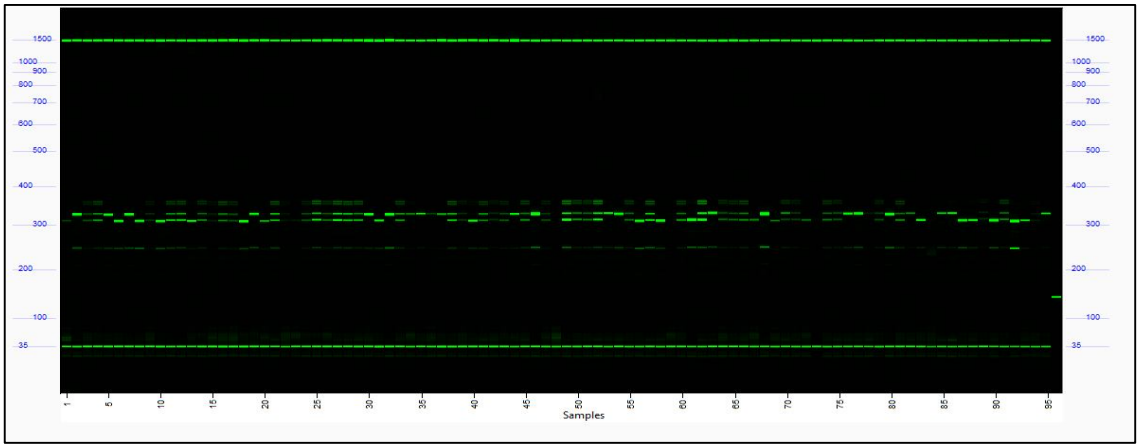

Figure 3.9 Fragment analyzer results of ScarN marker on F2 Population.

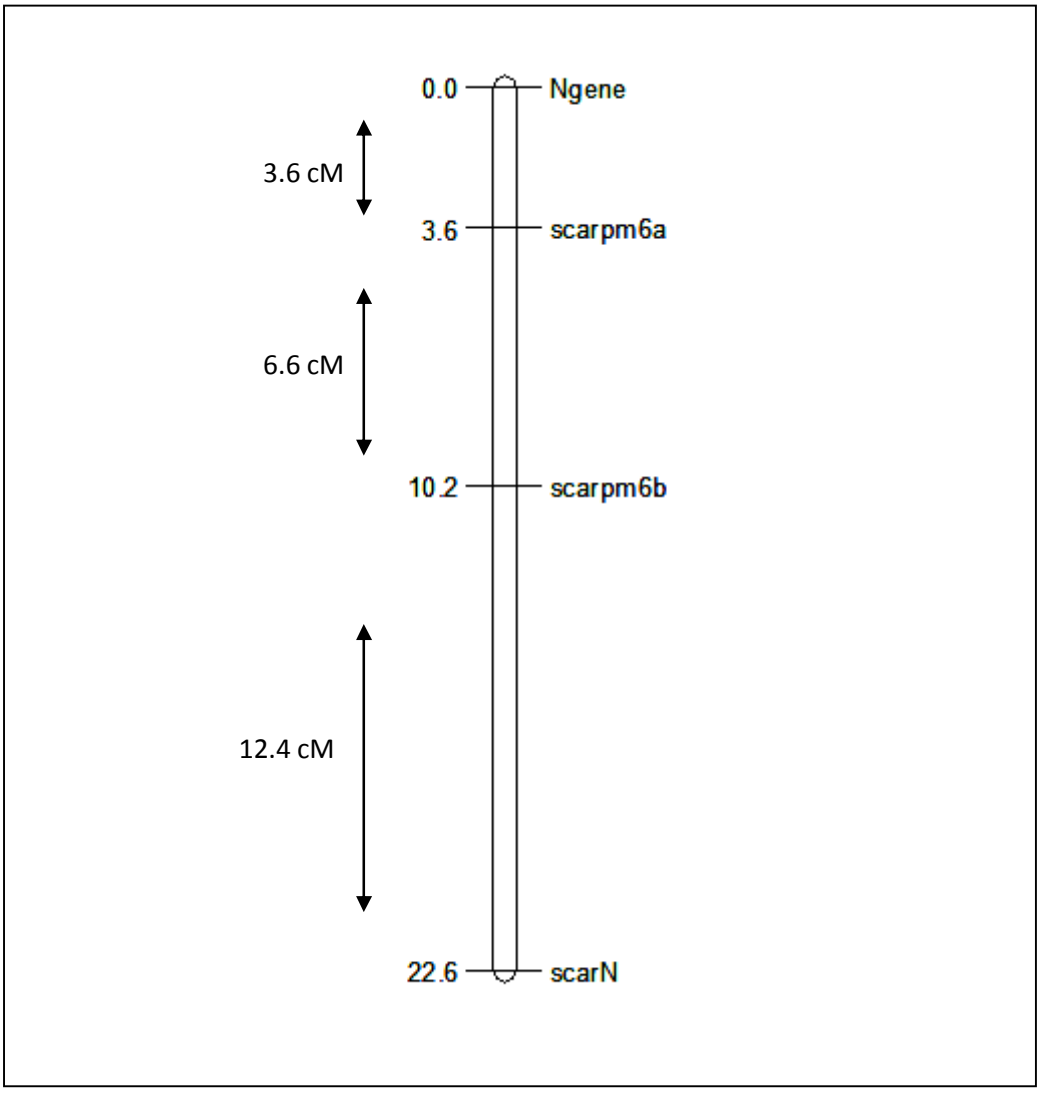

Figure 3.10 Linkage map of the N gene constructed by Kyazma JoinMap software constructed.

CHAPTER 4

CONCLUSION

C. *annuum* (pepper) is one of the most valuable agricultural crops worldwide and Turkey ranks third among all countries in pepper production. Pepper species have economical and also pharmaceutical importance so, it is very crucial to develop methods to increase pepper yields. One of the most important biotic factors that affect pepper growth and development is the root-knot nematode (*Meloidogyne* species) in Turkey. The dominantly inherited N gene which was mapped on chromosome P9, 7 cM from Me1 and 2 cM from Me3, confers resistance to pepper species against *Meloidogyne* species. The aim of this work was to develop a marker tightly linked to the N gene which can be used in marker-assisted selection.

To a construct linkage map and identify an N linked marker we used 132 SSR Hpms primers, 230 EST-SSR markers and 45 chromosome 9 specific primers. When we looked at all 407 analyzed markers, only 11.3% polymorphism was observed in our population. However, this was expected because we used an intraspecific population in our work.. Polymorphic markers were analyzed with JoinMap software and three markers were observed to link with the N gene. These markers are ScarPM6a (3.6 cM), ScarPM6b (10.2 cM) and ScarN (22.6 cM) which are located in the same segregation group with the N gene.

To increase the efficiency and effectiveness of pepper breeding for nematode resistance, use of MAS (marker assisted selection) must be universalized and developed. Marker analysis has a very important role in modern plant breeding thus, developing tightly linked markers and linkage maps are the building blocks of future plant breeding.

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