GENETIC DIVERSITY OF TURKISH SPINACH CULTIVARS (*Spinacia oleracea L.*)

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ABSTRACT

GENETIC DIVERSITY OF TURKISH SPINACH CULTIVARS
(*Spinacia oleracea L.*)

In this study, 95 Turkish spinach (*Spinacia oleracea L.)* accessions were used for genetic diversity analysis by using the SRAP (Sequence Related Amplified Polymorphism) marker system and also 81 of the spinach accessions were used for morphological data analysis using data from the Centre for Genetic Resources, Wageningen University, The Netherlands. In the study, a total of 25 SRAP marker combinations were used and 19 of these were suitable for genetic analysis because they gave amplification and they were polymorphic. For the 19 SRAP markers, 123 bands were amplified and 67 were polymorphic. These polymorphic bands were used to construct a dendogram for spinach cultivars and to determine the genetic distance. Dendrogram analysis was done UPGMA method which was calculated using DICE matrix. Genetic similarity ranged between 0.30 and 0.95. Group B had most related accessions, 0.77 similar, and Group A, C and D had more distinct accessions, 0.60 similarity.

For the morphological data PCA(Principal Component Analysis) was performed and no any apparent group was seen so high diversity was indicated by the 2D plot. All these results showed that the SRAP marker system was a suitable marker to determine genetic diversity, that Turkish spinach germplasm is diverse and that accessions should be preserved. Another importance of this results is that it is the first and only study that has been done with Turkish spinach germplasm.

ÖZET

Türk Ispanak (*Spinacia oleracea L.*) Çeşitlerinde Genetik Çeşitliliğin İncelenmesi

Bu çalışmada, 95 tane Türk ıspanak hattı PCR tabanlı bir moleküler işaretleyici yöntemi olan SRAP (Sequence Related Amplified Polymorphism) ile incelenmiştir.

Yine aynı çalışma içerisinde 81 tane Türk ıspanak hattının Wageningen Üniversitesi Genetik Kaynaklar Merkezinden alınan bilgiler ile morfolojik analizleri yapılmıştır. Çalışma içerisinde 25 adet SRAP işaretleyicisi kombinasyonu kullanılmış ve bunlardan 19 işaretleyici iyi sonuçlar vermiştir. Bu 19 tane işaretleyici ile toplamda 123 SRAP fragmenti elde edilmiş ve bu fragmenlerin 67 tanesi polimorfik bulunmuş ve bunlarla genetik uzaklığı ve hatların birbirleriyle olan ilişkisini gösteren ağaç çizilmiştir. Genetik uzaklık belirten ağaç analizi DICE martriks oluşturulmasına dayanan UPGMA (Unweighted Pair Group Method) yöntemi ile yapılmıştır. Yapılan analizler sonucunda Türk ıspanak hatlarının genetik olarak birbirlerine olan uzaklarının aralığı 0.30 ve 0.95 olarak bulunmuştur. Genetik ağaç içerisindeki gruplardan olan B grubundaki bireylerin benzerlik oranı 0.77, A, C ve D grubu bireylerinin benzerlik oranı ise 0.60 olarak belirlenmiştir. Yine PCA (Principal Component Analysis) yöntemiyle yapılan genetik ve morfolojik analizlerde de genetik çeşitliliğin fazla olduğu bireylerin iki boyutlu grafik alanında oldukça fazla bir dağılım gösterdiği belirlenmiştir. Bir başka deyişle tüm bu analizlerde bireyler arasında belli bir gruplaşma gözlenmemiştir. Tüm bu sonuçlar SRAP işaretleyici yöntemiyle Türk ıspanak türlerinde genetik çeşitlilik analizlerin yapılabilirliğini göstermiştir. Genetik çeşitlilik açısından zengin olan bu ıspanak türleri ülkemizde korunmalıdır. Bu çalışma Türk ıspanak türleri ile yapılan ilk ve tek çalışma olarak önem kazanmıştır.

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CHAPTER 1

INTRODUCTION

1.1. General Properties of *Spinacia oleracea L.*

Spinach (*Spinacia oleracea L.*) is an edible flowering plant that belongs to the family *Amaranthaceae*. It is an annual plant that grows quickly and has the ability to survive over winter in temperate regions. For example, in Turkey, it is found throughout the winter season. Spinach leaves are variable in size from about 2–30 cm long and 1–15 cm broad. Larger leaves are found at the base of the plant and small leaves are found higher on the flowering stem (LeStrange, et al. 1999). The flowers of spinach are usually yellow-green, 3–4 mm in diameter and mature into several small seeds. Plants may flower in as little as two weeks provided that they are grown in ideal conditions.

Three different types of spinach are generally available (LeStrange, et al. 1999): Savoy type has crisp, creased, curly leaves and is primarily used for fresh market purposes (LeStrange, et al. 1999). The smooth-leaf type has flat, unwrinkled, spade-shaped leaves and is preferred for processing (LeStrange, et al. 1999). The baby spinach type is preferable for use in salads owing to its taste and delicate texture (LeStrange, et al. 1999). Spinach is also classified according to seed type: prickly or smooth. Commercially, smooth-seeded types are used because they are much easier to handle and plant accurately (LeStrange, et al. 1999).

1.2. Origin and Distribution of *Spinacia oleracea L.*

Spinach originates from central and southwestern Asia. It is thought that it was first cultivated in ancient Persia (Iran) but it has a longer history in Europe than many other vegetables. For example, spinach was recognized by Arab traders in India and China where it was known as ‘Persian vegetable’. The Moors introduced it into Spain and spinach became known as ‘the Spanish vegetable’ in England, and then spread to other parts of the world. After it first appeared in England and France in the 14th

century, it was consumed in large amounts because it appeared in early spring, when other vegetables were scarce (Boswell, 1949; Rolland, et al. 2006)

Today, China, the United States and Japan are among the largest commercial producers of spinach. Turkey is the fourth largest producer of spinach with about 225 thousand tons per year based on FAO statistics (Food and Agriculture Organization of the United Nations, 2009) (Table 1.1.)

Table 1.1. World spinach production in 2009 based on FAO statistics. Countries are ordered by their total production

Country	Production (in tones)
China	12,862.005
U.S.	369,770
Japan	288,000
Turkey	225,343
France	130,000
Italy	100,000
Albania	10,577
Austria	10,109

1.3. Nutritional Profiles of *Spinacia oleracea L.* and Effects on Health

Spinach contains high levels of minerals and vitamins, especially calcium and magnesium which are excellent bone-supportive nutrients; phosphorus; iron; potassium; vitamin A which is a free radical-scavenger; vitamin K; vitamin B; vitamin E and vitamin C (Table 1.2). Spinach also contains potassium, vitamin B2, vitamin B6, folate, betaine, copper, protein, manganese, zinc, niacin, selenium and omega-3 fatty acids. Spinach has a more delicate texture and more nutrients than most other green-leaf vegetables. It is also extremely rich in antioxidants which scavenge free radicals and adjust blood pressure. These antioxidants are especially high when spinach is consumed fresh, steamed, or quickly boiled.

Table 1.2. Nutritional profile per 100g raw spinach

Energy	97 kJ (23 kcal)
Carbohydrates	3.6 g
Sugars	0.4 g
Dietary Fiber	2.2 g
Fat	0.4 g
Protein	2.2 g
Vitamin A	9400 mg
Folate	194 µg
Vitamin C	28 mg
Vitamin E	2 mg
Vitamin K	483 µg
Calcium	99 mg
Iron	2.7 mg

Because of its rich content, spinach has many positive effects on health. In 2005, studies showed that spinach was the most protective nutrition of all against cancer risks (Isoko, et al. 2005). In addition to protective effects, it has the strongest effects on cancer cell proliferation (Isoko, et al. 2005). For instance, it was found that spinach could help to prevent breast cancers because of its high content of lutein and other carotenoids (Longnecker, et al. 1997).

1.4. The Importance of Genetic Diversity

Genetic diversity refers to the total number of different genetic characteristics in species. For all living organisms, genetic diversity is very important for species to survive and adapt to new environments and gene diversity in species is essential for evolution. Genetic diversity can be assessed in various ways such as by looking at the proportion of polymorphic loci in the genome (by looking at heterozygosity levels and alleles per locus). In plants, genetic diversity is important to develop varieties tolerant to heat, salinity, drought and pathogen attack. Thus previously known resistant genes can be identified and transferred to susceptible lines thanks to conservation of genetic diversity in plants.

Suitable molecular markers are used for genetic diversity research. After numerous PCR experiments by using suitable marker systems, data collected from different varieties are analyzed by software program and their genetic divergence can be

assessed throughout their evolution. This procedure is also called ‘dendogram analysis’. This dendogram is frequently used to visualize the relationship of the different lines and varieties produced by hierarchical clustering. It is beneficial to design crosses between diverse individuals and to store the data for future studies.

1.5. Genetic Analysis with Marker Systems

Genetic markers are present at specific locations on a chromosome and act as ‘tags’ for genome analysis experiments. Genetic markers are divided into two types: morphological and molecular (DNA) markers (Kumar, 1999).

a. ***Morphological markers***

Morphological markers determine the morphology of a plant (height, grain colour, etc.). The inheritance of these markers can be monitored visually without using specialized biochemical or molecular techniques. Morphological traits that are controlled by a single locus can be used as genetic markers provided their expression is reproducible over a range of environments (Kumar, 1999). However, the number of morphological markers is very limited and they may not be suitable for distinguishing heterozygous individuals from homozygous individuals because it shows only phenotypic effects of alleles (Kumar, 1999).

b. ***Molecular markers***

With the advent of DNA marker technology, several types of DNA markers were developed for molecular analysis. Marker technology gained in popularity because it enables breeders to use the genetic composition of plants as a criterion for selection and to develop breeding processes. DNA marker systems also help plant breeders or geneticists to develop plants that have the desired properties (Kumar, 1999).

Molecular markers (DNA markers) reveal neutral sites of variation at the DNA sequence level (Jones, et al. 1997). ‘Neutral’ means that, unlike morphological markers, these variations do not show themselves in the phenotype (Jones, et al. 1997). Molecular markers are stable, easily detectable in all tissues and independent of environmental conditions. In other words, they are not affected by environmental effects such as growth, differentiation or development (Agarwal, et al. 2008).

1.5.1. Molecular Marker Systems in *Spinacia oleracea L.*

In spinach, some studies were done using molecular marker systems to determine pathogen resistance and classification or to determine sex determination genes. All these studies contain different molecular marker systems which were chosen according to the aim of the study. This is important because different marker systems show different polymorphism, therefore the aim of the research is an important factor in selecting a suitable marker system. For example, random markers are suitable for studies which focus on evolution or historical processes.

In spinach studies, simple sequence repeats or microsatellites (SSR), target-region amplified polymorphism (TRAP) and sequence-related amplified polymorphism (SRAP) marker systems were used according to the aim of the study. In this study, SRAP markers were used but all related studies and marker systems are explained in the next sections.

1.5.2. Simple Sequence Repeat (SSR) Marker Systems

SSRs are a group of repetitive DNA sequences. They occur abundantly in most eukaryotic genomes. Polymorphism arises mostly by replication slippage or non-equal crossing over events. Plant genomes generally contain large numbers of SSRs or microsatellites. These repetitive sequences are generally found at many different loci and they can be polyallelic. Dinucleotide repeats are the most abundant type of SSR (Morgante, et al. 1993). These markers have some advantages such as they are codominantly inherited, reproducible, simple, PCR-based, extremely polymorphic and highly informative because of the number and frequency of detected alleles. SSR markers have the ability to distinguish between closely related individuals. However, they are expensive to develop, require sequence information and specific primers for one species usually do not work in other species (Morgante, et al. 1993).

In recent years, SSRs have been widely used for cultivar discrimination and genetic mapping in several species including spinach (Gupta, et al. 2000). For example, 15 microsatellite primer pairs were studied for spinach (Groben and Wricke 1998; Khattak, et al. 2006), nine of which were mapped to five of the six chromosomes in spinach (Khattak, et al. 2006). This study showed that genic microsatellite markers for

spinach may be efficiently used for cultivar discrimination and classification. In addition, these markers were useful for genetic mapping of the sex determination locus (Khattak, et al. 2006), and this finding may become a useful tool for marker-assisted selection studies in the future.

1.5.3. Target-Region Amplified Polymorphism (TRAP) Marker System

Another study was done using TRAP markers to determine the genetic diversity between spinach accessions. This technique uses two primers of 18 nucleotides to generate markers (Jınguo, et al. 2003). One of the primers, the fixed primer, is designed from a targeted expressed sequence tag (EST) sequence in the database and is used as a forward primer. The second primer, the arbitrary primer, is an arbitrary sequence with either an AT-or GC-rich core to anneal with an intron or exon, respectively that is used as a reverse primer (Jınguo, et al. 2003). After PCR, many amplified fragments can be seen on the gel (usually polyacrylamide gel) because of primer binding to all possible expressed sequence tags (ESTs). All fragments are detected and compared with each other in a dominant manner.

This technique is rapidly developing and is used for genotyping germplasm collections and marking genes for desirable agronomic traits of crop plants. The fixed and arbitrary primers are created based on the availability of sequence information for the species (Jınguo, et al. 2003).

1.5.4. Sequence-Related Amplified Polymorphism (SRAP) Marker System

The SRAP (sequence-related amplified polymorphism) marker system is used because of its simplicity and reliability. The aim of the technique is to amplify open reading frames (ORFs) (Li, et al. 2001). The SRAP system is based on amplification from two primers that are about 17-18 nucleotides long and consist of the following elements: core sequences that are not specific (filler sequences), followed by the sequence CCGG in the forward primer and AATT in the reverse primer (Li, et al. 2001). The filler sequences must be different from each other and can be 10-11 bases long (Li, et al. 2001). After the PCR (polymerase chain reaction) process, many bands

can be seen on the gel (agarose gel can be used) like in the TRAP marker system. The images can be a little complicated but the variations of the bands on the gel can be seen and scored. The differences between the bands represent the 'variability' or 'diversity' between individuals.

SRAP has several advantages over other systems: simplicity, reasonable throughput rate, similar properties of other co-dominant markers, and it allows easy isolation of bands for sequencing because it targets ORFs (Li, et al. 2001).

1.6. Goals

The primary aim of this study was to determine the genetic diversity of Turkish spinach cultivars which will be the first study in the literature. For this, 95 accessions were used to examine genetic variation with SRAP markers. Dendrogram analysis allowed an assessment of the genetic relationships among Turkish spinach cultivars and their diversity. Findings from this study may be useful for reorganization of Turkish spinach germplasm resources and for targeting new collection efforts.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

In this study 95 spinach accessions were analyzed for their genetic diversity. Of these, 81 accessions were obtained from the Centre for Genetic Resources,Wageningen University, the Netherlands and 14 spinach accessions were obtained from Yüksel Tohumculuk in Antalya. Each accession was given a different pedigree number. There was no information about whether the lines were inbred or outbred. Eight seeds of each accession were planted in seedling plates that contained torf and perlite. Seeds were germinated in a growth chamber at optimum temperature and humidity conditions. All accessions are listed in Table 2.1.

Table 2.1. Table of spinach accessions with their pedigree number,source and location and genotype numbers

Pedigree Number	Source	Location	Genotype Number
CGN09483	CGN	Turkey	56
CGN09488	CGN	Turkey	59
CGN09489	CGN	Turkey	70
CGN09490	CGN	Turkey	40
CGN09499	CGN	Çanakkale	29
CGN09500	CGN	Burdur	39
CGN09501	CGN	Turkey	71
CGN09502	CGN	Ayvalık, Balıkesir	41
CGN09503	CGN	Trabzon	33
CGN09504	CGN	Diyarbakır	36
CGN09507	CGN	Niğde	20
CGN09508	CGN	Deresakarı, Bilecik	18
CGN09509	CGN	Turkey	50
CGN09543	CGN	Biga, Çanakkale	24
CGN09556	CGN	Turkey	54
CGN09557	CGN	Turkey	62
CGN09558	CGN	Turkey	8
CGN09559	CGN	Turkey	13
CGN09560	CGN	Turkey	11
CGN09561	CGN	Turkey	35
CGN09562	CGN	Turkey	30

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Table 2.1. (cont.)

CGN09563	CGN	Turkey	1
CGN09564	CGN	Turkey	55
CGN09565	CGN	Turkey	77
CGN09566	CGN	Turkey	81
CGN09567	CGN	Turkey	46
CGN09568	CGN	Turkey	43
CGN09570	CGN	Turkey	76
CGN09571	CGN	Turkey	42
CGN09572	CGN	Turkey	48
CGN09573	CGN	Turkey	78
CGN09574	CGN	Turkey	74
CGN09575	CGN	Turkey	27
CGN09576	CGN	Turkey	21
CGN09577	CGN	Turkey	7
CGN09578	CGN	Turkey	65
CGN09579	CGN	Turkey	28
CGN09580	CGN	Turkey	63
CGN09581	CGN	Turkey	5
CGN09582	CGN	Turkey	10
CGN09583	CGN	Turkey	69
CGN09584	CGN	Turkey	47
CGN09585	CGN	Turkey	32
CGN09586	CGN	Turkey	25
CGN09587	CGN	Turkey	80
CGN09618	CGN	Ulaş, Sivas	23
CGN09619	CGN	Kayseri	6
CGN09620	CGN	Malatya	26
CGN09621	CGN	Turkey	79
CGN09623	CGN	Turkey	49
CGN09624	CGN	Turkey	52
CGN09625	CGN	Eskişehir	58
CGN09626	CGN	Trabzon	16
CGN09627	CGN	Bolu	53
CGN09628	CGN	Elmadi, Antalya	19
CGN09629	CGN	Bornova, İzmir	22
CGN09632	CGN	Turkey	67
CGN09664	CGN	Istanbul	68
CGN09665	CGN	Maraş	31
CGN09666	CGN	Ankara	4
CGN09667	CGN	İstanbul	3
CGN09668	CGN	Kilis	60
CGN14160	CGN	Turkey	15
CGN14161	CGN	Turkey	17
CGN14162	CGN	Turkey	62
CGN14163	CGN	Turkey	66
CGN14164	CGN	Turkey	12
CGN14165	CGN	Turkey	75
CGN14166	CGN	Muğla	72
CGN14174	CGN	Turkey	64
CGN14175	CGN	Turkey	44
CGN14176	CGN	Giresun	45
CGN14201	CGN	Turkey	51
CGN14202	CGN	Turkey	9
CGN14203	CGN	Turkey	37
CGN14204	CGN	Turkey	34
CGN14205	CGN	Turkey	38
CGN14206	CGN	Turkey	2
CGN14210	CGN	Kayseri	73

(cont. on next page)

Table 2.1. (cont.)

CGN18785	CGN	Turkey	57
CGN21753	CGN	Biga	14
S-11	Yüksel Tohumcu.	Turkey	82
S-119	Yüksel Tohumcu.	Turkey	83
S-130	Yüksel Tohumcu.	Turkey	84
S-131	Yüksel Tohumcu.	Turkey	85
S-134	Yüksel Tohumcu.	Turkey	86
S-145	Yüksel Tohumcu.	Turkey	87
S-25	Yüksel Tohumcu.	Turkey	88
S-274	Yüksel Tohumcu.	Turkey	89
S-309	Yüksel Tohumcu.	Turkey	90
S-315	Yüksel Tohumcu.	Turkey	91
S-319	Yüksel Tohumcu.	Turkey	92
S-5	Yüksel Tohumcu.	Turkey	93
S-57	Yüksel Tohumcu.	Turkey	94
S-65	Yüksel Tohumcu.	Turkey	95
S-75	Yüksel Tohumcu.	Turkey	96

2.1.2. Plant Morphological Data

For some of the 81 plant accessions, morphological data were collected from the Centre for Genetic Resources, Wageningen University, the Netherlands. In addition to this, the last 14 accessions which were collected from Yüksel Tohumculuk were eliminated because no data were available for them. In all 12 morphological parametres were selected and the analysis was done.The definitions of scores are were shown in Table 2.2.

Table 2.2. Scoring criteria for the morphological traits. Definitions of skores were shown in the table. Different scoring criteria was established by Wageningen University.

Stem anthocyanin content	1=very low 3=low 5=intermediate 7=high 9=very high
Leaf shape	1=elliptic, 2=broad elliptic, 3=circular, 4=ovate, 5=broad ovate, 6=triangular
Leaf colour	1=yellow-green, 2=grey-green, 3=blue-green
Leaf blistering	3=weak, 5=medium, 7=strong
Bolting time	Count days from sowing till 10% of plants have stem of 5 cm
Petiole length	3=short, 5=medium, 7=long, 9=very long
Leaf colour intensity	3=light, 5=medium, 7=dark
Leaf glossiness	3=weak, 5=medium, 7=strong

(cont. on next page)

Table 2.2. (cont.)

Leaf lobing	1=absent, 3=weak, 5=medium, 7=strong
Female plant number	1=none, 3=25%, 5=50%, 7=75%, 9=all
Monocieoussinous	1=no monoecious plants, 3=25%, 5=50%, 7=75%, 9=only monoecious plants
Branching Flowering Plant	1=very low 3=low 5=intermediate 7=high 9=very high

2.2. Methods

2.2.1. DNA Extraction

DNA extraction was performed by taking young leaves when the seedlings were at the 4-5 leaf stage. For extraction protocol, different methods were applied to choose the best one and the Promega Wizard Genomic Purification kit was chosen. After extraction process, the genomic DNAs were quantified by Nanodrop ND-1000 spectrophotometer and the 96 genomic DNAs were stored at -20°C in DNA Rehydration Solution until molecular characterization.

2.2.2. SRAP Marker Analysis

Marker combinations were first tried on a subset of the population to find good combinations for applying on the whole population. Therefore, 12 accessions were randomly chosen from the population and nearly 100 marker combinations were analyzed. After this analysis, the 25 best marker combinations were selected and applied to the rest of the population.These markers and their sequences are listed in Table 2.3.

For PCR amplification, the solution contained 2µl 10X buffer, 2 µl $MgCl_2$, 0.7 µl dNTP, 0.3 µl Taq polymerase, 9.5 µl dH_2O, 2 µl ME (forward primers), 2 µl EM (reverse primer) and 1.5 µl 50-100ng DNA. The PCR amplification conditions for efficient SRAP marker analysis were: 5 min. initial denaturation at 94°C, then 5 cycles composed of denaturating at 94°C for 1 min, annealing at 35°C for 1 min,extension at 72°C for 1 min and followed by 35 cycles; heating at 94°C for 1 min, annealing at 55°C

for 1 min, extension at 72°C for 1 min and 10 min final extension at 72°C. After PCR process, PCR products were run in 3 % agarose gels in 1X TAE buffer and they were visualized under Bio-Rad UV light system.

Table 2.3. SRAP primers and their sequences used in this study.

Forward Primers (5'-3')	Reverse Primers (3'-5')
ME3: TGAGTCCAAACCGGAAT	EM1: GACTGCGTACGAATTAAT
ME4: TGAGTCCAAACCGGACC	EM2: GACTGCGTACGAATTTGC
ME5: TGAGTCCAAACCGGAAG	EM4: GACTGCGTACGAATTTGA
ME6: TGAGTCCAAACCGGTAG	EM5: GACTGCGTACGAATTAAC
ME8: TGAGTCCAAACCGGTGT	EM6: GACTGCGTACGAATTGCA
ME9: TGAGTCCAAACCGGTCA	EM7: GACTGCGTACGAATTATG
ME11: TGAGTCCAAACCGGGTA	EM8: GACTGCGTACGAATTAGC
ME12: TGAGTCCAAACCGGGGT	EM9: GACTGCGTACGAATTACG
ME2: TGAGTCCAAACCGGAGC	EM10: GACTGCGTACGAATTTAG
	EM11: GACTGCGTACGAATTTCG
	EM12: GACTGCGTACGAATTGTC
	EM13: GACTGCGTACGAATTGGT
	EM14: GACTGCGTACGAATTCAG
	EM15: GACTGCGTACGAATTCAG
	EM16: GACTGCGTACGAATTCGG
	EM17: GACTGCGTACGAATTCCA

2.2.3. Data Analysis

The PCR products obtained from SRAP marker analysis were scored as present (1) or absent (0) for each fragment. These data were used to calculate the genetic distance between accessions and draw a dendogram. Dendogram construction was done using NTSYS-pc version 2.2 (Numerical Taxonomy Multivariate Analysis System, Exeter Software, Setauket, N.Y.) software program. DICE matrix and Unweighted Pair Group Method with Arithmetic Averages (UPGMA) methods were used for dendrogram construction. UPGMA is the simplest method for constructing trees, it generates rooted trees. However, the great disadvantage of UPGMA is that it assumes the same evolutionary speed on all lineages, i.e. the rate of mutations is constant over time and for all lineages in the tree. DICE similarity index calculates the similarity between two samples. Unweighted Pair Group Method with Arithmetic Averages (UPGMA) and the SAHN clustering program cluster the similarity data for construction

of similarity dendogram. Correlation between tree and data matrix was compared with the Mantel test by NYSYS-pc version 2.2 (Mantel 1967).

In the second part, for the morphological data analysis, multivariate principal component analysis (PCA) was done using JMP 7.0 software (SAS Institute, Cary, NC, USA). PCA is one of the statistical methods to reduce the dimensionality of a data set. This analysis is used for showing how different variables work together, decreasing redundancy of data set and filtering some of the noise.

CHAPTER 3

RESULT AND DISCUSSION

3.1. Morphological Data Analysis

Morphological data for 12 parameters were collected from the Centre for Genetic Resources,Wageningen University, the Netherlands. The morphological characters were: stem anthocyanin content, leaf shape, leaf colour, leaf blistering, bolting time, petiole length, leaf colour intensity, leaf glossiness, leaf lobing, female plant number, monoeciousness, and branching flower plant. These data and their scores are shown in Table 3.1.The JMP 7.0 software program was used for multivariate PCA of the morphological data.According to this analysis, no apparent grouping was observed on the plot.

Table 3.1. Morphological data and its scores

Accessions	Genotype Number	Stem Anthocyanin Content	Leaf Shape	Leaf Colour	Leaf Blistering	Bolting Time	Petiolee Length	Leaf Colour Intensity	Leaf Glossiness	Leaf Lobing	Female Plant Number	Monoecioussinouss	Branching Flowering
CGN09483	56	3	5	1	5	44	6	5	4	5	5	1	6
CGN09488	59	5	4	1	4	41	5	5	3	5	6	1	6
CGN09489	70	5	4	3	3	41	6	5	3	7	6	1	6
CGN09490	40	5	5	3	4	43	6	5	3	6	3	2	7
CGN09499	29	2	4	2	4	43	6	5	7	3	5	2	5
CGN09500	39	2	4	2	4	43	5	5	5	5	4	2	6
CGN09501	71	5	4	3	4	34			7	5	4	2	5
CGN09502	41	3	3	2	4	43	6	5	3	9	5	1	6
CGN09503	33	2	3	2	3	43	5	5	3	9	6	1	6
CGN09504	36	4	3	2	4	40	5	5	3	9	5	2	5
CGN09507	20	2	4	2	4	47	5	5	5	5	3	1	6
CGN09508	18	2	3	2	4	40	4	5	5	5	3	2	3
CGN09509	50	3	3	2	4	33	6	5	5	7	5	1	4
CGN09543	58	1	5	2	4	47	5	5	5	5	5	1	6
CGN09556	24	5	3	3	4	47	4	5	5	7	5	1	6
CGN09557	54	5	3	3	3	40	4	5	3	7	3	3	5
CGN09558	62	5	4	1	5	50	3	6	4	3	5	1	7
CGN09559	8	3	3	1	6	50	4	5	5	3	6	1	6
CGN09560	13	5	3	3	3	43	5	6	3	7	6	1	6
CGN09561	11	3	4	1	4	47	5	6	5	3	5	1	6
CGN09562	35	3	3	1	3	43	4	6	3	7	3	3	5
CGN09563	30	3	4	1	4	43	5	5	3	5	4	1	6
CGN09564	1	5	3	1	4	43	5	6	3	5	3	5	5
CGN09565	55	3	6	2	3	41	5	6	5	7	5	1	6
CGN09566	77	3	4	1	4	40	5	6	3	5	5	1	7
CGN09567	81	3	4	3	4	43	6	6	3	7	6	1	6
CGN09568	46	3	4	5	4	43	6	6	3	7	4	1	5
CGN09570	43	5	3	5	3	40	5	6	4	7	5	1	6
CGN09571	76	5	4	5	4	43	5	6	3	7	5	1	6
CGN09572	42	5	4	5	4	43	6	5	3	7	6	1	6
CGN09573	48	7	3	5	3	33	4	6	3	5	5	1	5
CGN09574	78	5	3	5	3	29	4	6	3	7	4	1	5
CGN09575	74	5	4	1	4	47	5	6	3	7	5	1	7
CGN09576	27	5	4	1	5	43	5	5	4	3	6	3	7
CGN09577	21	5	3	1	3	40	4	6	3	5	3	3	4
CGN09578	7	7	3	3	4	47	4	6	4	5	6	1	6
CGN09579	65	5	3	3	3	43	4	6	4	5	3	2	6
CGN09580	28	5	2	5	3	33	3	6	3	7	3	1	4
CGN09581	63	3	3	1	3	40	4	5	3	7	4	1	5
CGN09582	5	3	3	1	4	40	5	5	3	9	5	1	6
CGN09583	10	5	4	1	4	47	4	5	3	7	6	1	7
CGN09584	69	3	3	1	4	47	5	5	3	5	4	1	6
CGN09585	47	3	3	1	3	43	5	5	3	5	6	1	6
CGN09586	32	3	3	1	3	33	4	6	3	7	4	2	4
CGN09587	25	3	3	3	4	40	5	5	4	5	5	2	5
CGN09618	80	1	4	2	3	43	6	5	3	6	5	1	5
CGN09619	23	2	4	2	4	43	5	5	3	6	5	1	5
CGN09620	6	3	3	2	3	43	5	5	3	7	4	1	6
CGN09621	26	3	3	2	3	43	5	5	3	7	5	1	5
CGN09623	79	3	3	2	3	33	4	5	5	6	5	2	3
CGN09624	49	4	3	2	4	43	5	5	5	7	3	3	5
CGN09625	52	2	6	2	4	40	5	5	4	6	7	2	4

(cont. on next page)

Table 3.1. (cont.)

CGN09626	16	2	4	2	3	40	5	5	7	6	3	2	5
CGN09627	53	2	4	2	5	40	4	5	7	6	6	1	5
CGN09628	19	3	4	2	4	43	6	6	3	6	5	1	6
CGN09629	22	3	4	2	4	40	5	5	7	7	3	1	5
CGN09632	67	6	2	2	2	29	5	6	5	6	3	1	5
CGN09664	68	3	3	2	3	36	5	5	7	6	5	2	4
CGN09665	31	2	4	2	4	43	4	5	5	5	4	2	5
CGN09666	4	2	3	2	4	40	5	5	5	6	4	1	6
CGN09667	3	3	3	2	3	40	4	5	5	6	4	2	6
CGN09668	60	2	3	2	3	43	5	5	5	6	5	2	5
CGN14160	15	1	5	2	5	47	5	5	6	5	2	6	6
CGN14161	17	1	5	2	4	47	5	6	6	6	4	1	7
CGN14162	61	3	4	2	4	47	5	6	5	6	6	1	7
CGN14163	66	3	3	2	4	47	5	6	4	6	6	1	7
CGN14164	12	1	4	2	4	47	5	6	6	5	5	1	7
CGN14165	75	2	5	2	4	54	5	6	5	5	6	1	7
CGN14166	72	3	3	2	3	36	5	5	5		5	1	5
CGN14174	64	2	4	2	4	43	5	6	6	5	5	2	5
CGN14175	44	3	3	2	4	43	5	6	5	6	6	1	6
CGN14176	45	2	3	2	3	36	5	5	5	5	4	3	5
CGN14201	51		8	2	5	57	5	7	6	3	7	1	6
CGN14202	9	3	2	2	3	40	3	5	4	6	3	2	5
CGN14203	37	2	4	2	3	47	5	6	5	6	5	1	7
CGN14204	38	2	3	2	4	43	4	6	5	6	6	1	6
CGN14205	2	3	3	2	4	43	4	6	4	6	5	1	6
CGN14206	73	3	2	2	3	33	4	6	4	6	5	3	5
CGN14210	57	2	4	2	3	34	6	5	5	6	5	1	7
CGN18785	14		8	2	5	57	5	6	6	4	6	1	6
CGN21753	34	3	3	2	4	46	7	5	5	5	4	1	3

Multivariate PCA analysis of the morphological data showed that the first, second and third components. Eigen values were 25.38, 18.33, and 11.89% of the variance among accessions, respectively. Therefore the first three components explained 55.60% of variance. Eigenvalues have statistical meaning that they are quantitative values of how much a component represents the data. The higher the eigenvalues of a component, the more representative it is of the data. The percent of variance shows how well all the components summarize the data. From the results, it was seen that the important traits in the first component were bolting time, leaf shape, leaf blistering and branching flower plant. All of these parameters were positively correlated with the first component (r=0.45 to 0.35). For the second component, female plant number, monoeciousness, stem anthocyanin content and leaf glossiness properties were most important (r=0.43 to -0.36). These traits correlated both positively and negatively with the second component. The third component had the highest correlation with petiole length (r=-0.55), leaf colour intensity (r=0.45985) and leaf lobing (r=-0.41339)

When plotted in two dimensions, the score plot showed separation among spinach accessions (Figure 3.1). This meant that the accessions had good variability for morphological parameters. Some accessions were more similar to each other than other accessions such as genotypes CGN14163 (66) and CGN09571 (42). On the other hand, CGN09580 (63) was the most distinct accession and was most separated from the others. In another study, Iranian spinach genetic diversity was studied using morphological traits such as petiole length, leaf number, and female plant number (Eftekhari, et al. 2010). In this study they used some of the same morphological properties of spinach that we did. However, this article was not accessible to give detailed information.

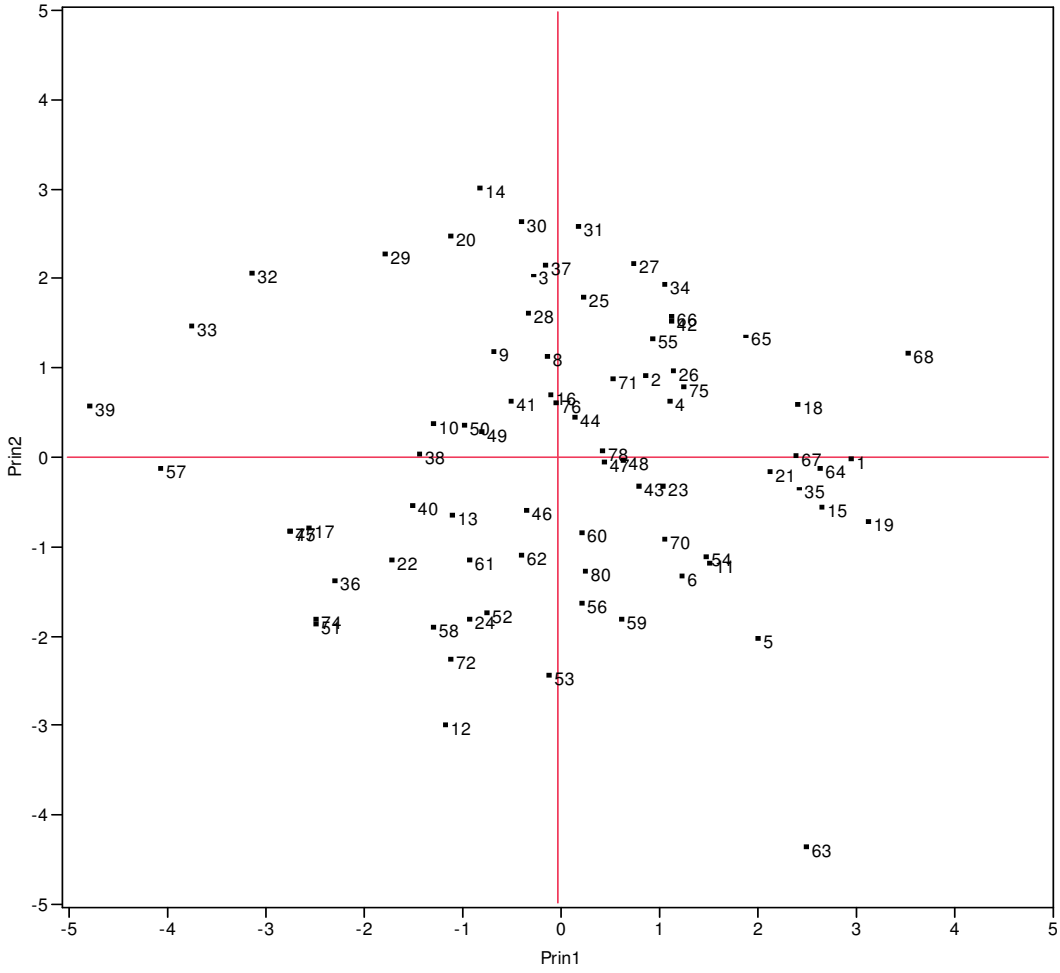

Figure 3.1. Score Plot for PCA analysis of morphological parameters. Prin1 dimension contains bolting time, leaf shape, leaf blistering and branching flower plant data set. Prin2 dimension contains female plant number, monoeciousness, stem anthocyanin content, leaf glossiness

3.2. Genotypic Data Analysis

3.2.1. SRAP Marker Data Results

In the study 95 Turkish spinach cultivars were used for SRAP marker analysis. A total of 25 marker combinations were applied and 19 marker combinations amplified well and were used as polymorphic marker combinations in this study. The other six primer combination did not amplify well. For the polymorphic marker combinations, a total of 123 bands were found to amplify and 67 of these bands were polymorphic (61%). Of these marker combinations, ME3-EM6, ME5-EM5, ME4-EM1 and ME11-EM7 combinations gave the best polymorphic results. Amplification quality was determined by taking into account average of total bands. Results are shown in Table 3.2

Table 3.2. Suitable SRAP marker combinations in the end of PCR analysis, well-amplified marker combinations and number of polymorphic bands found in this study.

SRAP Marker combinations	Amplification Quality	Number of Polymorphic Bands
ME3-EM11	Poor	2
ME8-EM2	Poor	2
ME6-EM10	Average	3
ME12-EM14	Poor	2
ME3-EM6	Very good	6
ME5-EM5	Very good	10
ME6-EM7	Average	3
ME12-EM7	Poor	2
ME5-EM17	Average	3
ME11-EM14	Poor	1
ME11-EM2	Poor	2
ME2-EM9	Poor	2
ME4-EM1	Very good	7
ME11-EM7	Average	3
ME3-EM8	Very good	5
ME3-EM13	Good	4
ME12-EM8	Poor	2
ME11-EM15	Poor	2
ME11-EM4	Poor	1
ME6-EM12	No amplification	**Average: 3.26**
ME3-EM5	No amplification	
ME8-EM14	No amplification	
ME11-EM16	No amplification	
ME6-EM2	No amplification	
ME9-EM13	No amplification	

Partial results for two of the well-amplified marker combinations, ME5-EM5 and ME4-EM1, are shown in Fig 3.2. and 3.3. respectively.

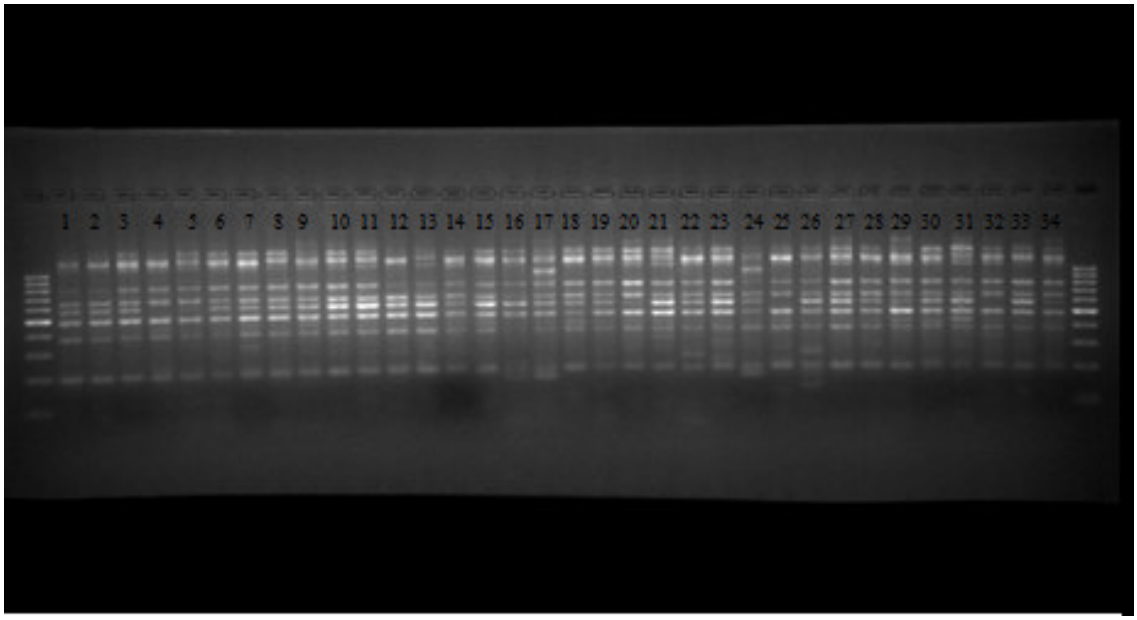

Figure 3.2. ME5-EM5 SRAP marker combination gel image. First and the last columns are 100 bp ladder and the other samples are first 34 spinach accessions.

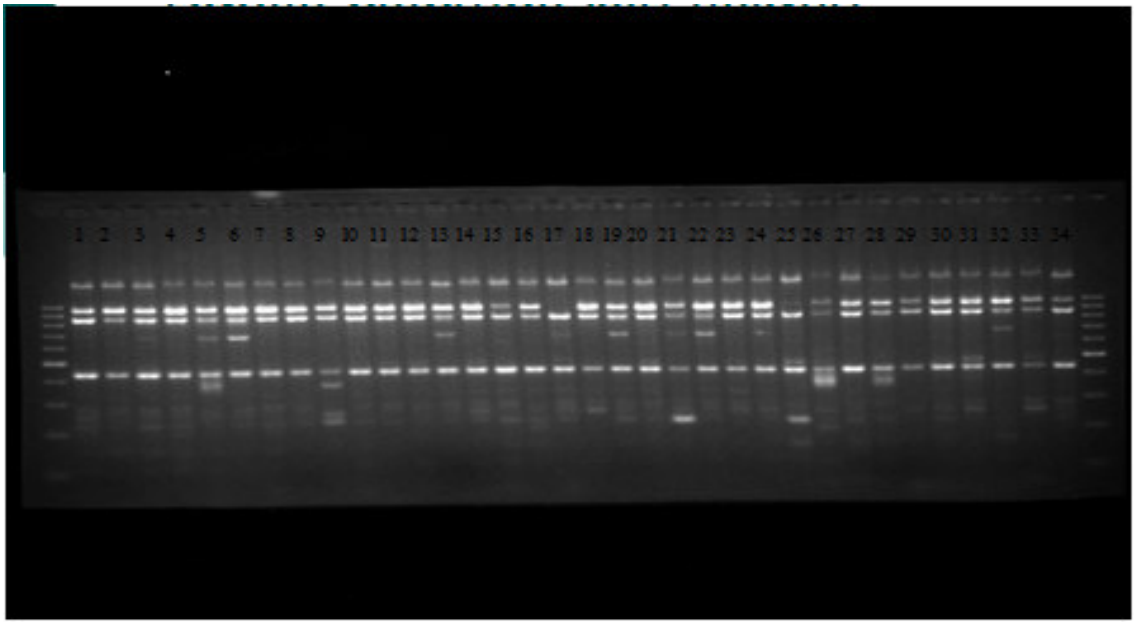

Figure 3.3. ME4-EM1 SRAP combination gel image. First and the last columns are 100 bp ladder and the rest are 34 spinach accessions

According to Mantel test, the correlation between the genotypic data distance matrix and the dendogram was very high (r=0.96). The dendogram scale varied between 0.30 and 0.95. Thus, the minimum similarity between spinach cultivars was 30% whereas the highest similarity between them was 95%.

According to the spinach dendogram, the tree could be divided into five groups: A, B, C, D and E. The largest group was A. It contained 75 spinach accessions with minimum similarity of 0.60. Group B had 5 accessions of spinach and the minimum similarity value was 0.77. Group C and D had only two accessions and the similarity value was the same as for Group A: 0.60. The last group was E and it had 11 accessions and the similarity value was 0.66. An additional accession, genotype 82, grouped away from the rest of the accessions with similarity of only 0.30. Overall, the results indicate moderate genetic diversity in the spinach accessions.

The whole dendogram and its individual clusters are shown in figures (Figure 3.4, Figure 3.5 and Figure 3.6).

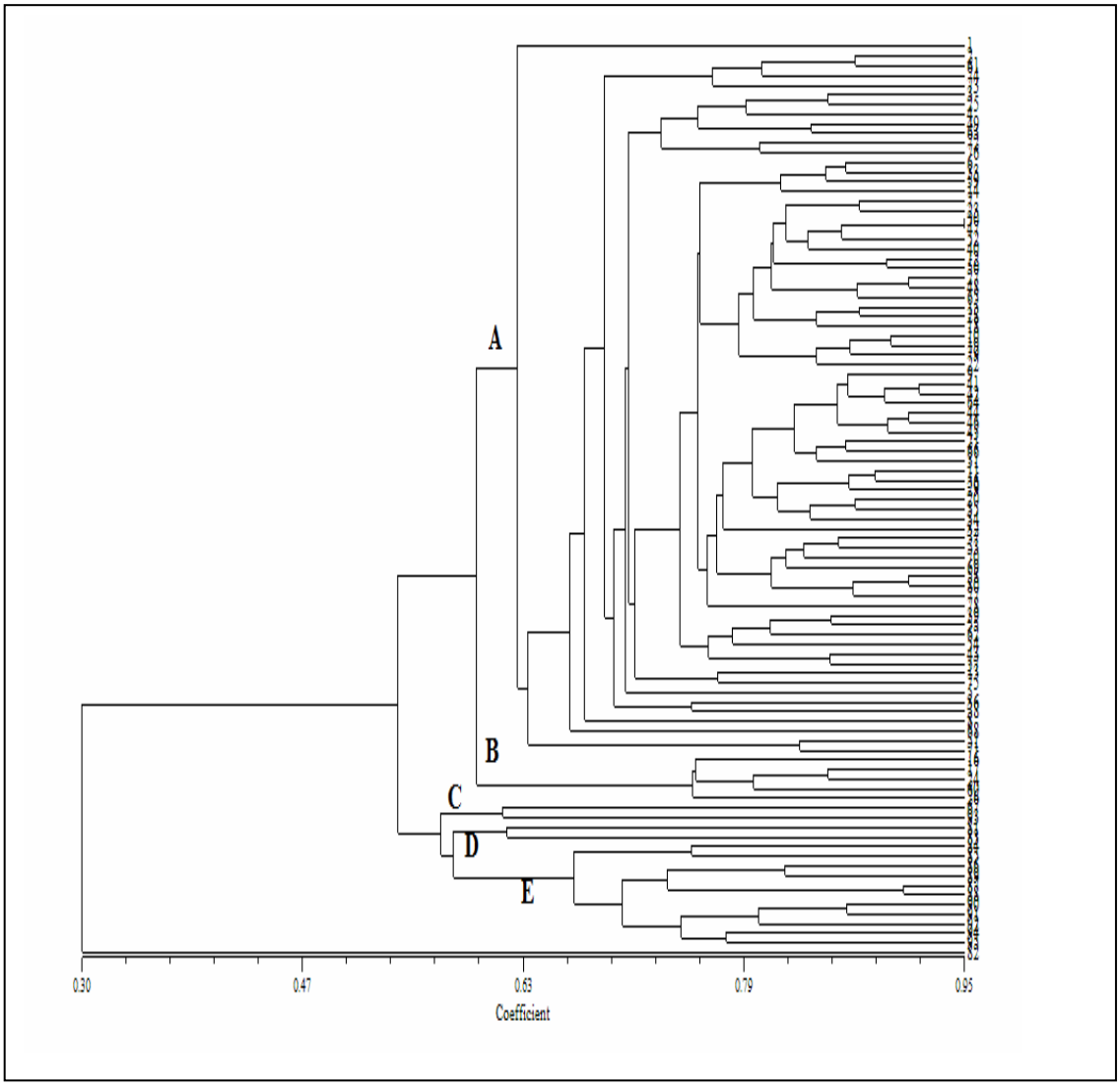

Figure 3.4. Whole dendogram with its individual clusters.

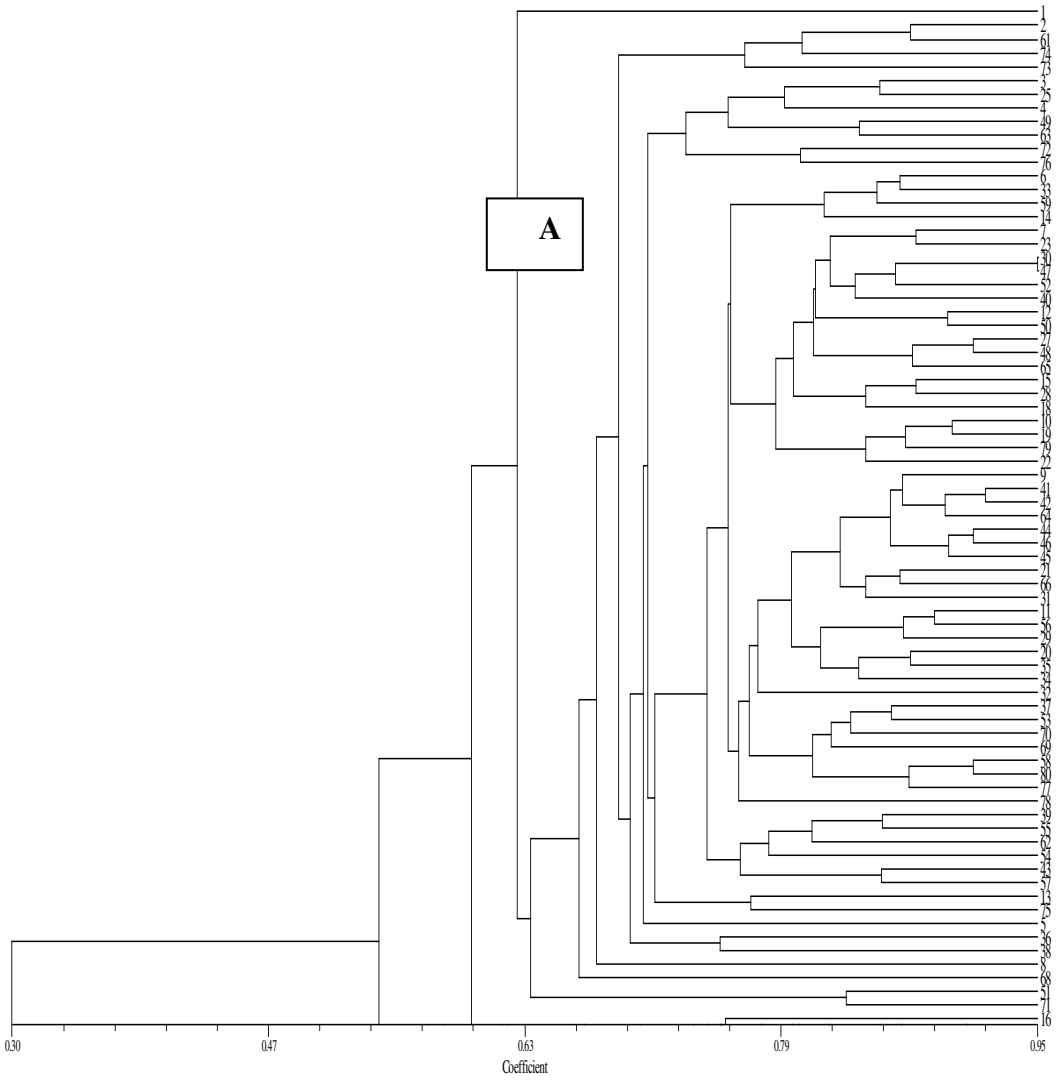

Figure 3.5. Cluster A and its accessions

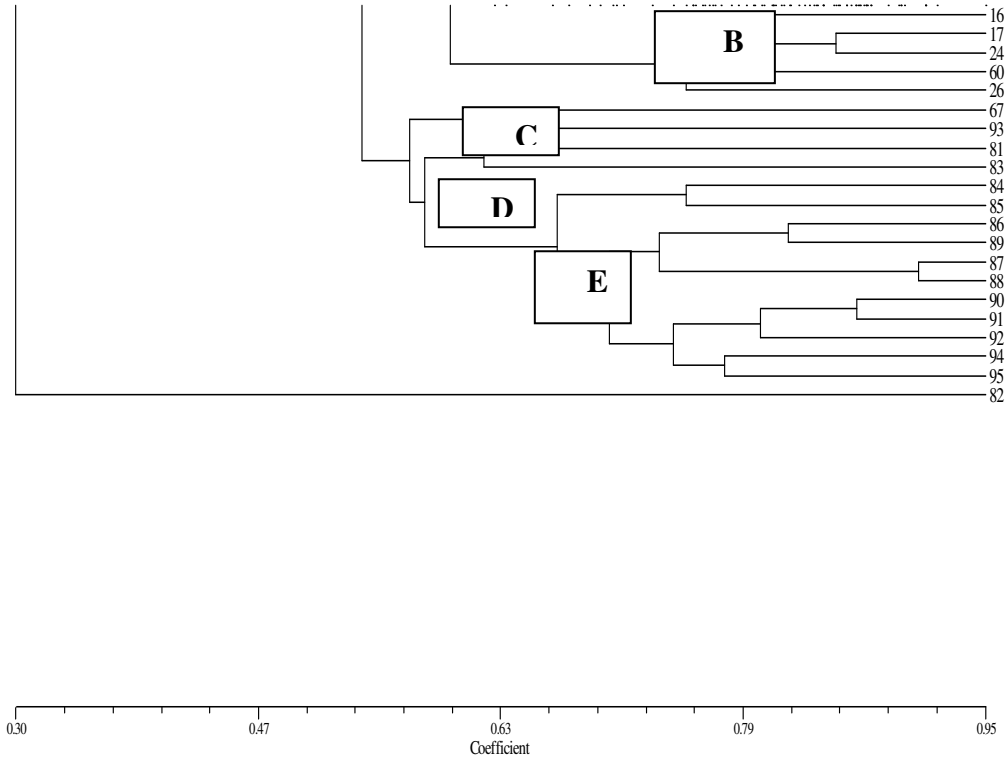

Figure 3.6. Clusters B, C, D and E and their accessions.

PCA (Principal Component Analysis) was also used to examine the molecular diversity of the spinach accessions. PCA is a summary of high dimensional genetic data into plots with minimum loss of the data. For our data, the first, second and third components accounted for 49.79%, 6.71% and 3.80% of the total variance, respectively. 2D and 3D plots of the PCA analysis gave only one group of accessions. This cluster had more accessions than the others (Figure 3.7 and Figure 3.8.). The remaining accessions were more diverse. The accessions in this cluster are thought to be genetically more similar accessions. It was seen that some genotypes were nearly the same because they plotted nearly on top of each other. This analysis pinpointed the similar accessions which could be eliminated from the Turkish spinach genebank as they are redundant germplasm and it may not be necessary to conserve them if resources are limited. On the other hand, the diverse accessions that were seen on the plot should be conserved in the genebanks for future studies. Overall, it was seen that SRAP markers are suitable for molecular diversity analyses in spinach.

Other spinach studies were done using SSR marker systems for finding a sex determination locus. In addition, the TRAP marker system which requires sequence information for finding genetic diversity was applied in commercial hybrids (Hu, et al.

2007). TRAP was also found to be a reliable marker to assess the diversity among germplasm. Both of them can be useful because they are generated from plant sequence data.

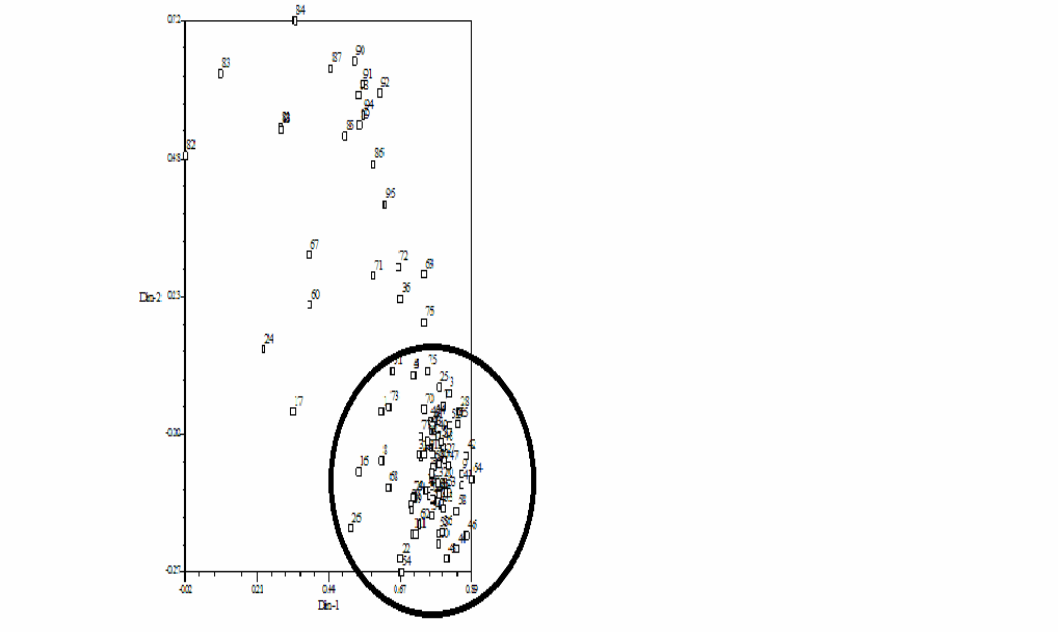

Figure 3.7. 2D PCA plot

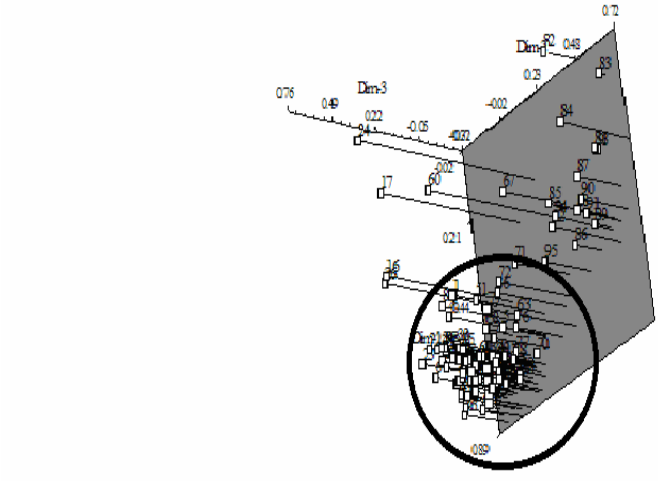

Figure 3.8. 3D PCA plot

CHAPTER 4

CONCLUSION

Spinach *(Spinacia oleracea L.)* which originated from Persia is a very important source of nutrients and is dispersed throughout Turkey and all over the world. This plant material was chosen because there are not many studies on spinach. Our aim was to see the degree of genetic diversity among Turkish cultivars and for this aim SRAP marker system was used. This system provided many advantages such as simplicity, reasonable throughput rate, and similar properties of other dominant markers (Li, et al. 2000). In addition, this is the first time that SRAP markers were used to determine genetic diversity in spinach cultivars. A total of 95 spinach accessions were collected from Wageningen University, The Netherlands which also provided morphological data for the accessions. The dendogram analysis for the molecular marker data showed five groups: A, B, C, D and E. These results were supported by the PCA test and its plots because of their good distribution through the plot. Multivariate PCA analysis of morphological data was performed for 12 parameters and the analysis showed good separation of the accessions on the plot. In other words, there were no apparent clusters on the plot.

Our study provided information that may be useful to geneticists and breeders who wish to find the most morphologically or molecularly distinct spinach accessions. For germplasm collections, the results of this study may help to conserve more distinct accessions and to eliminate similar accessions from the Turkish spinach genebank to save limited resources. In future studies, a breeder may select two distinct accessions and hybridize them to create a new line with desired properties such as high antioxidant level, pathogen resistance etc.

In conclusion, it was seen that the SRAP marker system was suitable to assess the genetic diversity between spinach cultivars. It should be emphasized that this study was the first and only study that has used SRAP marker systems for determination of molecular genetic diversity of spinach accessions.

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