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MASTER THESIS

NIĞDE ÖMER HALISDEMIR UNIVERSITY
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T.R.
NIĞDE ÖMER HALISDEMIR UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL GENETIC ENGINEERING

MOLECULAR CHARACTERIZATION OF *VACCINIUM* SPECIES FROM TURKEY

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Supervisor

Prof. Dr. Sedat SERÇE

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Olivet Delasi GLEKU tarafından **Prof. Dr. Sedat SERÇE** danışmanlığında hazırlanan "**Molecular Characterization of *Vaccinium* species from Turkey**" adlı bu çalışma jürimiz tarafından Niğde Ömer Halisdemir Üniversitesi Fen Bilimleri Enstitüsü **Tarımsal Genetik Mühendisliği Anabilim Dalı**'nda Yüksek Lisans (İngilizce) tezi olarak kabul edilmiştir.

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Prof. Dr. Murat BARUT
MÜDÜR

THESIS CERTIFICATION

It is certified that I have written this thesis by myself. I further confirm that the contents of this thesis write-up were obtained and presented within the framework of scientific and academic rules, and that this study was prepared in accordance with the university's rules of writing thesis, all expressions and information that are not mine are duly cited.

Olivet Delasi GLEKU

SUMMARY

MOLECULAR CHARACTERIZATION OF *VACCINIUM* SPECİES FROM TURKEY

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Blueberry production has recorded an accelerated rise in production among all small fruit species. The production is mainly conducted with three species which are all native to America: 1) *Vaccinium corymbosum* L. (highbush blueberry); 2) *V. ashei* Reade (rabbit eye blueberry); and 3) *V. angustifolium* Ait. (lowbush blueberry). Most blueberry cultivars have several *Vaccinium* species in their background. Turkish flora has several *Vaccinium* species like *V. arctostaphylos* L., V. *myrtillus* L., *V. vitis-idaea* L., *V. uliginosum* L. exhibiting potential for utilization in cultivar development. In this study, genotypes of *V. arctostaphylos*, *V. myrtillus, V. uliginosum* species and genetic diversity of 'Jubilee' and 'Misty' varieties were investigated with iPBS marker system. A total of 10 primers yielded 274 bands 271 of which were polymorphic. In the dendrogram obtained as a result of the research, these three species separated clearly. *Vaccinium arctostaphylos* and *V. myrtillus* were found closer to each other as compared to *V. uliginosum* species. 'Jubilee' and 'Misty' varieties showed the highest similarity with *V. myrtillus.* These results shed light on the diversity and evolution of related species and their use in blueberry breeding.

Keywords: Vaccinium, Blueberry, Molecular Characterization, Polymorphism, Germplasm

ÖZET

ÜLKEMİZDEN ÖRNEKLENEN *VACCİNİUM* TÜRLERİNİN MOLEKÜLER KAREKTERİZAZYONU

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Tarımsal Genetik Mühendisliği Anabilim Dalı

Danışman : Prof. Dr. Sedat SERÇE

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Maviyemiş üretimi, tüm üzümsü meyve türleri üretimi arasında en hızlı artış gösteren üretimdir. Üretim esas olarak tamamı Amerika'ya özgü üç tür ile gerçekleştirilmektedir: 1) *Vaccinium corymbosum* L. (yüksek çalı maviyemiş); 2) *V. ashei* Reade (tavşan gözü maviyemiş); ve 3) *V. angustifolium* Ait. (alçak çalı maviyemiş). Çoğu maviyemiş çeşidinin ebeveynleri arasında birkaç *Vaccinium* türü bulunmaktadır. Türkiye florasının, kültür gelişiminde kullanım potansiyeli sergileyen *V. arctostaphylos* L., *V. myrtillus* L., *V. vitis-idaea* L., *V. uliginosum* L. gibi çeşitli *Vaccinium* türleri vardır. Bu çalışmada, *V. arctostaphylos, V. myrtillus, V. uliginosum* türlerine ait genotipler ile 'Jubilee' ve 'Misty' çeşitlerinin genetik çeşitliliği iPBS markör sistemi ile araştırılmıştır. 10 primer kullanılan çalışmada 271 tanesi polimorfik toplam 274 band skorlanmıştır. Araştırma sonucunda elde edilen dendrogramda bu üç tür ayrı gruplar oluşturuştur. *Vaccinium arctostaphylos* ve *V. myrtillus, V. uliginosum* türü karşılaştırmasına göre birbirine daha yakın bulunmuştur. 'Jubilee' ve 'Misty' çeşitleri ise en yüksek benzerliği *V. myrtillus* türü temsilcisi bireyleri ile göstermiştir. Bu sonuçlar, ilgili türlerin çeşitlilik ve evrimleri ile maviyemiş ıslahında kullanımları konusunda ışık tutmaktadır.

Anahtar Sözcükler: Vaccinium, Maviyemiş, Moleküler Karekterizasyon, Polimorfizm, Gen Kaynakları

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Blueberry production has recorded an accelerated rise in production among all small fruit species. Most blueberry cultivars have several *Vaccinium* species in their background and the Turkish flora has several *Vaccinium* species like *V. arctostaphylos* L., V. *myrtillus* L., *V. vitis-idaea* L., *V. uliginosum* L. exhibiting potential for utilization in cultivar development. *Vaccinium* species have enormous benefits. These berries are rich in anti-oxidant compounds, they can alter the lipid metabolism, beneficial for dietary purposes, and are rich in potassium and fiber. They are also beneficial in dealing with cardiovascular and urinary diseases, and can improve brain function and cognitive ability. Many *Vaccinium* species are as well used for ornamental purposes. Due to their diverse usefulness, they have become relevant in breeding programs. This thesis molecularly characterized some genotypes of wild *Vaccinium* species in the Turkish flora to ascertain their molecular properties which can further the course of breeders.

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

SYMBOLS AND ABBREVIATIONS

Symbols	Descriptions
%	Percentage
°C	Degree Celcius
cm	Centimetre
m	Metre
µl	Microlitre
bp	Base Pair
min	Minutes
h	Hours
s	Seconds
gr	Grams
kb	Kilo Base
rpm	Runs per minute
ml	Milliliter
ng	Nanogram
pg	Picogram
V	Voltage

Abbreviations	Description
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
ESTs	Express Sequence Tags
EST-PCR	Express Sequence Tag-Polymerase Chain Reaction
GS	Genome Size
iPBS	İnter Primer Binding Site
LTR	Long Term Repeat
LARD	Large Retrotransposon Derivative

NTSYS	Numerical Taxonomy and Multivariate Analysis System
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PBS	Primer Binding Site
RAPD	Random Amplified Polymorphic DNA
RFLPs	Restriction Fragment Length Polymorphisms
SAS	Statistical Analysis System
SSR	Simple Sequence Repeat
TRIM	Terminal Repeat Retrotransposons in Miniature
tRNA	Transfer Ribonucleic Acid
UPGMA	Unweighted Pair Group Method using Arithmetic Average
USDA	United States Department of Agriculture

CHAPTER I

INTRODUCTION

Molecular characterization is a study that seeks to describe the distinct nature of molecules in a species (in this case *Vaccinium* species) without necessarily considering the environmental, developmental and physiological state of the species. It can simply be referred to as the classification of species at molecular levels.

Vaccinium species are of the primeval genus of shrubs found in the Ericaceae plant family. Although there are assorted *Vaccinium* species of marketable value, major productions come from the species in the *Cyanococcus* section such as, the highbush blueberry (*V. Corymbosum* L.), lowbush blueberry (*V. angustifolium* Ait.), and rabbit eye blueberry (*V. ashei* R. syn. *V. virgatum* Ait.). The other valuable relations of the *Vaccinium* species (blueberries) are species of cranberries (*V. macrocarpon* Ait.) from the *Oxycoccus* section, lingonberry (*V. vitis idaea* L.) which belongs to the *Vitis-idaea* section, bilberry (*V. myrtillus* L.) from *Myrtillus* section, and lastly the Caucasian whortleberry (*V. arctostaphylos* L.) which is a member of the *Hemimyrtillus* section. Whereas these crops are globally popular, in many instances, their individual spread are quite narrow. Almost all *Vaccinium* plants require acidic soils for growth and as wild plants they live in habitats such as bog and acidic woodlands. Many *Vaccinium* species have edible fruits and are cultivated for commercial use (Çelik 2009).

Over 450 varied species are contained in the *Vaccinium* genus however few are for human consumption, such as; lowbush blueberry, cranberry, bilberry, bearberry (*V. erythrocarpum* Michx.), rabbit eye blueberry, Blue Ridge blueberry (*V. pallidum* Ait.), and Caucasian whortleberry (Özgen et al., 2014). All *Vaccinium* species are perennial, exhibiting both self and cross-pollination abilities and have small and pulpy berries as fruits which are edible in many instances (Song et al., 2011). Most *Vaccinium* species naturally prefer the cool regions of the Northern hemisphere hence abounding in Europe, Asia and North America whereas non-existent in New Zealand, Australia and most parts of Africa; yet some species are also present in tropical regions such as Madagascar or Hawaii (Powell et al., 2002). Global berry fruits consumption is

increasing daily and currently several species of the genus *Vaccinium*, commonly known as blueberries, form part of most berry diets.

The recent rise in interest for these species are due to the numerous health properties that are related to them. These berries are rich in anti-oxidant compounds, they can alter the lipid metabolism, beneficial for dietary purposes, and are rich in potassium and fiber (Carbone et al., 2008). They also have beneficial effects on cardiovascular and urinary diseases, and can improve brain function and cognitive ability (Szajdek et al., 2008). Many *Vaccinium* species are of ornamental relevance as well (Retamales and Hancock, 2012).

The major type of cultivated blueberries *Vaccinium corymbosum* was developed in the last century (Ballington, 2001) and several other *Vaccinium* species have only been recently domesticated (Hancock et al., 2008; Česonienė et al., 2013). Interspecific hybridization has been a productive breeding strategy to boost *Vaccinium* crops (Sakhanokho et al., 2018). There are conservation projects involving wild *Vaccinium* to preserve traits that are potentially useful for breeding purposes, such as disease resistance, winter hardiness, low chilling requirement, adaptation to high pH soils, early ripening, or late bloom among others (Ehlenfeldt and Ballington, 2012).

Species in the genus *Vaccinium* show a certain level of evolutionary complexity looking at their karyology with species having many ploidy levels derived from a single base chromosome number, x = 12. *Vaccinium* species exist in nature as diploids through to hexaploids, however they also have some as autoploids and allopolyploids. Interspecific triploids, pentaploids, octoploids and nanoploids have been utilized for domestication purposes (Vorsa and Ballington, 1991). The most extensively studied of the *Vaccinium* species are those from section *Cyanococcus* (including different ploidy levels) and section *Oxycoccus* (only diploids) (Lobos and Hancock, 2015). Other sections such as *Myrtillus* and *Vitis-idaea* (only diploids), section *Hemimyrtillus*, including *V. arctostaphylos* (only tetraploids), and section *Vaccinium*, including *V. uliginosum* L. (different ploidy levels) have also been largely researched into (Retamales and Hancock, 2012). Chromosome numbers are relatively well documented for *Vaccinium*, an example is the Chromosome Counts database (Rice et al., 2015) where there is available information on more than 100 species of the genus.

Fruits, particularly those with wild backgrounds show great morphological and biochemical diversity. They are a rich source of organic acids, sugars, fibers, minerals, etc. Wild edible fruits are also rich in ascorbic acid, tocopherol, anthocyanins, phenolics, and carotenoids such as β-carotene and the presence of these compounds contribute significantly to their possession of antioxidant properties. Among fruits, berries are one of the richest groups which serve as powerful antioxidants due to the wide variety of anthocyanins and high phenolic contents. Red, blue, purple and black coloured berries indicate high anthocyanins and they exhibit a wide variety of biological activity and promote health (Milivojevic et al. 2012).

Presently, the interest of consumers in *Vaccinium* species has risen due to increasing relevant proofs of health benefits and antioxidant properties of these berries. Health benefits connected to the consumption of some *Vaccinium* species have been identified over centuries gone by, but their anticancer, antioxidant, cardio protective, and other bioactive properties have only over the past decade been scientifically proven (Monavar et al. 2011). The anthocyanins and proanthocyanidins are some of the specific polyphenols that have been reported as the bioactive ingredients of the *Vaccinium* species (Stintzing et al. 2002). Identification and quantification of anthocyanins, phenolics and antioxidant properties of *Vaccinium* species are well defined (Çelik et al. 2008). The naturally occurring phenolic compounds in *Vaccinium* species are redox-active antioxidants as well as iron chelators and are found in red, blue and purple coloured flowers, fruits and vegetables. The usual daily dietary intake of anthocyanins is approximately 200 mg (Zafra-Stone et al., 2007). Together with the growing popularity of wild berry consumption, cultivation area of berries will broaden as well as breeding programs (Koca and Karadeniz, 2009). Pomological features of berries are highly influenced by the species and variety within species and the ecological conditions of the plants (Mikulic-Petkovsek et al., 2014; Veberic et al., 2015).

Blueberries became well known around the world due to the high levels of phenolic compounds and that made it the world's number one small fruit presently (Kim et al., 2010; Routray et al., 2014). These compounds found in the blueberries have been reported to have numerous valuable health benefits including superb antioxidant, anti-hypertensive, anti-diabetic, anti-leukemia, anti-obesity, anti-inflammatory, and anti-microbial activity, as well as neuroactive properties, to protect against cancer and stroke

(Ehlenfeldt and Prior, 2001; Deng et al., 2014). Blueberries are considered to be one of the richest sources of phenolic compounds and antioxidant phytochemicals among fruits and vegetables, and they contain significant levels of anthocyanins, flavonols, flavonons, proanthocyanidins, and phenolic acids (Castrejón et al., 2008; Wang et al., 2012).

Genetic differences, the cultivar type, growing location and season, agronomic factors, the degree of maturity at harvest, and postharvest storage conditions are some factors that have an impact on the total phenolic content, total anthocyanins, and the antioxidant capacity of blueberry fruit and leaves (Ehlenfeldt and Prior, 2001; Deng et al., 2014). For the past two decades since their potential role in the prevention of chronic diseases was realized they have received much attention in research into antioxidant studies as per their high levels of beneficial nutrients and bioactive phytochemicals, especially anthocyanins. Investigations of these non-nutrient biologically active compounds have revealed that wild or improved blueberry varieties with high quality phenolics are associated with high antioxidant activity. Phenolic contents of *Vaccinium* berries (e.g. anthocyanins, flavonoids, coumarins, lignans and benzoic acids) are well documented in the literature (Yuan et al., 2011) and many studies have investigated the contents and composition of the phenolic acids, anthocyanins and flavonoids of *Vaccinium* berries (Su, 2012). Anthocyanins are one of the major constituents in blueberries and are responsible for their red, blue, purple and black colours.

The health benefits of *Vaccinium* berries, and in part of the leaves, attributable to their polyphenols and high natural anthocyanin contents include reducing the risk of coronary heart disease, inhibiting platelet aggregation, protecting arterial endothelial cells, reducing the risk of cancer and inflammatory disorders, modulating the immune system, enhancing eye function and retarding neurological disorders (Primetta et al., 2013). Apart from the health benefits, the berries of *Vaccinium* are an important source of food colorants and pharmaceutical ingredients (Li et al., 2011).

However, there are still many knowledge gaps regarding the evolution and systematic variation in this genus, which are relevant for conservation programs and development of new cultivars. A proper molecular characterization of the *Vaccinium* species will go a

long to provide a linking bridge to close part of the knowledge gap and this is what this research seeks to achieve.

CHAPTER II

REVIEW OF LITERATURE

2.1 Taxonomy of the Genus *Vaccinium*

Vaccinium is a major finance generating genus in the Ericaceae family with about 30 different sections and 450 species (Vander Kloet and Dickinson, 2009; Trehane, 2004). Ericaceae is a family in the order Ericales of flowering plants growing mainly in acid and infertile conditions. Their growth in this kind of environment is due to the fact that the species possess mycorrhizal fungi which assists them in extracting nutrient from the infertile soils and evergreen foliage that helps in conserving the absorbed nutrients (Keddy, 2007). South America is considered the origin of this genus nevertheless species are spread in several other regions of North America, Europe, and Asia (Vander Kloet, 1988).

Blueberry, an economically pertinent and carefully considered species belong to the section *Cyanococcus*. It has a varying taxonomical group mostly with genotypes from assorted species like *V. corymbosum* L. (highbush blueberry), *V. angustifolium* Aiton. (lowbush blueberry) and *V. virgatum* Aiton. (rabbit -eye blueberry). Section *Cyanococcus* however has no proper species partitioning hence under natural circumstances there exists a notably incessant interspecies hybridization (Qu and Hancock, 1995). Blueberry cultivars of marketable value are mostly hybrid in origin (Qu and Vorsa, 1999; Brevis et al., 2008).

Some other species of the genus with market value but not popularly cultivated include the bilberry (*V. myrtillus* L., 2x, Section: *Myrtillus*), lingonberry (*V. vitis-idaea* L., 2x Section: *Vitis-idaea*), Caucasian whortleberry (*V. arctostaphylos* L., 4x, section: *Hemimyrtillus*) and bog bilberry (*V. uliginosum* L., 2x, 4x, 6x, Section: *Vaccinium*). These sections are geographically distributed along the high latitudes of the northern hemisphere with species that both self and cross-pollinate (Retamales and Hancock, 2012).

2.2 Habitat and Geographical Distribution

Species of the genus *Vaccinium* are "acid-loving" growing in a pH of 5.8 or less. Species are diversely distributed in their habitats based mainly on the section they belong to (Vander Kloet, 1988). Most blueberry species are native to North America and require chilling of several hundreds of hours to enhance cultivation. This requirement has however been reduced by hybridizing the northern blueberry species with the southern ones. Blueberries require good drainage even though they can grow in infertile soils (Retamales and Hancock, 2012).

Pine and spruce heath forests, plateau areas near mountains and old peat bog areas in Europe, North America, Greenland and northern part of Asia are the natural growth areas of bilberry (*V. myrtillus)* (Vander Kloet, 1988). They can be propagated sexually by the seeds and asexually by rhizomes (Zoratti et al., 2015). Bog bilberry *(V. uliginosum)* also normally grows in cooler parts of the northern hemisphere in similar environments like the bilberry or in bog environments (Wang et al., 2014). The Caucasian whortleberry (*V. arctostaphylos)* is majorly spread across Iran, Turkey, Caucasus, Bulgaria and regions around this area. They grow preferably in forests with fagus, firs, pine or *Rhododendron* in highly elevated areas with enough rain (Nickavar and Amin, 2004).

2.3 Importance of *Vaccinium*

Humans for over several thousands of years have had an intuitive understanding of the relevant benefits they can derive from *Vaccinium* species hence included it in their diets and medicines (Cseke et al., 2016). Local people across the globe have picked *Vaccinium* species (blueberries) from nature before they were domesticated about the1900s. These berries were mostly enjoyed as fresh fruit or processed into jam, juice, pies, jelly or wine (Çelik, 2012).

For almost two decades there has been a drastic rise in the demand and consumption of this berry as a result of the discovery of the several medicinal and nutritional properties it possesses and it has been called by many as a super food (Mudd et al., 2013). Anthocyanin, carotenoids, flavonoids, polyphenols, galactosides, glucosides, and low

amounts of ascorbic acids among others are some rich and beneficial secondary plant metabolites contained in fruits of *Vaccinium* species (Moyer et al., 2002; Nickavar and Amin, 2004).

There are substantive evidences from research indicating that the natural components of blueberries have high beneficial relevance to the human body in taking care of their health. These components have anticancer, antioxidant and antidiabetic abilities which help curtail illnesses like cancer, diabetes among others (Trehane, 2004; Mudd et al., 2013). Moreso, these compounds have produced desired results when used in handling issues related to cardiovascular diseases, improving brain function and cognitive ability as a substitute of artificial drug or medicine. Further research in this area is still ongoing (Mudd et al., 2013; Hou, 2003).

Modern science has come up with biochemical defences to the health upgrading properties of *Vaccinium* species. Pharmaceutical industries have included these berries in nutraceuticals (functional food) or dietary supplements and in recent times, there are over 180 *Vaccinium* phytopharmaceutical products available globally (Cseke et al., 2016). The phenolic acid content of some choice Caucasian whortleberry species were ascertained by Ayaz et al. 2005. Seven hydroxybenzoic acids and four hydroxy cinnamic acid derivatives were identified of which Caffeic acid was the most abundant phenolic acid. The prime anthocyanins according to Latti et al. 2009 were delphinidin (41%), petunidin (19%) and malvidin (19%).

The United States produces half of the world's total production of blueberry and cranberry and making great economic gains and improving the nation's economy (FAOSTAT, 2017). Even most profitable cultivars of blueberries are developed and patented by this country. Presently, production of the berry fruits has increased globally and common in some other countries of South America and Europe. (Lobos and Hancock, 2015).

2.4 Cultivation of Vaccinium

Vaccinium species are propagated using viable seeds, canes or rhizomes. Blueberries and cranberries are the two main *Vaccinium* crop plants cultivated for profit generating

purposes. The initial domestication of blueberries were carried out by the US Department of Agriculture (USDA) in 1908 from the indigenous shrubs of *Vaccinium corymbosum* L. 4x Section: *Cyanococcus* (highbush blueberry) (Lyrene, 2008). There are two types of highbush blueberry cultivars, the northern and southern type. Blueberries generally have chilling requirements (exposure of plants to required cold temperatures and duration before the plants break dormancy), the southern type as compared to the northern type have less chilling requirement, winter hardiness and wider adaptability. The primary southern highbush blueberry cultivar came into being by cross-breeding the northern type with distinct native evergreen species of *V. darrowi* camp, 2n = 2x Section: *Cyanococcus* and was launched in the 1950s in Florida (Sharpe and Darrow, 1959). Wild *Vaccinium* species of several distinct origins have been crossed with the northern highbush cultivars to integrate profitable genes and increase their versatility (Williamson and Darnell, 1997). 'Jubilee' is an example of a financially beneficial southern highbush variety that was released in 1994 by USDA and 'Misty' by the University of Florida in 1992 (Spiers et al., 1996).

For cranberries however, the preliminary cultivation record dates back to 1816 in Cape Cod Massachusetts. Cranberries of the *Oxycoccus* section were first domesticated from wild species in Cape Cod Massachusetts (Trehane, 2004). Their disease and pest resistance properties has resulted in the selection of over 100 different clones from the wild species for breeding purposes (Hancock et al., 2008).

In recent times, the global interest of researchers has also been in the area of species from the other sections of the order Ericales like *Myrtillus*, *Vaccinium*, and *Hemimyrtillus* (Wang et al., 2014). Species from these groups are being gathered and safeguarded in several countries of Europe in addition to that of the United States. The whortleberry (*V. arctostaphylos*) of section *Hemimyrtillus* is regarded a tertiary gene pool for blueberry crop improvement because it possesses a relevant adaptive trait, the ability to produce fertile offsprings from being crossed with species from section *Cyanococcus*. (Ballington, 2001; Ehlenfeldt and Ballington, 2012).

2.5 Markers

Molecular markers are very relevant in breeding, biodiversity applications, forensics and map-based cloning of genes as a result of their use in giving recognition to a specific sequence of DNA in a pool of unknown DNA. Restriction fragment length polymorphisms (RFLPs) was the initial marker used to study the natural genetic variations among *Vaccinium* species, to come up with the levels in which the mitochondrial DNA can be sorted out and to differentiate between the various high-bush blueberry cultivars (Haghighi and Hancock, 1992). After the initial study with (RFLPs), other PCR centered molecular markers have been used in blueberry like the random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and express sequence tag-polymerase chain reaction (EST-PCR) markers (Levi and Rowland, 1997; Dhanaraj et al., 2004; Rowland et al., 2010;). Sequencing technologies have seen great improvements over the last decade with the inception of several thousands of expressed sequence tags (ESTs) and a few hundred SSRs markers and as a result breeders and researchers have easy access to transcriptome sequences (Dhanaraj et al., 2007; Bian et al., 2014). The marker study was aimed at establishing the genetic linkage map of relevant genes responsible for cold hardiness, disease resistance, pest resistance, tolerance to high pH conditions and other relevant revenue generating breeding traits (Dhanaraj et al., 2004; Rowland et al., 2012; Bian et al., 2014). Schlautman et al. (2017) investigated the creation of a composite map from a high density multigene pedigree connecting mapping by sequencing genotypes.

2.6 The iPBS Approach

The iPBS amplification procedure requires the availability of a tRNA complement as a reverse transcriptase primer binding site (PBS) in LTR (long term repeat) retrotransposons. The iPBS amplification procedure is germane to endogenous retroviruses and retroviruses, *Gypsy* and *Copia* LTR retrotransposons and also to non-autonomous LARD and TRIM elements, both in the plant and animal kingdom as opposed to erstwhile retrotransposon isolation procedures. This approach has proven to be a robust technology when it comes to DNA fingerprinting even for a novice in DNA sequencing (Kalendar et al, 2010).

The iPBS procedure is an answer to the challenges encountered in erstwhile procedures with its ability to both isolate LTR retrotransposons in almost all organisms and its outright use as a general marker system. Generally, retroviruses and LTR retrotransposons of cellular tRNAs both use iPBS as primers for reverse transcription in their duplicating stages. The tRNA attaches to the primer binding site (PBS) next to the 5_ LTR and primes synthesis of minus-strand cDNA by reverse transcriptase (Kalendar et al, 2010). Instead of using a tRNA primer, *Tf1*/*sushi* group of fungi and vertebrates and *Fourf* in maize represent a special class of LTR retrotransposons that are able to self-prime cDNA synthesis (Hizi 2008). To quickly see polymorphism between individuals, rapidly clone LTR segments from genomic DNA, and to conduct in silico database searches, a complete set of the safeguarded portions of PBS sequences are to be utilized. All organisms with retrotransposons containing PBS sites complimentary to tRNA can employ this procedure for apt results (Kalendar et al, 2010).

The iPBS marker system has the following advantages over other marker systems; it has the capacity to project large regions of plant genomes, the procedure is easy to perform and can be used for any organism. It is a cheap procedure to perform since it requires just the basic laboratory facilities to conduct and for fingerprinting and genetic similarity studies in plants, they have been proven to be a sturdy marker system (Guo et al., 2014).

2.7 *Vaccinium* Gene Resources in Turkey

Vaccinium arctostaphylos L. (whortleberry), *Vaccinium myrtillus* L. (bilberry), *Vaccinium uliginosum* L. (bog bilberry) and *Vaccinium vitis-idaea* L. (cowberry or lingonberry) are the four species of the genus *Vaccinium* present in Turkish flora (Davis, 1978). Properties of the different *Vaccinium* species in the Black Sea Region of Turkey have been reported by (Baytop, 2004, Ebcioğlu, 2009; Ekim, 2007; Güner, 2012; Karol et al., 2000; Sarıbaş, 2010; Tekin, 2007; Torlak, et al. 2010).

V. arctostaphylos:

The forests of the North-eastern Black Sea region most predominantly, Rize is home to *V. arctostaphylos* a perennial and deciduous plant with purple-black blackberries. Growing in acidic and rainy soils these species naturally abound in this region as it

possesses this requirements richly (Ozgen et al., 2014; Figure 2.1). *Vaccinium arctostaphylos* contains high phenolic compounds and anthocyanins hence it is used for medicinal purposes. It gained popularity and is used in Turkish local medicine as an antidiabetic and antihypertensive agent (Baytop, 1999). Turkey has an immense variety of *V. arctostaphylos* and some studies have been carried out to examine some morphological and biochemical properties of its content (Capocasa et al., 2008).

Tea currants, locally known as; "Ayı Üzümü" ,"Anatolian grass", "hunter grape", "mehobah", "libade", "lifar", "lifor", "ligarba", "likaba","likapa","lycarba","forest lifor","forest ligarb", "Gamber cornflowers", or "Trabzon tea". Other areas of natural growth of these species in the Black Sea region include; Artvin, Trabzon, Ordu, Giresun, Samsun, Kastamonu, Zonguldak, Bartın, Sinop, Ardahan, Gümüşhane, Bayburt, Karabük, Düzce, Sakarya, Bolu, Kocaeli, Yalova, Çanakkale, İstanbul, Balıkesir, Bursa and Kırklareli (Davis, 1978; Çelik, 2008). This fruit with a high antioxidant content is picked from nature and eaten as fresh fruit, jam, marmalade, dried fruit or juice according to the desire of the local people (Özgen et al., 2014). The plant has smooth shoots which can grow 2-3 m long, dark red, green and stained or unspotted in colour, has large bright green leaves and straight edges. The flowers are hermaphoditic in nature, multicoloured (white, red, pink stripes) and bell-shaped with round black or dark blue fruits (Çelik, 2008; Çelik, 2009; Çelik, 2011 and 2012 a and b).

Figure 2.1. General view of flowers, fruits and plants of *V. arctostaphylos*

V. myrtillus:

The shepherd grapes growing together with rhododendron and spreading juniper in the Eastern Black Sea Region or alone cover the area where they are formed by forming rhizome. Known in Europe as “Bilberry”, “Alpine bilberry” or “European blueberry”. Shepherd grapes are popularly known as “bush blossom”, “gara gilik”, “currants”, “hencoyik”, “lifora”, “liforza”, “blueberry”, “highland liforu”, “highland lycopera”, “ground ligar”, or “ground liforu” (Figure 2.2). Naturally it is spread across the provinces of Artvin, Rize, Trabzon, Ordu, Giresun, Bayburt, Erzurum-Şenkaya, Gümüşhane, Ardahan, Kastamonu-Ilgaz Mountain, Bursa-Uludağ and Balıkesir (Davis, 1978; Çelik, 2008). It is a perennial, 10-60 cm tall, dwarf and has thin bushes. It also has spreader-crawler properties and sheds its leaves in winter, the edges of the leaves are indented-protruding and toothed, the leave is bright green and the lower face is covered with sparse veins. Blossoms occur individually or in pairs on the leaf seat and the fruits are round, hazy blue in colour. (Çelik, 2012 a and b).

Figure 2.2. General view of flowers, fruit and plants of *V. myrtillus*

V. uliginosum:

V. uliginosum, a less well-known species is spreading in Rize, Trabzon, Giresun-Karagöl, Gümüşhane and Bursa Uludağ of the Turkish flora (Çelik, 2012; Figure 2.3). It is a perennial deciduous shrub and a wild plant of great market value naturally growing in the northern regions of Turkey, Europe, North America, and some parts of Asia (Jacquemart, 1996). Fruits of *V. uliginosum* possess high quantities of anthocyanin (glucosides, galactosides, and arabinosides of delphinidin, peonidin, petunidin, cyanidin, and malvidin), flavonols (galactosides of myricetin, quercetin, and syringetin; glucosides of quercetin and syringetin; and rhamnosides and arabinosides of quercetin), and flavonoids (Cüce and Sökmen, 2017).

Figure 2.3. General view of flowers, fruit and plants of *V. uliginosum*

V. vitis-idaea:

Species of *V. vitis-idaea* are evergreen shrubs located in Rize in the mountains of Kaçkar. The cultured blueberry were introduced to Turkey in the 2000s (Çelik, 2012; Figure 2.4). Lingonberries have several nutritional benefits hence are consumed on a large scale in the human diet, its leaves also possess preventive and treatment properties for urinary tract infections, stomach disorders, rheumatic diseases and hypercholesterolemia (Bujor et al 2018).

Figure 2.4. General view of flowers, fruit and plants of *V. vitis-idaea*

2.8 Use of Gene Resources in Breeding Programs

Various varieties of *Vaccinium* have contributed to the development of blueberry varieties. There are many types of *Vaccinium* that have adapted to different ecological conditions of the world, originating in different regions and at different ploidy levels. Of these, the species in the *Cyanococcus* section are particularly important. The species involved can be crossed with each other; even different levels of ploidy do not interfere.

Many researchers have used the species in the *Cyanococcus* breeding program through hybridization (Hancock et al., 2008, Table 2.1). 'US 75' and 'Fla 4B' genotypes developed by cross-species hybridization studies and found in the genealogy of many varieties as a source of low cooling requirement. These species have always contributed to breeding programs. For example, *V. angustifolium* cold resistance, adaptation to high pH conditions; *V. ashei* resistance to drought, low cooling requirement, upright growth property; *V. constablaei* resistance to cold; *V. darrowii* low cooling requirement, adaptation to high pH conditions, heat resistance; *V. elliottii* has been used in breeding programs due to properties such as drought resistance (Ballington, 1990 and 2001; Lubby et al., 1991; Galetta and Ballington, 1996; Lyrene, 2008). As a result, the varieties developed carry the genes of many species. For example, the genetic structure of 'O'Neal' contains *V. corymbosum, V. darrowii, V. ashei* and *V. angustifolium* genes.

Table 2.1. Important *Vaccinium* species used in blueberry breeding (Hancock et al., 2008)

Section	Type	Ploidy	Location
Botadendron	*V. arboreum* Marsh	2 *X*	Southeast North America
Cyanacoccus	*V. angustifolium* Ait	4 *X*	Northeast North America
	V. ahei Reade	6 *X*	Southeast North America
	V. boreale Hall & Aald	2 *X*	Northeast North America
	V. constablaei Gray	6 *X*	South American Mountains
	V. corymbosum L.	2 *X*	Southeast North America
	V. corymbosum L.	4 *X*	East And Mid-North North America
	V. darrowii Camp	2*X*	Southeast North America
	V. fuscatum Ait	2 *X*	Florida
	V. myrtilloides Michx	2 *X*	Central north America
	V. pallidum Ait	2 *X*, 4 *X*	Mid-Atlantic North America
	V. tenellum Ait	2 *X*	Southeast North America
	V. elliottii Chapm.	2 *X*	Southeast North America
	V. hirsutum Buckley	4 *X*	Southeast North America
	V. myrsinites Lam	4 *X*	Southeast North America
	V. simulatum Small	4 *X*	Southeast North America
Oxycoccus	*V. macrocarpon* Ait.	2 *X*	North America
	V. oxycoccos L.	2 *X*, 4 *X*, 6 *X*	Polar Circle
Vitis-idaea	*V. vitis-idaea* L.	2 *X*	Polar Circle
Myrtillus	*V. cespitosum* Michx	2 *X*	North America
	V. chamissonis Bong.	2 *X*	Polar Circle
	V. deliciosum Piper	4 *X*	Northwest North America
	V. membranaceum Dougl. Ex Hook	4 *X*	West North America
	V. myrtillus L.	2 *X*	Polar Circle
	V. ovalifolium Sm.	4 *X*	Northwest North America
	V. parvifolium Sm.	2 *X*	Northwest North America
	V. scoparium Leiberg ex Coville	2 *X*	Northwest North America
Polycodium	*V. stamineum* L.	2 *X*	Central and Eastern North America
Pyxothamus	*V. consanguineum* Klotzch	2 *X*	South Mexico and Central America
	V. ovatum Pursh	2 *X*	Northwest North America
	V. bracteatum Thunb	2 *X*	East Asia, China and Japan
Vaccinium	*V. uliginosum* L.	2 *X*, 4 *X*, 6 *X*	Polar Circle

CHAPTER III

MATERIALS & METHODS

3.1 Plant Material

This research was carried out in the Department of Agricultural Genetic Engineering, Faculty of Agriculture Science and Technologies, Niğde Ömer Halisdemir University Niğde, Turkey. The plant materials consisted of tissues of *V. arctostaphylos*, *V. myrtillus*, *V. uliginosum* species and the *V. corymbosum* cultivars 'Jubilee' and 'Misty'. 'Jubilee' and 'Misty' were sampled from the greenhouse while the others were sampled from the Black Sea Region in September 2019 (Figure 3.2; Figure 3.3). Five (5) samples each of *V. arctostaphylos*, *V. myrtillus*, *V. uliginosum* were studied with a sample each for 'Jubilee' and 'Misty' as the control group. Some of the leaf samples were dried hence seeds were grown in MS media under tissue culture conditions to get fresh tissues for genomic DNA extraction (Figure 3.1).

Figure 3.1. Seeds from *Vaccinium arctostaphylos*, *V. myrtillus*, *V. uliginosum* species sampled from the Black Sea Region of Turkey and grown in MS media

Figure 3.2. Plant samples from *Vaccinium arctostaphylos, V. myrtillus, V. uliginosum* species sampled from the Black Sea Region of Turkey

Figure 3.3. Plant materials of 'Jubilee' and 'Misty' cultivars grown in the greenhouse of Faculty of Agriculture Science and Technologies, Niğde Ömer Halisdemir University, Turkey

3.2 Genomic DNA Extraction

The procedures followed in this analysis were adopted and modified from Dellaporta et al. (1983). DNA was extracted from 0.4 g leaf tissues of *Vaccinium* species. Most of the leaf tissues were used in their preserved form (stored in -80 ºC freezer) grinding them into a fine powder before carrying out the extraction. Liquid N_2 was used to aid the proper grinding of the leave tissues in a mortar with a pestle. A buffer mixture containing extraction buffer with 0.1 g of sodium bisulfite dissolved in it, nuclei lysis buffer and 5 % sarkosyl was prepared before crushing the leave tissues hence 750 µl of the buffer was added to the crushed leaves in the mortar and thoroughly mixed then transferred into a falcon tube (2 ml). The mixture was then incubated for 30 min at 65 ºC and shaken thoroughly. All detectable polysaccharides, denatured protein, and cell wall debris were eliminated by adding 700 µl of phenol/chloroform/isoamyl alcohol (25:24:1) to the mixture and centrifuged at 14,000 rpm for 5 min. The upper phase of the mixture was transferred into a new tube adding 500 µl of chloroform/isoamyl

alcohol (24:1) to emulsify the extract and the sample allowed to go through centrifugation at 10,000 rpm for 5 min. The supernatant was transferred into a new falcon tube and 500 µl of cold isopropanol added, inverting the tubes for about 20 times or until DNA precipitates then it was incubated for 30 mins at room temperature after which it was centrifuged at 10,000 rpm for 5 min and the supernatant discarded. 500 µl of cold ethanol was then added to the pellet for washing and placed in a -20 ℃ refrigerator over-night. The sample was recovered the next morning and centrifuged at 10,000 rpm for 5 min to defreeze it, discarding the ethanol and drying the pellet at room temperature or at 37 ℃ in an incubator. The DNA was resuspended in 50 µl TE buffer and thoroughly mixed, incubated at 65 ℃ for 30–60 mins and stored at -20 until use. DNA concentrations were checked to determine the quality of the samples using the Quawell Q5000 UV-Vis Spectrophotometer and gel electrophoresis. DNA was further diluted in TE buffer to a final concentration of 50 ng/µl and stored at -20 °C until use.

3.3 Polymerase Chain Reaction (PCR) Amplification

PCR was performed in a 25 µl reaction mixture containing 5 µl DNA, 2.5 µl of 10× DreamTaq PCR buffer, 2 µl of $MgCl_2$, 0.375 µl dNTPs, 3 µl of iPBS primer for 18 nt primers or 5 µl of iPBS primer for 12/13 nt primers, 11.925 µl of PCR water or 9.925 µl of PCR water respectively and 0.2 µl Taq DNA polymerase (DreamTaq, Thermoscientific). The amplification was carried out in a thermo cycler. After an initial denaturation at 95 °C for 3 min, the PCR was performed in this order: amplification for 35 cycles with denaturation at 95 °C for 15 s, annealing at 50-55 °C (depending on the primer) for 60 s, and extension at 72 °C for 2 min. After amplification, a final extension step of 72 °C for 7 min was performed and products stored at 4 °C before analysis. The PCR products were analyzed by 1.8 % agarose gel using 1× TAE buffer at 90 V/cm for 2 h. For identifying the band sizes on the gel, a DNA ladder, GeneRuler DNA Ladder Mix (Thermo Scientific), was loaded into the first well on the gel. The gel was stained with ethidium bromide and bands visualized using the Gel Doc™ XR+ gel imaging system (Bio-Rad). All PCR and electrophoresis analysis were repeated at least twice and only sharp and clear bands were scored. A 1 kb DNA ladder was used as the molecular standard in order to validate the pertinent iPBS markers (Figure 3.4).

Figure 3.4. A photograph of the researcher conducting PCR using the thermal cycler

3.4 Statistical Analysis

iPBS (inter Primer Binding Site) retrotransposon markers were used for the characterization of *Vaccinium* species. The iPBS molecular data were documented in binary form; 1 indicating the presence of a band and 0 for its absence generating a binary matrix in effect. The PCR reactions were repeated twice and only the replicable accessions of all products examined.

The analyses were carried out where each accession is considered an entity. First, a similarity matrix was generated using Similarity coefficients. This matrix was then used for multivariate analyses of clustering and principle coordinate analysis (PCoA). For cluster analyses, the UPGMA (Unweighted Pair Group Method using Arithmetic Average) method was used to construct dendrograms. The similarity matrix data were also subjected to PCoA analysis using the NTSYSpc program version 2.11V (Exeter Software, Setauket, NY). The genotypes were plotted on first three dimensions using the G3D procedure of SAS program.

CHAPTER IV

RESULTS

The genotypes of *V. arctostaphylos*, *V. myrtillus*, *V. uliginosum* species, sampled from Turkey, and genetic diversity of 'Jubilee' and 'Misty' varieties were investigated with the iPBS marker system. Amplification of the specific regions of the genomes of these samples were carried out successfully. An example of the amplified products after gel electrophoresis is given in Figure 4.1.

Ten primers were employed in the study. A data matrix was constructed based on the presence (1) or absence (0) of iPBS bands. Missing data were scored as '9'. Bands with size between 3500 and 150 bp were scored manually by three people, cross-checking scores at least twice. In total 274 bands were scored of which 271 were polymorphic with primer 2391 giving the greatest scorable bands while the least was from primer 2277 (Table 4.1; Table 4.2).

These bands were used to evaluate pairwise comparisons of the genotypes. Two of these pairwise comparisons pairs, *V. uliginosum*-2 vs. *V. uliginosum*-5 and *V. uliginosum*-2 vs. *V. uliginosum*-11 were calculated as 100% because of the identical band patterns. This was possibly caused by the relatively low numbers of bands to compare because of missing values. For most of the other pairs, the similarity indexes varied between 47-98% (Table 4.3).

The similarity indexes were subjected to the cluster analysis (Figure 4.2). The cluster analysis revealed that the individual genotypes from each species grouped together. *V. myrtillus*-1, *V. myrtillus*-8, *V. myrtillus*-12 and *V. myrtillus*-14 were positioned at an identical place. When the results were evaluated holistically, it was found that the species of *V. myrtillus* and *V. arctostaphylos* were more closely related than the other species. It was also found that *V. uliginosum* genotypes in the study exhibited a higher level of diversity. Finally, the cultivars 'Jubilee' and 'Misty' were close to the *V. myrtillus*.

The similarity indexes were also subjected to the PCoA analysis (Figure 4.3). Overall, the results of PCoA were similar to those of the cluster analysis. The first, second and third dimensions explained 28, 21 and 16% of the total variation making a total of 65% (Table 4.4).

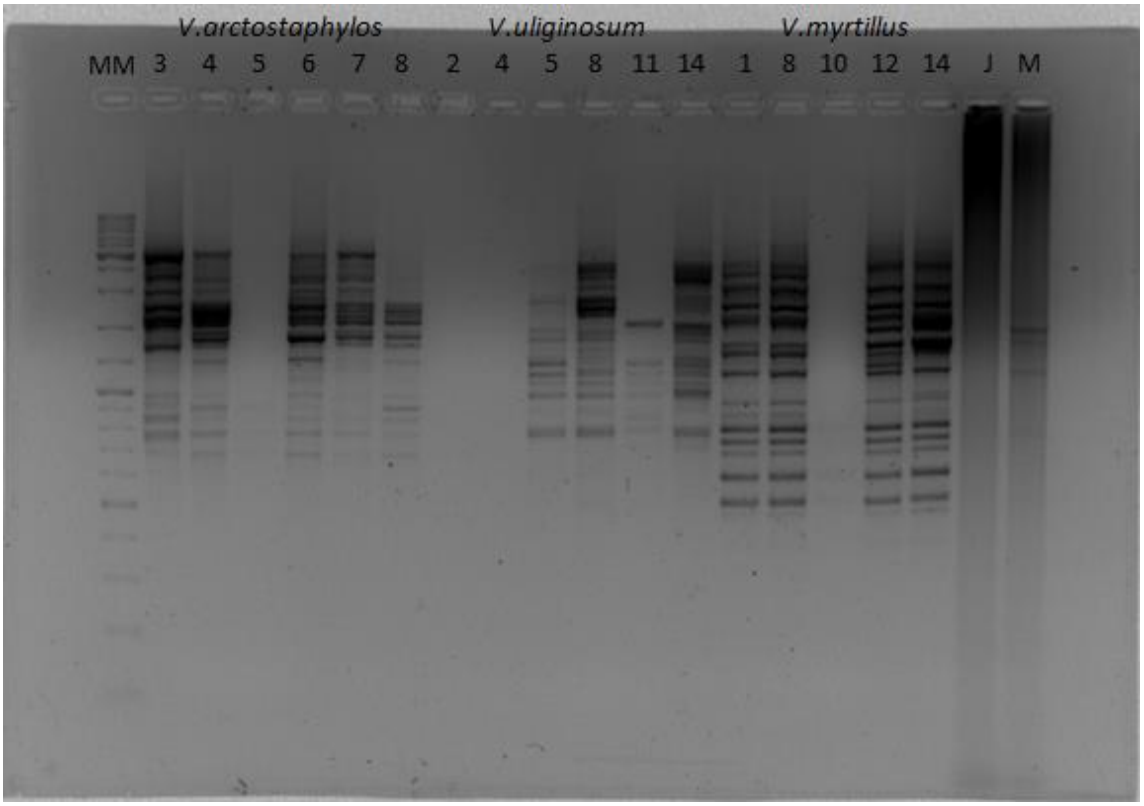

Figure 4.1. Amplified products by iPBS marker system of plant samples from *Vaccinium arctostaphylos*, *V. myrtillus*, *V. uliginosum* species sampled from Black Sea Region of Turkey. The image was generated using primer 2402.

Table 4.1. Summary of amplified products by iPBS marker system of plant samples from *Vaccinium arctostaphylos, V. myrtillus, V. uliginosum* species sampled from Black Sea Region of Turkey alongside 'Jubilee' and 'Misty' cultivars

Primer	Scored band sizes (bp)	Number of total scored bands	Number of total polymorphic bands	Percentage of Polymorphism (%)
2228	525 - 3000	25	25	100%
2272	250 - 3500	25	24	96%
2277	350 - 3000	18	18	100%
2376	150 - 2500	35	35	100%
2391	200 - 2000	42	42	100%
2398	250 - 3250	32	32	100%
2402	500 - 3000	32	32	100%
2095	400 - 2000	21	21	100%
2373	325 - 3500	25	24	96%
2387	350 - 3000	19	18	96%
Total / Mean	---	274	271	99%

Table 4.2. Summary of iPBS markers used in analyzing the samples of *Vaccinium arctostaphylos, V. myrtillus, V. uliginosum* species sampled from Black Sea Region of Turkey alongside 'Jubilee' and 'Misty' cultivars

Primer Name	Annealing Temperatures °C	Sequence	Base Pairs
2228	53	CATTGGCTCTTGATACCA	18
2398	51	GAACCCTTGCCGATACCA	18
2402	50	TCTAAGCTCTTGATACCA	18
2373	55	GCTCATCATGCCA	13
2272	55	GGCTCAGATGCCA	13
2277	52	GGCGATGATACCA	13
2376	52	TAGATGGCACCA	12
2391	52	ATCTGTCAGCCA	12
2095	53	GCTCGGATACCA	12
2387	52	GCGCAATACCCA	12

Table 4.3. Pairwise similarity index comparison matrix of *Vaccinium arctostaphylos*, *V. myrtillus*, *V. uliginosum* species sampled from Black Sea Region of Turkey alongside 'Jubilee' and 'Misty' cultivars

Genotype	Jubilee	Misty	*V. arctostaphylos*-3	*V. arctostaphylos*-4	*V. arctostaphylos*-6	*V. arctostaphylos*-7	*V. arctostaphylos*-8	*V. uliginosum*-2	*V. uliginosum*-5	*V. uliginosum*-8	*V. uliginosum*-11	*V. uliginosum*-14	*V. myrtillus*-1	*V. myrtillus*-8	*V. myrtillus*-10	*V. myrtillus*-12	*V. myrtillus*-14
Jubilee	1.00																
Misty	0.84	1.00															
V. arctostaphylos-3	0.55	0.54	1.00														
V. arctostaphylos-4	0.51	0.47	0.90	1.00													
V. arctostaphylos-6	0.51	0.52	0.83	0.79	1.00												
V. arctostaphylos-7	0.56	0.51	0.84	0.83	0.89	1.00											
V. arctostaphylos-8	0.54	0.56	0.78	0.78	0.83	0.88	1.00										
V. uliginosum-2	0.57	0.57	0.64	0.62	0.57	0.57	0.57	1.00									
V. uliginosum-5	0.59	0.62	0.64	0.63	0.65	0.68	0.70	1.00	1.00								
V. uliginosum-8	0.58	0.60	0.67	0.64	0.65	0.66	0.65	0.88	0.84	1.00							
V. uliginosum-11	0.58	0.63	0.66	0.70	0.65	0.70	0.68	1.00	0.79	0.76	1.00						
V. uliginosum-14	0.55	0.56	0.65	0.66	0.66	0.69	0.67	0.69	0.82	0.88	0.75	1.00					
V. myrtillus-1	0.61	0.57	0.57	0.55	0.57	0.60	0.59	0.60	0.65	0.66	0.59	0.66	1.00				
V. myrtillus-8	0.60	0.56	0.56	0.53	0.57	0.58	0.57	0.60	0.63	0.65	0.57	0.65	0.98	1.00			
V. myrtillus-10	0.61	0.57	0.73	0.73	0.61	0.68	0.70	0.62	0.80	0.73	0.81	0.70	0.79	0.78	1.00		
V. myrtillus-12	0.61	0.56	0.62	0.56	0.60	0.61	0.58	0.62	0.62	0.65	0.60	0.64	0.89	0.90	0.79	1.00	
V. myrtillus-14	0.62	0.56	0.57	0.53	0.56	0.57	0.54	0.62	0.57	0.62	0.56	0.59	0.86	0.88	0.75	0.90	1.00

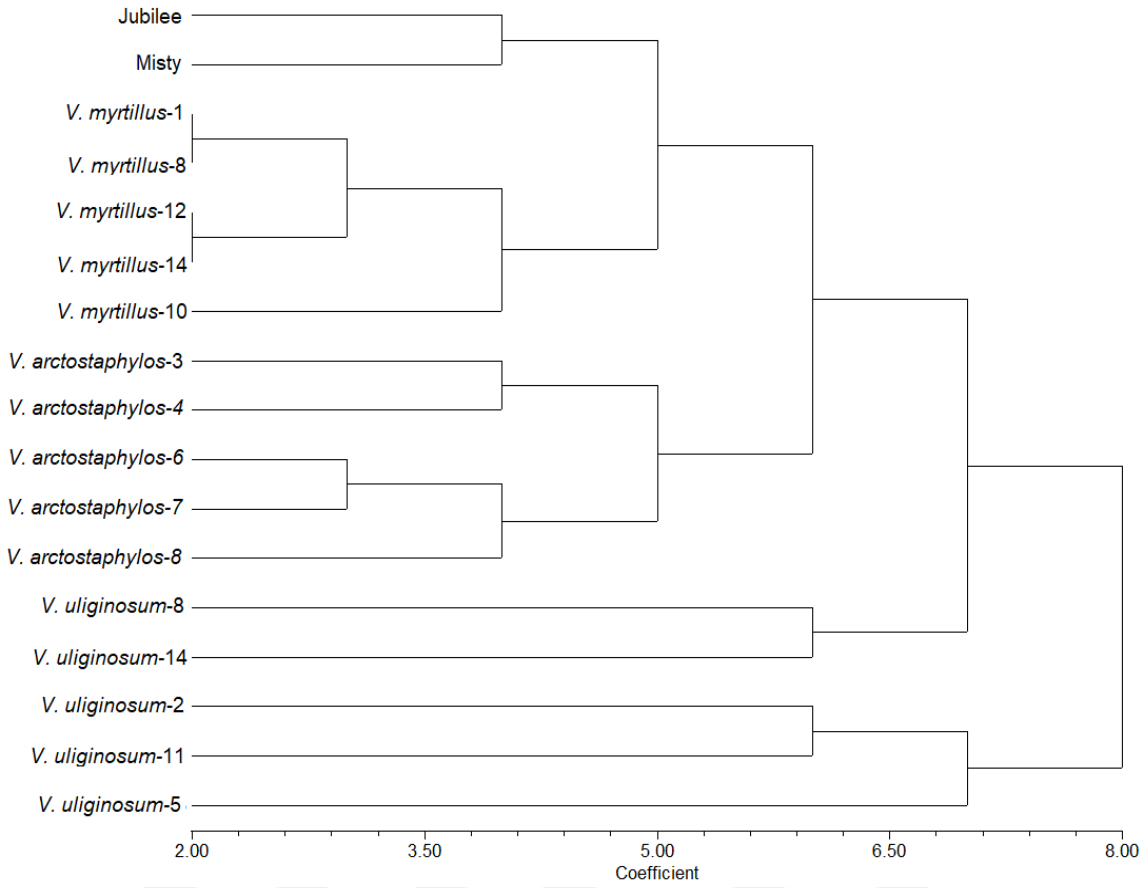

Figure 4.2. The UPGMA (Unweighted Pair Group Method using Arithmetic Average) dendrogram generated by the amplified products by iPBS marker system of plant samples form *Vaccinium arctostaphylos*, *V. myrtillus*, *V. uliginosum* species sampled from Black Sea Region of Turkey alongside 'Jubilee' and 'Misty' cultivars

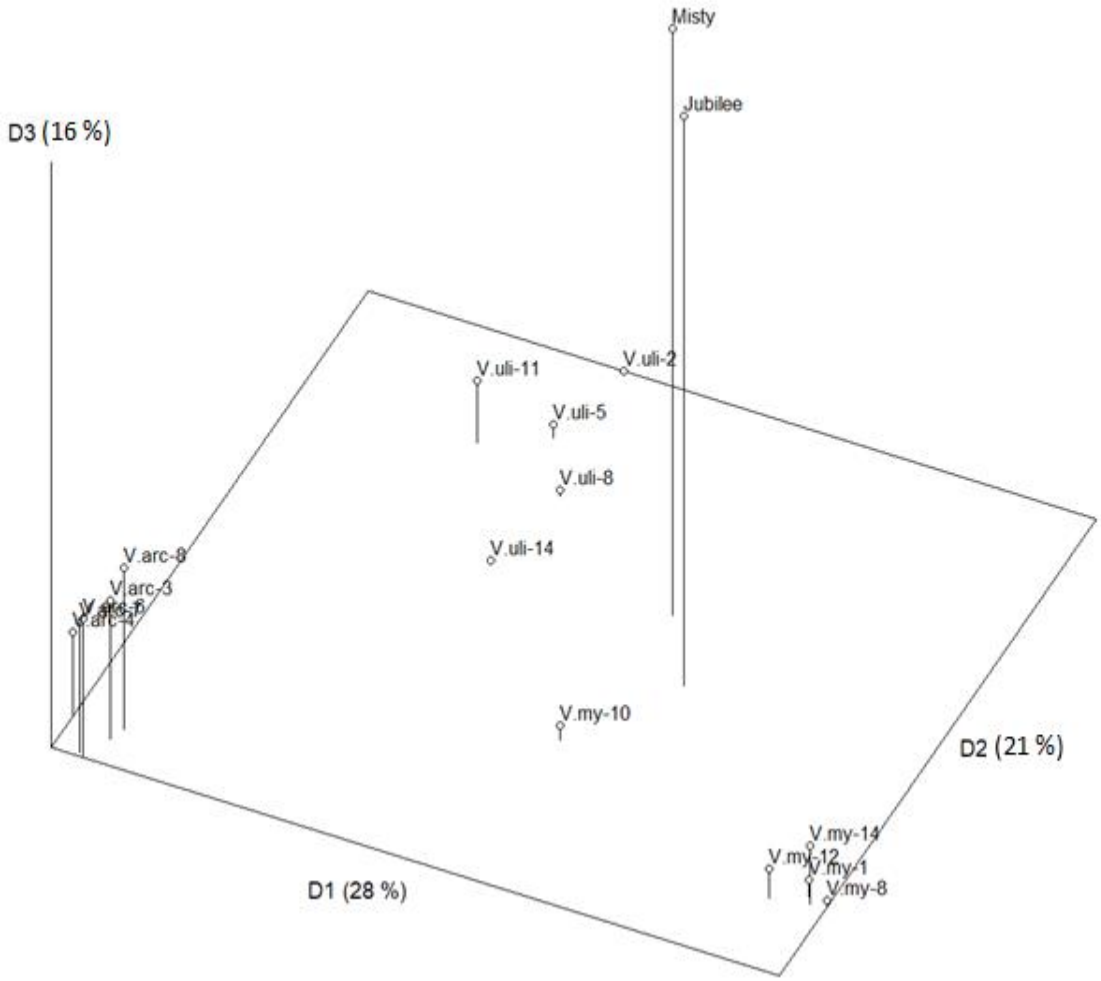

Figure 4.3. The three dimensional view of the amplified products by iPBS marker system after the principle coordinate analysis for the plant samples form *Vaccinium arctostaphylos, V. myrtillus, V. uliginosum* species sampled from Black Sea Region of Turkey alongside 'Jubilee' and 'Misty' cultivars

Table 4.4. The eigenvalue, percentage and cumulative variation three dimensional view of the amplified products by iPBS marker system after the principle coordinate analysis for the plant samples from *Vaccinium arctostaphylos*, *V. myrtillus*, *V. uliginosum* species sampled from Black Sea Region of Turkey alongside 'Jubilee' and 'Misty' cultivars

Dimensions	Eigenvalue	Percentage (%)	Cumulative
1	1.51	28.10	28.10
2	1.13	20.93	49.03
3	0.85	15.89	64.92
4	0.43	7.94	72.86
5	0.33	6.11	78.97
6	0.30	5.59	84.56
7	0.20	3.67	88.23
8	0.17	3.21	91.44
9	0.16	2.93	94.37
10	0.13	2.46	96.83
11	0.11	2.02	98.86
12	0.09	1.82	> 100%
13	0.08	1.56	> 100%
14	0.06	1.18	> 100%
15	0.02	0.32	> 100%
16	0.00	0.00	> 100%
…	…	…	…
Sum	5.37	99.98	
Average root	0.32		

CHAPTER V

DISCUSSION

With the current global rise in interest for *Vaccinium* species, it is important to ascertain the molecular behaviour of these species which will help breeders to make informed choices for selecting parents for their breeding programs so as to maximize profit and make progress.

Some earlier studies clearly showed that iPBS retrotransposons were very efficient for molecular studies in plants. However, this marker system has not been utilized for evaluating the molecular behaviour of *Vaccinium* species. Hence, this research particularly sought to investigate the applicability of iPBS retrotransposon markers for differentiating the *Vaccinium* species in the Turkish wild flora and two cultivated species.

One of the main concerns for the molecular characterization in plants is that selected marker systems must possess the ability of high reproducibility and should be the source of high polymorphism, even in closely related genotypes. For this purpose, reproducibility of the iPBS retrotransposon markers in *Vaccinium* species were tested using repeated polymerase chain reaction procedure. The results clearly showed that iPBS retrotransposon markers were highly reproducible in all tested cases.

The iPBS retrotransposons have several appealing features, such as high degree of polymorphism, technical simplicity, less time and minimum labour requirement, as well as reproducibility, which makes it a marker system of choice for cultivar molecular characterization. A disadvantage of iPBS retrotransposon marker system is difficulty in considering the presence or absence of faint bands hence, making scoring of the bands of iPBS retrotransposon markers relatively more difficult when compared to other markers.

In this study, genotypes of *V. arctostaphylos*, *V. myrtillus*, *V. uliginosum* species and genetic diversity of 'Jubilee' and 'Misty' varieties were investigated with the iPBS marker system. Several polymorphic bands were generated by this marker system.

Thus, the results exhibited that the iPBS marker system is a suitable system to use for studying the genetic diversity among *Vaccinium* species.

There are many *Vaccinium* species in the genus. Polyploidization is a common phenomenon in the *Vaccinium* genus. The representative genotypes of *V. arctostaphylos, V. myrtillus, V. uliginosum* species alongside cultivars of *V. corymbosum,* 'Jubilee' and 'Misty' were investigated by several scientists in the last decade using many other methods and studying several parameters.

One of these studies was Bozdag et al (2018) where they conducted a study that sought to investigate the characterization of morphological traits of wild *Vaccinium* species having potential horticultural importance, this lead to the collection of the species from the Eastern Black Sea Region of Turkey. Five morphologically different populations of wild *Vaccinium* species were collected from different habitats at diverse altitudes during the land expedition. After extensive literature search, focusing on their morphological characters, these *Vaccinium* populations were identified as whortleberry (*V. arctostaphylos*), bilberry (*V. myrtillus*) and bog bilberry (*V. uliginosum*). Twelve horticulturally important morphological traits like leaf weight, petiole length, leaf width, leaf length, leaf colour (upper side), leaf colour (lower side), fruit weight, fruit width, fruit length, fruit crown width, seed number and fruit colour were analyzed. Significant differences were observed among the populations and at species levels regarding analyzed traits. *V. uliginosum* has lower leaf weight, leaf width and leaf length values compared to the other two *Vaccinium* species. Considering all leaf traits, *V. arctostaphylos* species has the highest average values. The highest fruit weight values were measured in *V. arctostaphylos*(C), while the smallest fruits were measured in *V. uliginosum*. Similar tendencies were observed in fruit height. However, the highest value of fruit width (8.0 mm) and fruit crown width (4.7 mm) was found in *V. myrtillus* species which can be used as a distinctive character to identify *V. myrtillus* fruits. In terms of seed number, the species can be listed as *V. arctostaphylos* > *V. myrtillus* > *V. uliginosum*. From the result it is evident that fruit weight and seed number are directly correlated with the fruit size and shape. In addition, *V. myrtillus* have more round shaped fruit with wider fruit crown. Fruit colours were measured with L, a and b values. The highest value in terms of L value was in *V. uliginosum* species; the lowest value was determined in *V. arctostaphylos* (A). The highest values in terms of a and b values

were measured in *V. arctostaphylos*(C) sample, while the lowest value of b was measured in *V. myrtillus*. From this result it can be conclude that fruits of different *Vaccinium* species have unique colour composition and this variation is present at both interspecies and inter-population level. Therefore, fruit colour is not only genetically controlled but also effected by environmental condition. In addition, gene responsible for this character is segregating efficiently from population to population. It is evident that our results could be highly valuable for further characterization and improvement of wild or cultivated *Vaccinium* species in Turkey.

Sultana et al (2017) studied the *Vaccinium* genome analysing them through ploidy level estimation and repetitive DNA exploration using RepeatExplorer software. Diploid northern highbush blueberry (*Vaccinium corymbosum*) and American cranberry (*Vaccinium macrocarpon*) are the two most widely studied species of the economically important genus *Vaccinium* for which whole genome sequence data and draft assemblies have become recently available in the public database. It was found in this study that the total percentage of repetitive DNA sequence is 80 and 90% in blueberry (*Vaccinium corymbosum* L.) and cranberry genome (*Vaccinium macrocarpon* Ait.), respectively. The major portions belonged to Ty1/copia and Ty3/gypsy LTR-retrotransposons, followed by DNA transposon, non-LTR-retrotransposon, and satellite DNA fractions. Analysing the retrotransposon's key enzyme, the reverse transcriptase (RT), revealed that elements of the Ale and Ogre/Tat lineages have been predominant in the Ty1/copia lineages and Ty3/gypsy super families, respectively. Adding to this, the L1-type long interspersed nuclear elements (LINEs) were dominating the non-LTR retrotransposon fraction. *V. macrocarpon* contains remarkably higher amounts of LTR and non-LTR retrotransposon as compared to *V. corymbosum*. A total of seven different putative satellite families were identified and characterized. Among them VaccSat1, VaccSat4, VaccSat5 and VaccSat6 only occur in *V. corymbosum*, whereas VaccSat2, VaccSat3 and VaccSat7 were common in both species indicating that the percentage of satellite repeats in *V. corymbosum* were significantly higher than that in *V. macrocarpon*. Comparative phylogenetic analysis of these abundant repetitive sequences demonstrates the extent of sequence diversity and specificity between this two *Vaccinium* species. The results from this study are highly valuable for further genomic characterization of wild and cultivated *Vaccinium* species.

Bioinformatics and molecular characterization of satellite repeats was performed to understand the impact of their diversification on *Vaccinium* genome evolution by Sultana et al. (2020). Satellite repeat diversity was evaluated in four cultivated and wild species, including the diploid species *Vaccinium myrtillus* and *Vaccinium uliginosum*, as well as the tetraploid species *Vaccinium corymbosum* and *Vaccinium arctostaphylos*. Comparative characterization of six satellite repeat families using a total of 76 clones with 180 monomers was carried out. It was observed that the monomer units of VaccSat1, VaccSat2, VaccSat5, and VaccSat6 showed a higher order repeat structure, which is suspected to have resulted from the organization of two adjacent subunits with differing similarity, length and size. VaccSat1, VaccSat3, VaccSat6, and VaccSat7 were found to have sequence similarity to parts of transposable elements. Satellite-typical tandem organization for VaccSat1 and VaccSat2 in long arrays were detected, while VaccSat5 and VaccSat6 distributed in multiple sites over all chromosomes of tetraploid *V. corymbosum*, presumably in long arrays. On the contrary, very short arrays of VaccSat3 and VaccSat7 were spread across the chromosomes in the same species, likely as internal parts of transposable elements. The research also revealed that VaccSat1, 5 and 6 have some levels of species specificity, which can be used as markers for species identification, while VaccSat2, 3 and 7 are common satellite families in all studied species. This research provides a comprehensive overview on satellite species specificity in *Vaccinium*, which are potentially useful as molecular markers to address the taxonomic complexity of the genus, and provide information for genome studies of this genus.

Sultana et al. (2019) also studied the genome size (GS) data for several *Vaccinium* species with prevalence in Europe and Western Asia and analysing the global GS variation in the genus, considering available data and phylogenetic context using new GS assessments were obtained by flow cytometry and chromosome counts were verified. Phylogenetic analyses were performed by Bayesian inference and reconstruction of ancestral GS by maximum parsimony. They obtained GS data for five *Vaccinium* species (13 populations). Three species are reported for the first time. Values (2C) ranged between 1.16–1.47 pg at the diploid (2n=24) and between 3.13–3.16 pg at the tetraploid (2n=48) levels. The five species here investigated have been analyzed and placed in a reconstructed phylogenetic background (including 68 taxa).

Our results were in agreement with the studies summarized above with our results also clearly separating the species studied. The genotypes representing each species clustered together. *Vaccinium arctostaphylos* and *V. myrtillus* were found closer to each other as compared to *V. uliginosum* species. 'Jubilee' and 'Misty' varieties showed the highest similarity with *V. myrtillus*.

Other researches using iPBS marker system that have findings similar to this research include but not limited to the following;

Demirel et al. (2018) fingerprinted and identified the genetic similarity among 151 potato genotypes. They first, screened 16 potato genotypes using 45 iPBS retrotransposon markers to identify polymorphisms and further choose seventeen of these primers for fingerprinting the whole set of accessions as a result of the presence of strong, reproducible and polymorphic bands. The 17 iPBS primers selected produced 290 scorable bands with 224 being polymorphic. Individual primers produced bands ranging from 10 to 26 with 17.1 as average. The number of polymorphic bands per primer fell between 6 and 21 with a polymorphism percentage per primer ranging from 46.2 to 100.0% having an average of 77.2% per primer. The mean polymorphism information content (PIC) values of iPBS primers varied from 0.12 to 0.31 per primer. Genetic similarity based on Jaccard's coefficient of potato genotypes ranged from 0.61 to 0.93 with an average of 0.73.

Aydın et al. (2020) studied inter-primer binding sites retrotransposon marker system was employed to make simple the genetic variability at intra and inter species levels of 112 yeast strains belonging to eight species previously obtained from fermented foods. The molecular identification of yeast strains was first confirmed by sequencing the D1/D2 domain of the 26S rRNA. The eight primers used in the study produced 278 bands, all of which were polymorphic with an average of 34.75 polymorphic fragments per primer. The averages of polymorphism information contents and the resolving power values for the iPBS marker system were 0.23 and 10.11, respectively. The genetic parameters within each yeast species derived using iPBS markers were observed as; percentage of polymorphic loci for each species ranged from 19.23% to 71.21%, Nei's gene diversity from 0.085 to 0.228, while Shannon's information index values ranging from 0.125 to 0.349. The value of gene flow (0.09) and genetic variation among

the populations (0.85) showed higher genetic variation among the species. UPGMA analyses demonstrated considerable genetic variability in the yeast strains, clustered them according to their species, and revealed the intraspecific variation. Each of the selected iPBS primers provided enough species-discrimination. Present evaluations suggest the utility of iPBS marker system to estimate the genetic variation of yeast strains.

The efficiency of iPBS markers in detecting genetic differentiation in African Gnetum species was determined by Doungous et al. (2019). A set of 21 iPBS markers were employed and administered on 14 accessions including *G. africanum* Welw., *G. buchholzianum* Engl. and the recently identified species *G. latispicum*. Results of the best six out of the 21 selected primers generated 103 bands in G. africanum, 95 in *G. buchholzianum* and 24 in *G. latispicum*. A cluster analysis divided the accessions into two major groups with the first group comprising of all the accessions of *G. africanum*, whereas the second group was further divided in two subgroups representing accessions of *G. buchholzianum* and *G. latispicum*. In addition, the Jaccard similarity coefficient showed a close relationship between accessions of *G. buchholzianum* and *G. latispicum*. The iPBS marker system revealed genetic differentiation within African Gnetum and could be useful for evaluating genetic diversity, conservation, taxonomy and evolution studies they concluded.

Yildiz et al. (2020) characterized the genetic diversity and population structure of a collection of 94 pepper accessions using iPBS markers. A total of 20 iPBS primers were used that generated 172 bands (mean = 8.6 bands/primer), of which ~92% were polymorphic in the entire germplasm collection, whereas 83%, 69%, and 80% of the bands were polymorphic within the *C. annuum*, *C. chinense*, and *C. frutescens* subsets, respectively. All of the taxa analyzed were clearly differentiated by the iPBS markers. The polymorphism information content of the markers ranged between 0.15 and 0.99, with an average of 0.66. Cluster analyses by different methods (UPGMA, STRUCTURE, and principal coordinate analysis) revealed a clear separation of all of the *C. annuum* accessions from the other pepper species, with a few subclusters observed among the latter, including groups with accessions of both *C. frutescens* and *C. chinense*. At the interspecies level, the 3 clustering methods clearly discriminated *C. annuum* from *C. frutescens* and *C. chinense*. No clear association was found between

the iPBS-based clustering and geographical origin or fruit characteristics of the accessions.

CHAPTER VI

CONCLUSION

Turkey has a very rich flora. *Vaccinium* species, centred in Black Sea Region, are also among these species: *Vaccinium arctostaphylos* L. (whortleberry), *Vaccinium myrtillus* L. (bilberry), *Vaccinium uliginosum* L. (bog bilberry) and *Vaccinium vitis-idaea* L. (cowberry or lingonberry) (Davis, 1978). In this study, we investigated the genetic diversity of genotypes of *V. arctostaphylos*, *V. myrtillus*, *V. uliginosum* species sampled from Turkey. The results have a similitude with previous reports by Sultana et al. (Sultana et al., 2017; Sultana et al., 2019; Sultana et al., 2020). The high rate of polymorphisms and the high number of bands recorded per assay using a single primer shows that iPBS is an informative marker that can be used for studying about the populations, taxonomy, conservation and domestication of *Vaccinium* species. These results shed light on the diversity and evolution of related species and their use in blueberry breeding.

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