# COMPARISON OF MORPHOLOGICAL AND MOLECULAR STUDIES ON NATURAL *TRIFOLIUM* L. (CLOVER) SPECIES GROWN IN BOLU PROVINCE

by

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science of Philosophy.

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## ABSTRACT

### COMPARISON OF MORPHOLOGICAL AND MOLECULAR STUDIES ON NATURAL *TRIFOLIUM* L. (CLOVER) SPECIES GROWN IN BOLU PROVINCE

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In this study, two molecular marker technologies (RAPD and SSR) and morphological characters were utilized to determine the taxonomic relationships of 29 *Trifolium* L. taxa found in Bolu province. In molecular parts, 11 RAPD and 5 SSR primers were selected to reveal the genetic relationships of interspecies. After evaluations of band profiles, it was observed that all primers gave high polymorphism. Three UPGMA dendrograms were drawn in NTSYS program based on individual RAPD, SSR and combined molecular data. According to these dendrograms, all the members of *Trifolium* section were separated clearly from the remaining section members. Though, some differences were found in classification of taxa below section levels. In morphological study, 18 quantitative and 8 qualitative characters were selected. An UPGMA dendrogram was drawn in PHYLIP program with the data obtained from these characters. In the results of dendrogram based on morphological data, it was understood that the characters could not provide a clear separation in sectional ranks. Besides, using a large numbers of molecular markers in such kind of taxonomic studies were found to be more reliable.



**Key words:** *Trifolium*, RAPD, SSR, morphologic characters, Bolu

# ÖZET

## BOLU İLİNDE DOĞAL YETİŞEN *TRİFOLİUM* L. (ÜÇGÜL) TÜRLERİNİN MORFOLOJİK VE MOLEKÜLER YÖNLERİNDEN KARŞILAŞTIRILARAK İNCELENMESİ

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Bu çalışmada, Bolu ilinde bulunan 29 *Trifolium* L. taksonunun taksonomik ilişkilerini belirlemek için iki moleküler markör teknolojisi (RAPD ve SSR) ve morfolojik karakterler kullanılmıştır. Moleküler kısımlarda, türler arası genetik ilişkiyi ortaya çıkarmak için 11 RAPD ve 5 SSR primerleri seçilmiştir. Bant profillerinin hesaplamaları yapıldıktan sonra, bütün primerlerin yüksek polimorfizm verdikleri görülmüştür. Ayrı ayrı ve birleşmiş moleküler dataya dayanılarak üç UPGMA dendrogramı NTSYS programında çizdirilmiştir. Bu dendrogramlara göre bütün *Trifolium* seksiyon bireyleri diğer seksiyonlardan anlaşılır biçimde ayrılmıştır. Fakat, seksiyon altı taksonların sınıflamasında bazı uyumsuzluklar bulunmuştur. Morfolojik çalışmada, 18 nicel ve 8 nitel karakter seçilmiştir. Bu karakterlerden elde edilen verilerle PHYLIP programında UPGMA dendrogramı çizilmiştir. Morfolojik verilere dayanarak çizilen dendrogramda seksiyon kademesinde karakterlerin net bir ayrım yapamadıkları anlaşılmıştır. Çalışmanın sonucunda, türler arası sınıflamada çok sayıda moleküler markör kullanımının daha güvenilir sonuçlar verdiği bulunmuştur.

**Anahtar sözcükler:** *Trifolium*, RAPD, SSR, morfolojik karakterler, Bolu

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## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| AFLP | Amplified Fragment Length Polymorphisms |
| DNA | Deoxyribonucleicacid |
| FISH | Fuorescent In Situ Hybridization |
| ITS | Internal Transcribed Spacers |
| MAS | Marker Assisted Selection |
| NTSYS | Numerical Taxonomy and Multivariate Analysis System |
| PBS | Percentage of Polymorphic Bands |
| PCR | Polymerase Chain Reaction |
| PCR-RFLP | PCR-Restriction Fragment Length Polymorphism |
| PHYLIP | Phylogeny Inference Package Program |
| QTL | Quantitative Trait Locus |
| RAPD | Random Amplified Polimorphic DNA |
| rDNA | Ribosomal DNA |
| sect. | Section |
| SSR | Simple Sequence Repeats |
| UPGMA | Unweighted Pair Group Method of Analysis |

# 1. INTRODUCTION

Throughout history, humans have used plants for different purpose such as feeding, remedy of illnesses, making shelter and protecting from environmental conditions. Feeding and remedy were the main problems for ancient people. Humans have survived thanks to plant and animal productions. In todays world, people are still trying to solve the same problems; nourishment and diseases. Especially in last century, due to the increasement of world population, these problems became important and caused to make the requirement of plant and animal products greater than old one (Algan and Büyükkartal, 1999).

In food sources, animal proteins have important place in human feeding as much as plant nutritions. Thus, animal husbandry is important. The main important problem in animal husbandry is forage production. Qualified and economic production of forage plants is crucial for health and growing of farm animals (Algan and Büyükkartal, 1999). In our country, where is famous in agriculture and animal husbandry, requirement of forage plants have gradually increased because of the reduction of qualified, useful pastures and increasing of the livestocks numbers (Algan and Büyükkartal, 1999; Çakmakçı and Çeçen, 1999). Although the number of the forage plant kinds in our agriculture is limited, the studies and investigations showed that the superior examples of many forage plants are present in the Flora of Turkey (Algan and Büyükkartal, 1999). The genus *Trifolium* L. can be shown as an example of these superior plants. *T. repens* L., *T. pratense* L., *T. fragiferum* L., *T. nigrescens* Viv, *T. resupinatum* L., *T. hybridum* L. are examples for the forage plants members of *Trifolium* genus. These plants can solve our agricultural problem. Thus, they have become subjects for most of the studies (e.g Sürmen et al. 2013; Demiroğlu and Avcıoğlu, 2010; Örsdöven and Kendir, 2006; Başaran et al. 2006; Hakyemez and Sancak, 2005; Çakmakçı and Çeçen, 1999; Algan and Büyükkartal, 1999).

Beside of the agriculture, some of *Trifolium* plants have important role in medicinal aspects. They are known as folk medicine in various cultures. In traditional applications, they are used for several illness such as wounds, stomachache and diarrhea, eczema and psoriasis, sore throat, fever, pneumonia and meningitis, skin problems, lung illnesses, some disorders of nervous and reproduction system. Nowadays, therapeutic use of *Trifolium* plants is still based mainly on traditional medicine recommendations. Thus, most researches are proceeding to understand the biological activity of clovers and to reveal their possible therapeutic effects (Kolodziejczyk-Czepas, 2012).

This important genus, *Trifolium*, belongs to Leguminosae family and carries its family properties.

## 1.1 Fabaceae (Leguminosae)

### 1.1.1 The importance of Fabaceae family

Fabaceae is the third largest family of flowering plants with 727 genera and ca. 19,325 species (Ellison et al. 2006). The members of Fabaceae family are important economic plants. They are mainly used as crop plants and are grown on some 180 million Ha, or 12% to 15% of the Earth's arable surface. Also, they account for 27% of the world's primary crop production (Graham and Vance, 2003). Peas, beans, lentils, peanuts, alfalfa, sweet clover, wattle, carob are the famous members of legumes. The seeds and pods of some legumes are edible and highly nutritive food sources for human (Kaufman et al. 1989). Although alfalfa and sweet clovers are used as forage plants, they are used to make salad and oriental dishes in some cultures. Some legumes such as wattle and carob, are grown for commercial aspects. Wattle contains tannin and this chemical is used to make leather. Carob is a chocolate substitute (Stern et al. 2003). Except these plants, there are lots of legumes using for different aims such as medicinal aspects (isoflavones), preparing biodegradable plastics, making oil, gum source, making important dyes (indigo, logwood) and furniture (Sheokand et al. 2012; Stern et al. 2003). In addition to their economical importance, legumes are crucial for natural and agricultural ecosystems

(Choi et al. 2004). Nitrogen ($N_2$) is the primary nutrient for plant production. Legumes develop root nodules and fix $N_2$ by using symbiosis of compatible rhizobia. Agriculturally important legumes fix from 40 to 60 million metric tons of $N_2$ by annually, others fix from 3 to 5 million metric tons in natural ecosystems (Graham and Vance, 2003; Smil, 1999). Replacing this amount with fertilizer would cost $7 to 10 billion annually, but even modest use of alfalfa in rotation with corn could save farmers in the U.S. $200 to 300 million (Graham and Vance, 2003; Peterson and Russelle, 1991). Thus, using legumes is more cheaper and more environment-friendly way than consuming nitrogen fertilizers. Beside of this valuable traits, these plants can speed up the bioremediation process thanks to their rhizosphere. The plants release their exudates from their rhizosphere and activate microorganisms. As a result of this, microorganisms degrade heavy metals, pesticide residuals and petroleum hydrocarbon contamination (Sheokand et al. 2012).

### 1.1.2 Systematic properties of Fabaceae family

Fabaceae family is cosmopolitan and include many important plants (Stern et al. 2003). They are found in different forms such as herbs, shurbs, trees or climbing forms by twining or tendils, which forms by modification of leaflets. The leaves arrangement is usually alternate. Also they can found in spiral to two ranked, pinnately compound, trifoliolate, or untrifoliolate forms. Leaflets usually show sleep movements with pulvini (sing. pulvinus) (Judd et al. 2002).

Inflorescences are almost always indeterminate although they are reduced to a single flower. Flowers are usually bisexual and the symmetry changes from radial to bilateral. Petals usually have five parts. These parts are distinct or connate. Sometimes petals are all alike or become different from each other. The uppermost petals change in size, shape and color. These parts are called as banner or standard. The lower two petals fused together and form a keel or widely flaring. Stamen number shows variation from one to numerous. However, in usual they have ten stamens. Filaments can be distinct or connate which has two state such as monadelphous and diadelphous (9 connate, 1 adaxial or distinct). There are usually one carpel, rarely two or more carpels. Some species produce nectar. They have

mostly characteristic fruit structures called as legume. The Leguminosae name comes from this structure. Sometimes fruits can be found in different forms (e.g samara, loment) (Judd et al. 2002).

### 1.1.3 Systematic properties of subfamilies in Fabaceae

This family is divided into three subfamilies; Mimosoideae, Caesalpinioideae, and Faboideae (Papilionoideae). These subfamilies are generally identified with their characteristic flowers.

Mimosoideae plants are generally found in tree and shrub forms. They live mostly tropic and subtropic climates. They have small, radial symmetric (actinomorphic) flowers which are usually small and individually inconspicuous. The flowers are arranged in dense heads which resemble a pom-pom or clusters into spikes. The stamens can be found in the same number of petal or more than that and they can be free or all united into a tube or bound to the base of the petals. The leaves are predominately bipinnate, rarely pinnate. *Acacia* Mill. and *Mimosa* L. can be shown as examples for this subfamily (Leistner, 2005; Diggs et al. 1999; URL3).

Like Mimosoideae, Caesalpinioideae plants are mostly tropical and subtropical trees and shrubs. The flowers of them are bilateral symmetric (zygomorphic) and can be small or large. In usual, they are conspicuous individually and arranged in various types of inflorescences. Petals are differentiated and form a larger standard (i.e banner), wings, and keel structures. Stamens are externally visible and generally free from one another, not enclosed by a keel. They are found in ten or fewer counts. Leaves are mostly pinnate or bipinnate, rarely simple or one foliolate (Leistner, 2005; Diggs et al. 1999; URL3).

The Faboideae is the largest subfamily of Fabaceae with about two-thirds of all the genera and species. Unlike the plants of other two subfamilies, they live mostly in temperate regions. The majority of the species are herbaceous, although there are some trees and shrubs. They have characteristic (butterfly-like) flowers. The flower is zygomorphic and is made up of five petals; a ‘banner’ petal, two wing

petals, and two petals partially fused together to form a boat-shaped keel. The keel encloses the stamens, so they can not be seen externally. The number of the stamens are ten or fewer. The Faboideae contains most of the important leguminous crop species and have tribe Trifolieae in which *Trifolium* genus take place. (Leistner, 2005; Diggs et al. 1999; URL3). *Trifolium* species show the floral characteristics of Faboideae subfamily.

## 1.2 *Trifolium* L.

### 1.2.1 The importance of *Trifolium* genus

*Trifolium* (clovers) is one of the most important legume genera in livestock agriculture. The members of this genus are accepted as nutritive forages (Erdemli et al. 2007). For example, red clover (*Trifolium pratense* L.) is the second most important perennial forage legume, after alfalfa. Red clover has higher essential amino acids content (cystine, tryptophan, leucine) than that of corn and oat grains. This makes red clover a qualitive forage. Also, the high content of soluble carbohydrates provide a good energy food for farm animals. It contains large amounts of provitamin A and vitamins C, D, E, K, B1, B2 and B3 and rich in minerals (Vasiljevic et al. 2011). Beside of red clover, white clover (*T. repens* L.) are also cited with high nutritive value due to the presence of condensed tannin or proanthocyanids. These chemicals are important in animal health and nutrition (Foo et al. 2000; Jones et al. 1976). *Trifolium* species are used mostly for grazing of animals but they can be harvested as hay, silage and green-chop (Erdemli et al. 2007). In storage, grains can be mixed with clovers to provide balance of energy and protein. By this way, farm animals stay healthy in the rainless seasons (Demirel et al. 2010). These plants are also the favorite plants of *Apis mellifera* (European honey bee). Bees choose *Trifolium* plants for several reasons such as their nectars and predominant, wide distributions, presence of much species varieties (Koçyiğit et al. 2013).

Like the other members of legume family, clovers enrich the soil by making atmospheric nitrogen fixation and facilitate the root expansion of other species. Also,

they increase soil water retention capacity, and protect soil from mineral leaching (Fotiadis et al. 2010). Because of their benefits, clovers are sown by rotation with grains in agricultural fields. Furthermore, some *Trifolium* species (e.g *T. repens*) play important role in remediation of heavy metal-contaminated soils (Liu and Wang, 2014). Thus, they are essential plants for maintaining ecosystem.

Beside all of these benefits, clovers have significant place for humans health. Some of them are used as folk medicines in most culture. Generally, clovers have been used in the history to cure eczema and psoriasis in oriental and european cultures. However, some differences can be seen according to country (Kolodziejczyk-Czepas, 2012). In Pakistan, *T. pratense* and *T. repens* are used for treatments of sore throat, fever, pneumonia, meningitis and feverish feeling (Khan and Khatoon, 2008). In the Albanian Alps in Kosovo, *T. pratense* is used in stomach disorders and *T. repens* is used in anti-diarrhoeal remedy (Mustafa et al 2012; Kolodziejczyk-Czepas, 2012). In India the Naga tribes have used *Trifolium repens* as a medicinal plant for deworming remedy. Tangpu et al. (2004) investigated this activity of white clover. In their study, they evaluated its anticestodal activity by changing doses and used experimental *Hymenolepis diminuta* Rudolphi infections in albino rats. Finally, they revealed that the aerial shoots of *T. repens* bear anticestodal properties and supports its use in the traditional medicine system. In America, natives have used the flowers of red clover for feeding. Beside of this, they have used it in treatment of wounds and to cure cronic skin diseases by mixing with other plants such as *Arctium lappa* L. (great burdock) and *Rumex crispus* L. (curly dock). Floral parts of red clover were also used in lung illness because of its expectorant effect (Kadıoğlu, 2008). Except white and red clovers, *T. alexandrinum* L., *T. angustifolium* L., *T. arvense* L., *T. pannonicum* Jacq. are used because of their different properties such as antidiabetic, wound healing, eczema and psoriasis therapy, effects on stomachache and diarrhea in folk medicine (Kolodziejczyk-Czepas, 2012). In Turkey, people collect red, white clovers and haresfoot clover (*T. arvense*). They usually collect upper parts (stem and leaves) of white and haresfoot clovers. Flowers of red clover are edible. Turkish people have used red clover as

expectorant, antiseptic and sedative. *T. repens* with flowering branches is used as tonic and analgasic and *T. arvense* is used in treatment of constipation (Işık, 2005).

Nowadays, researchers have been investigating the biological active compounds of the medical plants to discover drug. *Trifolium* genus are attracted to attention of scientists because of their valuable compound ingredients. Species of *Trifolium* have isoflavones, phenolic and polyphenolic compounds such as flavonoids, saponins, clovamides (caffeic acid esters), phenolic acids and other substances (Kolodziejczyk-Czepas, 2012). In the study of Tham et al. (1998), they grouped isoflavones as phytoestrogens and cited that phytoestrogens may potentially confer health benefits related to cardiovascular diseases, cancer, osteoporosis, and menopausal symptoms according to the evidence from molecular and cellular biology experiments, animal studies and a limited extent, human clinical. Thus, especially isoflavones of *T. pratense* have been studying much more than those of others (e.g. Drenin et al. 2011; Coon et al. 2007; Occhiuto et al. 2007; Booth et al. 2006; Blakesmith et al. 2003; De Rijkea et al. 2001). Oleszek et al. (2007) analyzed 57 *Trifolium* species for their contents of isoflavones, phenolic acids, flavonoids, and clovamides. They also made cluster analysis to identify these species, which should be interested as potential sources of these four metabolites. As a result of this, they revealed that, the isoflavone contents of the three species (*T. heldreichianum* Hausskn., *T. scabrum* L., and *T. subterraneum* L.) had extremely high amounts of the other compounds although *T. pratense* are known as rich source of isoflavones. All of the species were found in high amount of concentration in terms of all four analyzed groups of phenolics (isoflavones, phenolic acids, flavonoids, and clovamides). Flavonoids and other phenolic compounds such as catechins, saponins, clovamides and phenolic acids may be reason for the antioxidative activity of some *Trifolium* species. Briefly, dose controls, the exact effects and mechanisms of isoflavones and phenolic compounds are not known very well in humans. Thus these issues are controversial. However it is known that *Trifolium* species are giving hope for anti-platelet and anti-cancer therapy, remedy in menopausal complaints, cardiovascular diseases preventation. Eventually, clinical investigations and experiments must be continued (Kolodziejczyk-Czepas, 2012).

### 1.2.2 Systematic properties of the genus *Trifolium*

*Trifolium* genus is found in tribe Trifolieae with other sister genera; *Parochetus* Buch.-Ham. Ex Don, *Trigonella* L., *Factorovskya* Eig, *Medicago* L., *Melilotus* Mill. (Zohary and Heller, 1984). The taxonomic position of the genus is summarized in Table 1. This genus with nearly 255 species, is one of the largest genera in Fabaceae family (Ellison et al. 2006). It has wide distribution on all over the world. The genus has eight sections; *Lotoidea* Crantz., *Paramesus* (C. Presl) Endl., *Mistyllus* (C. Presl) Godr., *Vesicaria* Crantz., *Chronosemium* Ser., *Trifolium* L., *Trichocephalum* Koch., *Involucrarium* Hook. (Zohary and Heller, 1984).

Table 1- The taxonomic position of the genus *Trifolium* (Herrmann, 2006)

| | |
|---|---|
| **Kingdom** | Plantae |
| **Division** | Spermatophyta |
| **Subdivision** | Angiospermae |
| **Class** | Dicotyledoneae |
| **Order** | Fabales |
| **Family** | Fabaceae |
| **Subfamily** | Faboideae |
| **Tribe** | Trifolieae |
| **Genus** | *Trifolium* |

Leaves of *Trifolium* plants are three foliolate. The name '*Trifolium*' comes from this property. However, this is not available character for all plants of the genus. Some members such as subsection *Lupinaster* (Fabr.) Ser. plants in *Lotoidea*, have 5-9 foliolated leaves. Generally, leaves are alternate. In some species, upper leaves can be opposite. The lower leaflets often show differences from upper leaflets in shape and size. For example, the lower leaflets are always broader than upper ones. Stipules can change in form according to the sections. They are mostly entire but in sect. *Paramesus* and *Involucrarium*, they become dentate or lobed or deeply laciniate. *Trifolium* plants can be annuals or perennials. Flowers of them are variously coloured and can be changed small to medium sized. The floral characters

are same as mentioned before in subfamily Faboideae. Five petals differentiate in wings, keel and standard. Corolla colour of species shows variety. In some sections such as *Lotoidea*, *Chronosemium* have persistent corolla colour. However, in some of the sections, colours change after anthesis. Thus, inflorecences appear bicoloured. Stamens are ten and diadelphous. The shape and form of calyx change in species to species. This organ protects seed and helps seed dispersal. Presence and length of pedicel show differences according to species. Pedicels are associated with seed dispersial. Inflorescences can be consist of one, few or many flowers and mostly arranged in heads or spike-like racemes, umbels or rarely solitary. They have commonly peduncle but sometimes, they can be sessile. Bracts can be absent or minute or conspicuous. Their shapes can sometimes change in terms of places where they are found on the same plant. Pods are oblong or obovoid and usually enclosed with calyx. Nevertheless, pods sometimes can be longer than calyx. They are one or two seeded, rarely many-seeded (Zohary and Heller, 1984).

Due to importance of *Trifolium* species, many studies have been carried with them in different fields. To ensure using the correct species and to use them effectively, making their taxonomic classification is essential. Like other plants, *Trifolium* plants were studied firstly in the field of classic taxonomy. The systematic positions of them were considered by many authors. Finally, Zohary and Heller (1984) made its classification between sister genera and within itself. Their classification, based on sections, has been protecting its currentness. Beside classic taxonomy, phylogenetic studies were also done. Some cladistic studies were about systematic positions of *Trifolium* between its sister groups and its upper ranks (e.g Ellison et al. 2006; Steele and Wojciechowski, 2003; Gazara et al. 2001; Endo and Ohashi, 1997). Some of them were about its section classification (e.g Ellison et al. 2006, Vizintin et al. 2006, Watson et al. 2000). As a result of these studies, researches have been produced some different views about origin of the genus, centres of diversity and migration. In classification of the genus, some morphologic and anatomic characters were used from leaf, stem, calyx, pollen, and seed to differentiate the sections of *Trifolium* (Koçyiğit et al. 2013; Zoric et al. 2012; 2010; 2009). In addition to taxonomic studies, many studies which reveal the genetic

variation of *Trifolium* species and populations, have done by using molecular techniques (e.g Vymyslicky et al. 2012; Bennett and Mathews, 2003; Kölliker et al. 2003; Olmos et al. 2003; Gustine et al. 2002). These studies are explained in details as follows.

### 1.2.3 Taxonomic studies on the genus *Trifolium*

The genus *Trifolium* was known by classical naturalists. Before Linnaeus (1753), many herbalists gave names to them and some different plants were classified as *Trifolium* incorrectly. Even Linnaeus was mixed the genus *Trifolium* with its sister genera. Linnaeus started the clear-cut group classification. After Linnaeus, Savi (1808-1810) revised the Italian clover. He divided the genus into two groups; bracteate and ebracteate. Nowadays, this is applied as diagnostic feature in taxonomy of these species. Seringe (1825) did comprehensive monographic revision of the genus. He used different markers such as form of head and calyx, inflation and structure of calyx, the venation of the leaflets. Presl (1832) constituted a landmark with his classification. After him, virtually no one followed his classification but, according to Zohary and Heller (1984), nearly all of his clusters are valid today and used as sections. Some of them were renamed (e.g *Amarenus*=*Chronosemium*, *Amoria*=*Lotoidea*, *Galearia*=*Vesicaria*). All of these studies were about old world *Trifolium*. The first person who was interested in new world *Trifolium* was Lojacono (1883a; 1883b). He classified species of American clovers and produce new identification key, Coombe (1968) was studied with *Trifolium* genus in the 20$^{th}$ century (Zohary and Heller, 1984).

Finally, Zohary and Heller (1984) made a comprehensive revision and published their book named as 'The Genus *Trifolium*'. This is a main information resource in terms of different aspects. They defined species and provided identification key for the genus in the book. Also they produce a new classification system and cited their views about some subjects such as evolutionary characters of the genus, geographical distribution and centers of diversity, origin, speciation and migration. They divided 237 species into eigth sections; *Lotoidea*, *Paramesus*, *Mistyllus*, *Vesicaria*, *Chronosemium*, *Trifolium*, *Trichocephalum*, *Involucrarium*.

The plants of these sections have some common characters. These are mentioned as follows.

Section *Lotoidea*: It is the largest and heterogeneous section. Thus, to identify and separate the species is more difficult than others. This creates confusion. (The confusion is mainly because of the American species.) The main characters in this section are the umbellate shape of the inflorescence, the pedicellate and bracteate flowers, two or many-seeded legume often protruding from the calyx. These features are not found in all other species so they considered this section as the most primitive group.

Section *Paramesus*: Plants in this section are different from that of sect. *Lotoidea* because of the structures of pod and the presence of gland-bearing teeth on the stipules and calyx teeth.

Section *Mistyllus*: They have unique structure of the symmetrically vesiculate calyx, persistent corolla, the manifestly bracteolate flowers and 2-4 seeded pod dehiscing.

Section *Vesicaria*: They are known with resupinate flowers and unique morphological and ontogenetical development of the asymmetrical vesicular calyx.

Section *Chronosemium*: They have peculiar bilabiate calyx, a persistent corolla with a spoon or boat shaped standard, a small generally stipitate, one seeded pod.

Section *Trifolium*: It is the second largest section after *Lotoidea*. It is heterogeneous in appearance but the species have distinct features such as flowers sessile and ebracteate, throat of calyx tube with hairy or callous ring or entirely closed by a bilabiate protrusion, calyx limb often with unequal teeth, one seeded pod enclosed in the calyx tube.

Section *Trichocephalum*: They are different sharply from other plants. Their most flowers become sterile in inflorescences and convert into a mass of hairs or bristles. Thus, they can be identify easily.

Section *Involucrarium*: Their distinctive properties are the dentation of the calyx teeth, the sharply dentate or incised stipules, and the presence of an involucre at the based of the inflorescence displaying dentation similar to that of the stipules.

In recent time, the relationships of *Trifolium* species were evaluated in terms of their genomic material by improvement of technology. Two of these studies were related with phylogenetic analyses. They are, Watson et al. (2000) and Ellison et al. (2006), the two comprehensive phylogenetic studies on this genus. They constructed their dendrograms based on Zohary's section.

Watson et al. (2000) investigated the nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) and three genic regions of chloroplast DNA. They used 118 accessions for 59 species, which are represented the eight sections of *Trifolium*, were predominantly old world species. The regions of ITS1 and ITS2 were searched by sequence analysis. The three gene regions (*rbc*L, *trn*K, *rpo*C1-C2) of chloroplast in 44 species were cut by different restriction enzymes. The results were evaluated separately and together.

Many parts of Zohary and Heller (1984)'s classifications were supported by Watson et al. (2000)'s results. Restriction site results showed five resolved groups of old world species. On the other hand, ITS results showed four distinct groups. The combined data separated them into five clades. Section *Chronosemium* were found to be monophyletic. However, there was a problem related with its replacement. To solve it, additional markers must be used. Also, they were found that except *Chronosemium* none of sections show monophylety. In three different data, sect. *Vesicaria* and sect. *Mistyllus* were resembled as sister groups and they were related with sect. *Lotoidea* members. Although in previous studies both sections were considered as quite distinct groups, they were found closely related with sect. *Lotoidea* (Zohary, 1972). In addition to this, ITS phylogeny showed that sect.

*Paramesus* was embedded within *Lotoidea*. These two results provide an evidence for Zohary's view. Sect. *Lotoidea* were formed two clades in three data. One of them was basal grade, another one was unsupported clade. This shows that *Lotoidea* is not monophyletic. Sect. *Trifolium* were divided into two or three clades depending on the two dataset. Thus, it is seen that *Trifolium* section can not be monophyletic. To sum up, Watson et al. (2000)'s study could not exactly resolve the sections but supported Zohary works in some views.

Ellison et al. (2006) studied *Trifolium* with 218 species representing nearly 86% of the genus. They researched ITS regions in nuclear rDNA and intron region of leucine (*trn*L) in chloroplast genome. They evaluated their data separately and together. As a result of this, they offered a new taxonomic classification. They divided the genus into two clusters as subgenus *Chronosemium* (Ser.) Hossain and subgenus *Trifolium* (C. Presl) Hossain. Subgenus *Chronosemium* is consist of the members in section *Chronosemium* and subgenus *Trifolium* includes the whole other sections. They united sect. *Vesicaria*, sect. *Mistyllus* and some members of sect. *Lotoidea* under a new cluster named as sect. *Vesicastrum* Ser. *T. alpinum* L. and *T. polyphyllum* C.A. Mey. from sect. *Lotoidea* were jointed to some members of sect. *Paramesus* and named as sect. *Glycyrrhizum* Bertol. *T. lupinaster* L., *T. eximium* Steph. ex Ser. and *T. gordejevii* (Kom.) Z. Weifrom sect. *Lotoidea* were made a new section called as sect. *Lupinaster*. Some members of sect. *Lotoidea* formed sect. *Trifoliastrum* Gray. and the remainers constructed sect. *Involucrarium*. Thus, Zohary's section *Lotoidea* were divided into four pieces. *T. glanduliferum* Boiss. and *T. strictum* L. remained in sect. *Paramesus* and members of section *Trichocephalum* stayed in the same cluster. In subgenus *Chronosemium*, sect. *Chronosemium* divided into two main groups. In a branch *T. campestre* Schreb., *T. dubium* Sibth. and *T. micranthum* Viv., *T. patens* Schreb. formed sister groups. In another one *T. aureum* Poll., *T. spadiceum* L. and *T. rytidosemium* Boiss. & Hohen. formed sister group. Subgenus *Trifolium* shows monophylety with its paraphyletic basement groups. In this subgenus, sect. *Trifolium* with sect. *Trichocephalum* and sect. *Vesicastrum* with sect. *Trifoliastrum*, sect. *Involucrarium* constructed sister clusters. Section *Lupinaster* remained outside of these groups. Sect. *Paramesus* and sect.

*Glycyrrhizum* (Bertol.) Bobrov bound them as outgroups. The Ellison's classification is given with the comparison of those Zohary's in Table 2. They found Zohary's sect. *Vesicaria* and *Mistyllus* to be polyphyletic. Also, they could not solve exactly the relationships of the members from sect. *Involucrarium* including American clovers with each other. The same problem in African species from sect. *Vesicastrum* emerged.

Table 2- Comparison of Ellison's classification with Zohary and Heller's taxonomy (Ellison et al. 2006)

| **Ellison's classification** | **Zohary and Heller's taxonomy** |
|---|---|
| subgenus *Chronosemium* (Ser.) Reichenb. | sect. *Chronosemium* |
| subgenus *Trifolium* | |
| sect. *Glycyrrhizum* Bertol. | *T. alpinum*, *T. polyphyllum* (sect. *Lotoidea*) sect. *Paramesus* |
| sect. *Paramesus* (C. Presl) Berchtold and J. Presl | |
| sect. *Lupinaster* (Fabricius) Ser. | *T. eximium*, *T. gordejevii*, *T. lupinaster* (sect. *Lotoidea*) |
| sect. *Trifolium* | sect. *Trifolium* |
| sect. *Trichocephalum* Koch. | sect. *Trichocephalum* |
| sect. *Vesicastrum* Ser. | sects. *Mistyllus*, *Vesicaria* and *Lotoidea* in part |
| sect. *Trifoliastrum* Gray. | sect. *Lotoidea* in part |
| sect. *Involucrarium* Hook. | sect. *Lotoidea* in part |

Except these two phylogenetic studies, there is one more study in which less accessions were used. It is Vizintin et al. (2006)'s study which used 31 *Trifolium* species from old and new world. They assessed the interspecies relationships based on sequences polymorphisms of nuclear ITS regions in ribosomal DNA and

calculating genomic sizes. As a result, they constructed dendrogram by using the data from ITS sequencing. In general their classification did not resemble to Ellison and Watson's ones in terms of sections. Also they could not provide homogenity for each sections. Thus, they could not obtain a good separation. On the other hand, they succeeded to collect the sections *Mistyllus*, *Vesicaria* and African *Lotoidea* in the same subgroup. This approach remind the Ellison's sect. *Vesicastrum*.

Beside of morphologic and phylogenetic classifications, chromosomal characters are also used in researches. These characters assist to resolve the phyletic relationships of species. They reveal chromosomal evolutionary history. *Trifolium* genus were assessed in terms of chromosomal characters (e.g Uslu, 2012; Kıran et al. 2010; Majumdar et al. 2004; Taylor et al. 1979). In addition to this, sometimes anatomical characters are preferred to resolve taxa. For *Trifolium* genus, different characters from seed, leaf epiderm were tested (Zoric et al. 2012; 2010; 2009).

The researchers whose studies were mentioned, provide some views about evolutionary characters, diversity centres, speciation, and migration of *Trifolium*. Before cited these, distributions and ecologic properties of the genus must be known.

### 1.2.4 Distribution of the *Trifolium* genus

According to Zohary and Heller (1984), *Trifolium* genus tends to spread all temperate regions of both hemispheres. There are three main distribution centres; America, Africa and Eurasia. The Eurasia contents 59% of the species and they mostly concentrate in the Mediterranean region. The countries which have board on the Mediterranean region, own some endemic species. Turkey has the greatest number of them with nine endemic species. Africa has 15% of the species and the North African species are Mediterranean ones. Remainder species are belong to America. When discussing the distribution of the genus in terms of sections, Mediterranean region is the main centre of sections *Trifolium* and *Paramesus*. The number of species decreases towards Central Europe and Sahara desert. Sect. *Chronosemium* centred in the North Mediterranean countries and concentrates in the direction of Central Europe. The majority of sect. *Trichocephalum* shows

concentration in the East Mediterranean, Iran and in Central Anatolia. The centers of *Vesicaria* are North Mediterranean region and the East part of the Irano-Turanian regions. Section *Mistyllus* is found in the East Mediterranean region and the highlands of the Eritreo-Arabian region (Ethiopia, Erirea, Kenya, Uganda, Tanganyika). The members of section *Involucrarium* are American. *Lotoidea* has three diversity centres; West coast of North America with 40 species, the highlands of the Eritreo-Arabian with 33 species and Mediterranean territory of Southern Europe with 26 species.

### 1.2.5 Ecology of the *Trifolium* genus

These plants live with different habitats in subtropical, tropical and equatorial areas of Africa and South America. The majority are restricted to specific habitats such as coastal plains, inundated soils, marshy habitats. Many perennial clovers live in asidic and alpinic habitats. At least two-thirds of the species are found in their limited natural habitats such as mountain peaks, rocky slopes, mountain forests, meadows, alpine zones with even 4,000 m elevation (Zohary and Heller, 1984).

### 1.2.6 Characters of the *Trifolium* plants in evolution

In this concept, there are three main opinions about archetype of *Trifolium*. According to Zohary's, *Lotoidea* is the most primitive and the oldest one with its rich diversity. Hence, it has the widest distribution extends from America to Africa. When comparing this section plants with others, they show primitive characters such as the loose racemose inflorescence, the bracteolate, long-pedicelled and non-elaborate flowers and typical leguminous pod. These characters form a basement for evolution of other section's plants. *Trifolium* genus tends to reduce the pod size and the number of seeds per pod, loses the separation tissue leading to indehiscence and use some floral organs as dispersal accessories in its evolutionary process (Zohary and Heller, 1984).

According to the molecular study of Watson et al. (2000)'s, the basal Lotoidean grade were formed. The plants which were found in this grade have some

common characters such as perennial life span, bracteate flowers with open throats, many seeded pods and campanulate calyx. This supported Zohary's views on the archetype of genus *Trifolium*.

According to Ellison et al. (2006), subgenus *Chronosemium* (sect. *Chronosemium*) comes from an annual ancestor. In subgenus *Trifolium*, the common ancestor of sect. *Trifolium* and sect. *Trichocephalum* is an annual plant. Also, the common ancestors of sect. *Vesicastrum* (sect. *Vesicaria* + sect. *Mistyllus* + some members of sect. *Lotoidea*), sect. *Trifoliastrum* (some members of sect. *Lotoidea*) and sect. *Involucrarium* (some members of sect. *Lotoidea*) are perennial plants. These evolutionary changes happened step by step, not a time, and they always did not ocur in one direction for some sections such as sect. *Trifoliastrum*.

### 1.2.7 Views on diversity centres, speciation and migration

Before Zohary (1972), many researchers provide some views about the centre of origin and migration possibilities of the genus. Like Zohary (1972), they also thought section *Lotoidea* as an ancestor of other species. Some of them claimed that the origin of genus is based on Mediterranean region where they show greatest diversity and they migrated into equatorial Africa. On the other hand, some of them claimed that the highlands of East Africa could be the centre of origin. They later migrated from this place to everywhere and finally, they reached Mediterranean region, evolved in there (Zohary and Heller, 1984). According to Zohary (1972). There are two opinions about the origin and migration of the genus *Trifolium*. First one is that tropical African clovers in subsections of *Lotoidea* originated independently from an older Lotoidean stock. This ancestor entered in Africa by Mediterraneo-Afro-Alpine Line during Early Miocene. Second one is that some of these species migrated from the Northwestern part of North America to East Asia by Bering Strait during the Tertiary. The reason that makes Zohary to think in this manner, is the concentration of species in sections *Lotoidea* and *Involucrarium* in North America is much more than that in section *Lotoidea* in Eastern hemisphere. Also, he provided some evidences to support the second assumption. To sum up his idea, he thougth that sect. *Lotoidea* migrated from the Northwestern part of North

America to Eurasia and differentiated here into white clover groups and its relatives. In turn, this is caused to evolution of different *Lotoidea* subsections and the other six sections. After that, some species migrate from here into Africa. In America, few species migrate from North to South (Zohary and Heller, 1984).

According to Watson et al. (2000) new world clade enter into the old world clade. Thus it did not support to Zohary's opinion. The molecular data showed that the origin of the genus is Mediterranean region. Also the East African species of *Lotoidea* formed a group with *Mistyllus* and *Vesicaria* rather than Mediterranean *Lotoidea*.

Ellison et al. (2006) agreed with the two geographic clades include African (sect. *Vesicastrum*) and American (sect. *Involucrarium*) species. This manner is opposite to Zohary's view. Subgenus *Chronosemium* and early forms of subgenus *Trifolium* are restricted in Mediterranean region that is consistent with Mediterranean origin hypothesis. Also they added another possibility to this idea. They mentioned that some species from sect. *Vesicastrum* (e.g *T. resupinatum* L., *T. fragiferum* L.) which lives Mediterranean region could be African origin, if the species are native to Africa. Due to poor resolution, they could not understand the North or South origin of American clovers. However, they accepted the possibilities of dispersal events from North to South America.

As mentioned before, the main reason for people to think about the taxonomic, phylogenetic relationships and evolution of *Trifolium*, is their importance in different aspects. Because of this, *Trifolium* plants are in great demand. To supply the demand, plant breeding is very essential. It provides obtaining the qualified, desired and cheap products, protecting natural species from extinction, forming cultivated species by seed production, and increasing the variation in agriculture (Yücel, 2011). There are many studies on this genus in plant breeding (e.g. Williams et al. 2008; Abberton, 2007; Joyce et al. 1999).

### 1.3 Breeding Studies on *Trifolium* Genus and Molecular Markers

Clovers were domesticated around the year 1000, in southern Spain. Then, they extended from these country into Europe. The first usage in agriculture is based on 17$^{th}$ and 18$^{th}$ centuries. In those years, people came across the problem of nitrogen deficiency in European agriculture. They solved this problem by using clovers in agricultural field (Kjaergaard, 2003). Until today, *Trifolium* have been used for different aims which were explained before. Now, they are taken in breeding programmes to obtain the plants with desired traits and to exploit them efficiently. The most studied taxa are *T. repens* (white clover) and *T. pratense* (red clover). In most clover breeding programs, persistence is accepted as an essential character because it is referred to adaptability in different ecologic conditions effecting by biotic and abiotic factors. To improve persistence, researchers mostly have been trying to gain the plants diseases and insects resistance (Taylor, 2008). Beside of this, leaf size, stolon architecture, density, seed yield, forage quality, tolerance to abiotic stress such as salinity and drougth are the other characters investigating in white clover breeding (Ravagnani et al. 2012; George et al. 2006). Besides persistence, disease and pathogen resistence, forage quality and yield, tolerance to abiotic stress such as cold, flooding, drougth are the desirable traits in red clovers too (Ravagnani et al. 2012; Herrmann et al. 2006).

In breeding programmes, firstly the diversity of species are revealed. Prior to the mid 1960's, plant breeders have utilized morphological, agronomical and physiological characters. In this manner, the evaluations of variations became bias arising from environmental variabilities. To escape from this, molecular genetic markers have been using together with phenotypic characters or singly. Also they allow the rapid estimation of whole genome and revealed intra- and inter-cultivar genetic variations at the DNA level (Khanlou et al. 2011; George et al. 2006; Kölliker et al. 2001b).

The genetic markers can be divided into two main classes; locus-specific hypervariable microsatellite markers, and multilocus arbitrary fingerprinting techniques (Hollingsworth and Ennos, 2004). Microsatellites or simple sequence

repeats (SSR) are short tandem repeats. They are found in non-coding regions of all eukaryote genomes and polymerase chain reaction (PCR) based genetic markers. SSRs shows high polimorphism even among closely related cultivars. Thus they are cited as more informative markers. Because of their co-dominancy, reproduciblity and reliablity, they are found as suitable markers for assessment of genetic diversity and taxonomic studies (Cholastova and Knotova, 2012; Fahima et al. 2002). Random amplified polimorphic DNA (RAPD) is an example of multilocus arbitrary DNA markers. RAPDs provide quick and inexpensive research. They are universal primers, so they can be used for any plant species that were investigated. To apply this method, whole genomic information is not necessary (Mengoni et al. 2000; Quiroz and Klose, 2001).

These markers have been used for *Trifolium* genus in various applications such as genetic and physical maps, quantitative trait locus (QTL), marker assisted selections (MAS) and evaluation genetic diversity of cultivars (Ravagnani et al. 2012). Researches are interested in these techniques to find out the desirable trait loci and to improve plants. SSR markers are usually preferred in genetic mapping and QTL researches.

Kölliker et al. (2001a) studied with white clover. They intended to develop and characterise a comprehensive set of SSR markers which can be utilized in QTL and mapping researches. They constructed enriched libraries from inbred *T. repens* genotype I7J and screened SSR primers for polymorhism across a set of 7 *T. repens* cultivars. Then, they tested the same SSRs with different *Trifolium* and sister taxa. As a result, their study succeeded the development of several *T. repens* DNA libraries highly enriched for SSR loci. They also indicated that their SSR primers can be used for the other *Trifolium* taxa. Jones et al. (2003) constructed a framework genetic map for *T. repens* by using SSR and Amplified Fragment Length Polymorphisms (AFLPs). This was the first molecular based genetic linkage map of white clover. They used 135 markers and identified 78 TRSSR loci with 68 SSR primers and proved the usage of SSR primers in mapping. Zhang et al. (2007) used SSR markers from several legumes such as white, red clovers to reveal the genetic map of allotetraploid white clover population of southern Great Plains of USA. At

last, they compared their map with *T. repens*, *T. pratense* and other taxa of studies which were made before. They found significant divergences in genome organization and rearrangements and suggested to use SSR markers together with FISH (Fuorescent in situ hybridization) analysis in finding marker location and gene relationships. Herrmann et al. (2006) focused on seed yield quality. To improve this character, they decided to find out the genes and their linkage genes that control the character by using SSR and AFLP primers. In their study, total of 42 SSR and 216 AFLP loci were used to construct a molecular linkage map and total of 38 QTLs were revealed for 8 seed yield components. Except these studies, many other researches have been made about QTLs, genetic linkage maps (e.g Wang et al. 2010; Zhang et al. 2008; Herrmann et al. 2008).

SSR and RAPD markers have been used in detecting genetic variation of *Trifolium* in infra-species level. Dolanska and Curn (2004) used RAPD, SSR, ITS regions in rDNA and PCR-RFLP (PCR-restriction fragment length polymorphism) methods to identify *T. repens* cultivars. They used 30 plants in cv. Hajek cultivar type and 12 plants in 4 other cultivar types for 6 RAPD primers. They metioned that RAPD gave high polymorphism for these plants within one population. Thus they found RAPD to be suitable in identification of indivudial level, not inter-populations level. On the contrary, they applied 5 SSR primers in 11 cutivars and 3 *Trifolium* taxa (*T. pratense*, *T. hybridum*, *T. incarnatum* L.) and suggested these primers to researchers for identification of *Trifolium* populations from each other. Dias et al. (2008) tested SSR markers and morphological characters in 58 *T. pratense* populations. They used 14 SSR primers and these markers gave high polymorhism on those taxa. Finally, they compared the result of SSR markers with morphologic markers to separate populations from each others. Greene et al. (2004) made a similar study with RAPD and morphological markers. They classified 33 wild red clover populations. In both studies (Dias et al 2008 and Greene et al. 2004), reserachers stated the incongruity of molecular and morphologic markers. Except red and white clover, there are some studies about different *Trifolium* taxa. Crowford et al. (1998) utilized RAPDs in *T. stoloniferum* Muhl. ex A. Eaton. They could get enough polymorphism between 21 populations. Arzani and Samei (2004) separated

20 *T. resupinatum* cultivars by using 8 RAPD primers. They found RAPDs to be successful in determination of cultivars. Beside these researches, there are some other clover studies in which RAPD and SSR markers were used (e.g Vymyslicky et al. 2012; Bortolini et al. 2006; George et al. 2006).

### 1.4 Aim of the Study

In Turkey, people have come across some problems in obtaining sufficient qualified forage. However, Flora of Turkey states rich sources especially in *Trifolium* genus with its nearly 100 species (Cocks, 1993; Davis, 1970). Thus, revealing their diversity and adding them to our agriculture is crucial. Also, breeding programmes can solve the problem in finding qualified products. On the other hand, as we know, studies which fasten on the solution have not been done yet. Bolu contains four sections of *Trifolium* genus. In other words, Bolu is one of the place to shelter quite large diversity of *Trifolium* plants. This opportunity makes inevitable to study *Trifolium* in this place. In the taxonomic point of view, some problems are known in classification of heterogenous section groups such as sect. *Trifolium* and *Lotoidea*. Also there are some arguments on subtaxa. Because of these, aims of the study are;

1) To reveal the inter-species diversity and draw lines of species,
2) To understand the relationships of the members in the same section and also among sections, to help the solution of classification in heterogenous sections,
3) To compare morphologic and molecular markers in terms of their effectiveness on separation of *Trifolium* species,
4) To light to the other studies in taxonomy and other research fields.

# 2. MATERIALS AND METHODS

## 2.1 Study Materials

The *Trifolium* genus and subgenus taxa are differentiated from each other according to their flower structers. Thus, plant sample collection was carried during the spring time. The distribution of *Trifolium* species in Bolu province were determinated based on the Flora of Turkey; Davis (1970). The field studies were made between 2011 and 2012. The first collection lasted from March to August. These studies were repeated in 2012 to obtain those lacking herbarium specimens from the same locations. During the field studies, some photos of *Trifolium* were taken as seen in Figure 1. Initial expectation from these studies was finding 15 species around the Bolu, that is written on the Flora, but nearly doubled number species were obtained in the field studies. The collected samples of informations are shown in the Table 3. Total 26 species and 3 varieties were included in this study (Table 3). The collected plants are represented the four sections of *Trifolium* genus and kept herbarium species in AİBU herbarium. Identification of the plants was made to based on the Flora of Turkey; Davis (1970). Beside this, a special database program named as DELTA (URL2) was used. This identification program is based on the *Trifolium* species from Turkey. Finally, an expert who studied on *Trifolium* species helped for identifications of samples.

After being certain of the taxa, plant materials separated for molecular and morphometric studies. For the molecular part of the research, the clean leaves were chosen to eliminate contamination, then dried with silica gels in small plastic package. In both studies, giving a specific number for each localites was ensured.


Figure 1- Representation photos of studied*Trifolium* taxa a) *T. hybridum* b) *T. physodes* c) *T. pratense*d) *T. medium* e) *T. pannonicum* f) *T. fragiferum*

Table 3- *Trifolium* taxa with collecting informations

| TAXON NAME | LOCALITY | COORDINATE | ALTITUTE |
|---|---|---|---|
| **Section *Lotoidea* L.** | | | |
| *T. repens* L.var. *repens* | Karacasu | 40° 40.920'N<br>31° 36.325'E | 740 m |
| *T. repens* L.var. *macrorrhizum* (Boiss.) | Kartalkaya | 40° 35.412'N<br>31° 48.208'E | 2050 m |
| *T. hybridum* L.var. *elegans* (Savi) Boiss. | Kızılcahamam | 40° 39.120'N<br>32° 27.779'E | 1425 m |
| *T. hybridum* L.var. *anatolicum* (Boiss.) | University | 40° 42.789'N<br>31° 30.890'E | 865 m |
| *T. nigrescens* Viv subsp. *petrisavii* (Clem.) | Kıbrıscık | 40° 27.107'N<br>31° 44.099'E | 1350 m |
| *T. retusum* L. | Kıbrıscık | 40° 28.672'N<br>31° 42.472'E | 1385m |
| **Section *Chronosemium* Ser.** | | | |
| *T. patens* Schreb. | Kartalkaya | 40° 35.823'N<br>31° 38.643'E | 1332 m |
| *T. campestre* Schreb. | University | 40° 42.858'N<br>31° 30.673'E | 861 m |
| *T. aureum* Poll. | Bolu-Seben | 40° 26.768'N<br>31° 43.765'E | 1300 m |
| *T. dubium* Sibth. | Kartalkaya | 40° 35.489'N<br>31° 48.195'E | 2010 m |
| *T. micranthum* Viv. | Kartalkaya | 40° 35.489'N<br>31° 48.195'E | 2010 m |
| *T. spadiceum* L. | Bolu-Seben | 40° 26.768'N<br>31° 43.765'E | 1300 m |

Table 3- *Trifolium* taxa with collecting informations (Continued)

| | | | |
|---|---|---|---|
| **Section *Fragifera* Koch.** | | | |
| *T. fragiferum* L. var. *fragiferum* | University | 40° 42.524'N<br>31° 30.490'E | 870 m |
| *T. physodes* Stev. ex Bieb. var. *physodes* | University | 40° 42.375'N<br>31° 31.119'E | 836 m |
| *T. resupinatum* L. var. *resupinatum* | Karacasu | 40° 40.920'N<br>31° 36.325'E | 740 m |
| **Section *Trifolium* L.** | | | |
| *T. scabrum* L. | University | 40° 42.510'N<br>31° 31.062'E | 860 m |
| *T. hirtum* All. | Kaynaşlı | 40° 46.415'N<br>31° 20.829'E | 363 m |
| *T. costantinopolitanum* Ser. | Kaynaşlı | 40° 46.415'N<br>31° 20.829'E | 363 m |
| *T. pallidum* Waldst & Kit. | Mudurnu | 40° 38.523'N<br>31° 27.388'E | 880 m |
| *T. arvense* L. | Gerede | 40° 39.195'N<br>32° 22.048'E | 1300 m |
| *T. ochroleucum* Huds. | Gerede | 40° 39.195'N<br>32° 22.048'E | 1300 m |
| *T. medium* L.var. *medium* | Gerede | 40° 39.195'N<br>32° 22.048'E | 1300 m |
| *T. medium* L.<br>var. *eriocalycinum* Hausskn. | University | 40° 42.789'N<br>31° 30.890'E | 865 m |
| *T. striatum* L. | Kaynaşlı | 40° 46.415'N<br>31° 20.829'E | 363 m |

Table 3- *Trifolium* taxa with collecting informations (Continued)

| | | | |
|---|---|---|---|
| *T. diffusum* Ehrh. | Kıbrıscık | 40° 28.672'N<br>31° 42.472'E | 1385 m |
| *T. pratense* L.var. *pratense* | University | 40° 42.789'N<br>31° 30.890'E | 865 m |
| *T. bocconei* Savi. | Gerede | 40° 39.195'N<br>32° 22.048'E | 1300 m |
| *T. pannonicum* Jacq. subsp. *elongatum* | University | 40° 42.938'N<br>31° 31.067'E | 880 m |
| *T. echinatum* Bieb. | University | 40° 43.122'N<br>31° 31.620'E | 814 m |

## 2.2 Molecular Methods

### 2.2.1 DNA isolation

Total genomic DNA isolation was made from young, fresh and clean leaves. The leaves were dried in a plastic bag with silica gel and DNA isolation was made by using Nucleospin Plant Kit. In this part of the research, two type of moleculer markers was used; SSR and RAPD.

### 2.2.2 Determination of primers

For application of RAPD-PCR, many articles were searched. 14 primers which showed polymorphism in the studies were selected from Ulloa et al. (2003) (OPAL-12, OPAB-14, OPAR-19, OPAV-08, OPBA-07); Bortolini et al. (2006) (OPH-02, OPH-07, OPH-12, OPH-15, OPAQ-09); Arzani and Samei (2004) (OPB-14). Nevertheless, 11 primers gave polymorhism and reproducible banding pattern for all the studied taxa. Thus, the study were continued with these 11 primers. Their names and sequences are given in Table 4. In the application of SSR-PCR, 7 primers were tried but 5 primers were found suitable for this study. These 5 primers were taken from Soldanova (2007) (TRSSRATS054, TRSSRATS055); Dolanska and

Curn (2004) (TRSSRAXX31, TRSSRA02C03, TRSSRA02C02). Names and sequences of SSR primers are shown in Table 5.

### 2.2.3 RAPD-PCR and visualization processes

DNA amplification was performed in a Progene Thermal Cycler (Techne-Promega). The RAPD reaction condition was as follows:

Each reaction was carried out in total volume of 25 µl. About 25 ng of genomic DNA, 2.0 mM $MgCl_2$, 1.0 mM of each dNTP, 1.0 µM of each primer, 1.0 U of *Taq* polymerase and 1X PCR buffer ($(NH_4)SO_4$).

The PCR cycle program was as follows:

Initial denaturation step was by 5 min at 93 °C, then 45 cycles at 92 °C for 1 min (denaturation), 35 °C for 2 min (annealing), and 72°C for 3 min (elongation), and a final extention at 72 °C for 10 min.

Table 4- Names and sequences of RAPD primers

| **Primers** | **Sequences (5'-3')** |
|---|---|
| OPAL-12 | CCCAGGCTAC |
| OPAB-14 | CTGATCGCGG |
| OPAR-19 | AACCCTTCCC |
| OPH-02 | TCGGACGTGA |
| OPH-07 | CTGCATCGTG |
| OPH-12 | ACGCGCATGT |
| OPH-15 | AATGGCGCAG |
| OPAV-08 | AAGTGCGACC |
| OPBA-07 | GGGTCGCATC |
| OPAQ-09 | AGTCCCCCTC |
| OPB14 | TCCGCTCTGG |

For optimization of primers, many temperature ranges were applied and the conditions which mentioned above were found suitable.

The RAPD products were separated by 1.5% agarose gel electrophoresis. When the DNA products were loaded, blank samples containing water instead of DNA were used for the negative control. 0.25–10 kb DNA ladder (Promega) was used to estimate the sizes of PCR products. Electrophoresis was performed at 100 W for approximately 1 hours. Each RAPD reaction was carried out twice. Then to make visable, the gel was stained in 0.5 μg/mL ethidium bromide. Images were photographed with a gel documentation system using UV ligth (Ultralum-Teknis).

### 2.2.4 SSR-PCR and visualization processes

DNA amplification was performed by the same machine used in application of RAPD-PCR.

SSR reaction conditions were as follows:

Reactions were carried out with a 25 μL final volume solution containing 1X buffer ($(NH_4)SO_4$), 1.0 mM of each dNTP, 1.0 μM of each SSR primer (forward and reverse), 2.0 mM of $MgCl_2$, 1.0 U of *Taq* polymerase, and 50 ng of genomic DNA. The reaction program of the primers was as follows:

Pre-denaturation step of 4 min at 95 °C, then 30 cycles with 30 s at 95°C (denaturation), 30 s at 55 °C (annealing), and 30 s at 72 °C (extension), followed further by 10 of the same cycles with annealing temperature 53 °C and 10min at 72 °C as the final extension (Herrmann et al. 2006).

Table 5- Names and sequences of SSR primers

| **Primer** | **Forward primers (F) (5'-3')** |
|---|---|
| TRSSRATS054 | (F) GACACCGATTATGTGCAAGA |
| | (R) AATCACGACGAGCGACAAC |
| TRSSRATS055 | (F) CAATACAATCACCGCACCAG |
| | (R) TCTCTGCTTCGCGTCTTCTC |
| TRSSRAXX31 | (F) TCTGTTTTGTTGGCCATGC |
| | (R) TTGCAAAGTGTTTGGAAGGA |
| TRSSRA02C03 | (F) TATGCTGGTAGATAAACTTAAA |
| | (R) TGCTCTGGAGATTGATGG |
| TRSSRA02C02 | (F) AAATAAAAACCACAAGTAACTAG |
| | (R) TATAGGTGATTTGAAATGGC |

PCR products were separated on 8% polyacrylamide gel with a 1X TBE buffer. Electrophoresis was performed at 70 W for approximately 3 hours on a 16 × 14 cm gel apparatus (Thermo Scientific-OWL P9DS) and was revealed through ethidium bromide staining. The amplification of the total number of taxa for each marker was performed in 2 gels that ran at the same time under the same conditions. PCR products were estimated using 100 bp plus a DNA ladder (Fermentas). Then to make visiable, the gel was stained in the same concentration of ethidium bromide which were used in RAPD applications. Finally, images were photographed.

### 2.2.5 Analysis of band profiles

All fragments obtained from both molecular markers for the 29 samples were scored manually. The band size were determined based on DNA ladders and were coded as absence (0) or presence (1). Faint and unreliable bands were not considered for the analysis. The genetic similarity between the *Trifolium* taxa was calculated according to Jaccard's coefficient (Jaccard, 1908). Unweighted pair group method

(UPGMA) dendrograms were constructed by using NTSYSpc program then the same data uploaded to the FreeTree program (Hampl et al. 2001). RAPD and SSR marker data were evaluated separately and finally combined for analysis.

## 2.3 Morphological Methods

### 2.3.1 Determination of morphological characters

In this part of the study, quantitative and qualitative characters from stem, leaves, flowers were used for comparing the taxa. Totally 26 characters which included 18 quantitative and 8 qualitative were selected for the study. The quantitative characters are shown in Figure 3, 4 and 5 and explained in Table 6. DELTA intkey database and the articles: Vymyslicky et al. (2012), Bulinska (2000), Williams et al. (2001) and Bennett (2000) were utilized to determine characters.

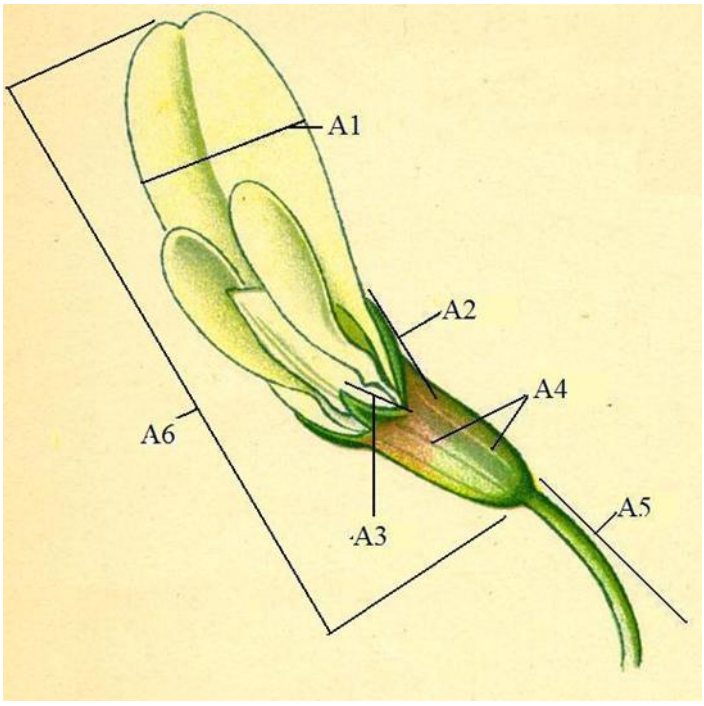


Figure 2- Quantitative characters on the flower (1) (URL3)

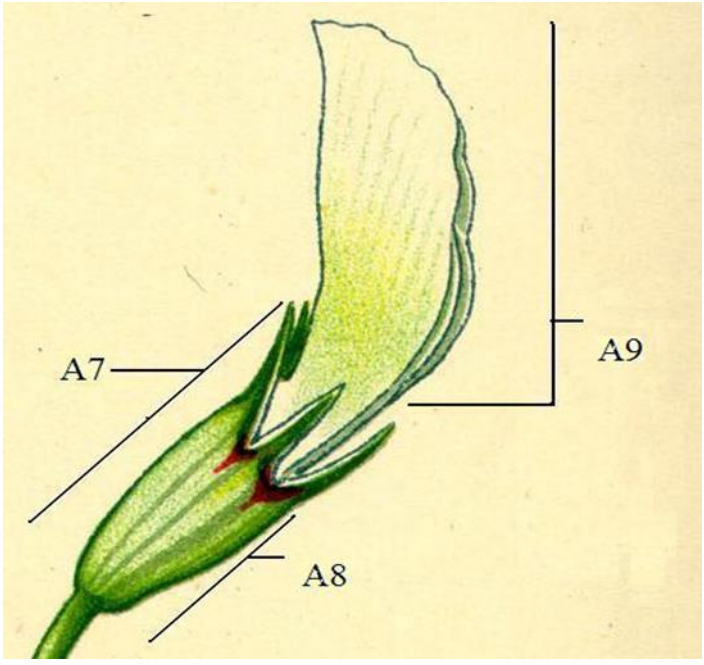


Figure 3- Quantitative characters on the flower (2) (URL3)

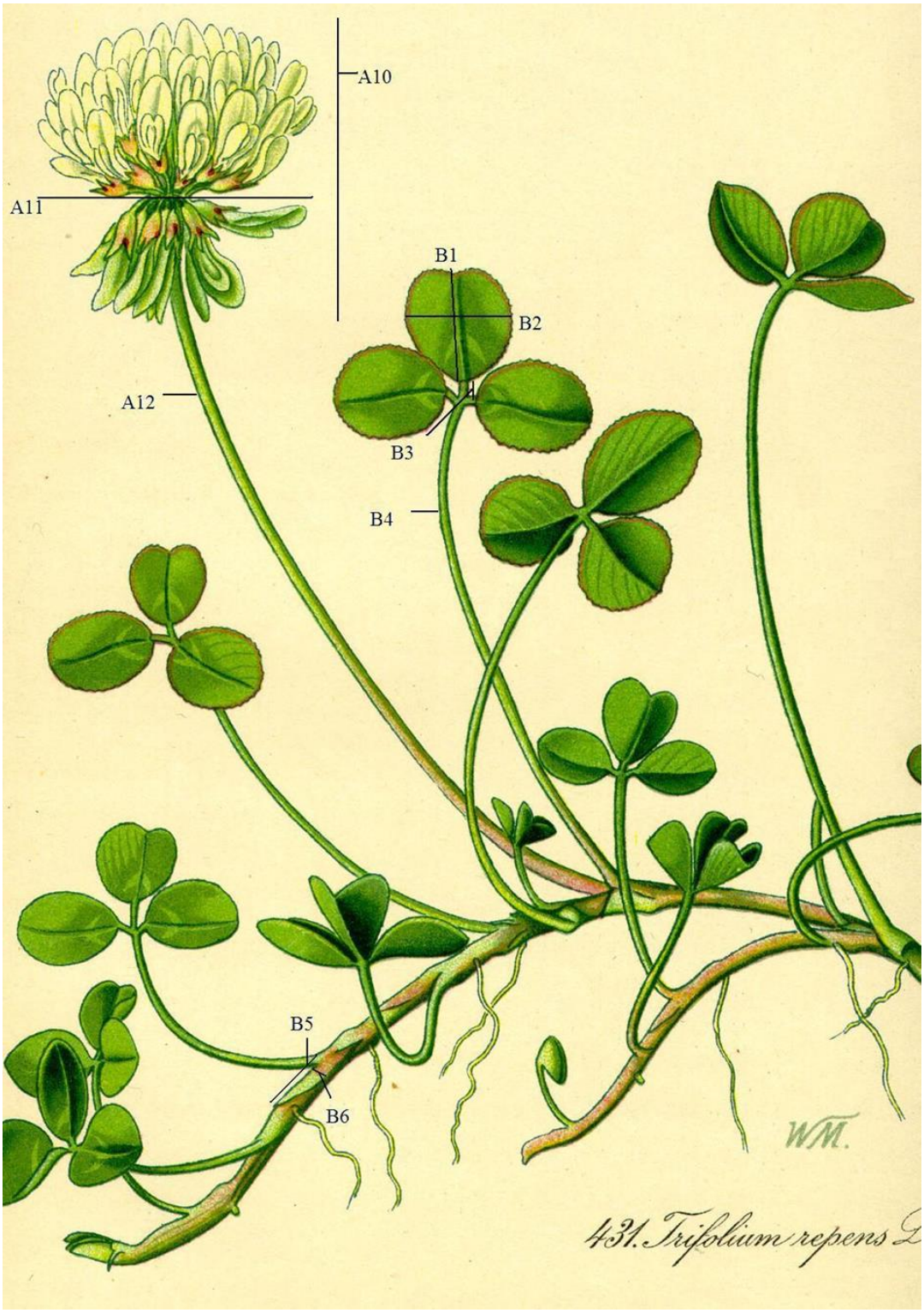


Figure 4- Quantitative characters on the flower and stem (URL3)

Table 6- Explaination of the characters shown in Figures 3,4, 5 with their codes

| A | FLOWER CHARACTERS | CODES | B | STEM CHARACTERS | CODES |
|---|---|---|---|---|---|
| A1 | Standard width | SDW | B1 | Terminal leaflet length | TLL |
| A2 | Long teeth calyx length | LTCL | B2 | Terminal leaflet width | TLW |
| A3 | Short teeth calyx length | STCL | B3 | Terminal petiolule length | TPL |
| A4 | Number of calyx veins | NCV | B4 | Petiole length | PTL |
| A5 | Pedicel length | PCL | B5 | Stipule length | SL |
| A6 | Individual flowering length | IFL | B6 | Stipule width | SW |
| A7 | Flowering calyx length | FCL | | | |
| A8 | Calyx tube length | CTL | | | |
| A9 | Standard length | SDL | | | |
| A10 | Inflorescence length | IL | | | |
| A11 | Inflorescence diameter | ID | | | |
| A12 | Peduncle length | PDL | | | |

### 2.3.2 Measurement and evaluation of characters

The measurements were done by selecting 10 individuals from each taxa and taking between 5 to 10 measures for each characters from these plants. To avoid seasonal and positional variation, flowers, leaves, stipules were selected from the same arrangements in the stem. For example, leaflets were chosen from the middle parts of the all plants. All flowers were taken from the upper part of the plants. All stipules were measured at the top of the plants. The base parts were preferred for petiole length in all measured plants. Well opened flowers were used for measurements in the flower characters and measured under the light microscope except peduncle length, inflorescence length and diameter.

After the measurements under the microscope, the units were transformed into centimeters. The other characters were measured by ruler. The means of the characters for each indvudials were calculated (seen as Appendix A). These mean values were used to draw boxplot graphics in the Minitab program for each quantitative characters (shown as Appendix B). Thus, these characters could be coded to analize together with qualitative characters.

Like quantitative characters, qualitative ones were considered over the same 10 individuals from each taxa. For each character state, a different code was given. These codes are shown with their states in Table 7. Finally, all of the morphological characters were analysed together in Phylogeny Inference Package (PHYLIP) program by constructing UPGMA dendrogram.

Table 7- Qualitative characters with their codes

| **CHARACTERS** | **CHARACTER STATES** | **CODES** |
|---|---|---|
| Calyx tube pubescence | Hairless | 0 |
| | Hairy | 1 |
| Presence of bracts | Absent | 0 |
| | Present | 1 |
| Stem pubescence | Hairless | 0 |
| | Hairy | 1 |
| Presence of bracteole | Absent | 0 |
| | Present | 1 |
| Life span | Annual | 0 |
| | Perennial | 1 |

Table 7- Qualitative characters with their codes (Continued)

| CHARACTERS | CHARACTER STATES | CODES |
|---|---|---|
| Leaflet shape | Obovate | 0 |
| | Obovate-oblong | 1 |
| | Obovate-elliptical | 2 |
| | Obovate-cuneate | 3 |
| | Broadly obovate | 4 |
| | Elliptical-oblong | 5 |
| | Broadly elliptical | 6 |
| | Linear oblong | 7 |
| | Cuneate-oblong | 8 |
| Type of leaflet margin | Entire | 0 |
| | Dentate | 1 |
| | Denticulate | 2 |
| | Serrulate | 3 |
| Shape of calyx tube | Elliptical | 0 |
| | Cylindrical | 1 |
| | Campanulate | 2 |
| | Obconical | 3 |

# 3. RESULTS

## 3.1 Molecular Results

### 3.1.1 RAPD marker results

In order to determine the genetic relationships of 29 *Trifolium* taxa from Bolu, 14 RAPD primers were initially tested. Only 11 primers out of the 14 were selected based on the quality and reliability of their amplification with all taxa. OPG-06, OPG-16, OPAP-01 were not found to be polymorphic for the studied taxa. The images of primer patterns are seen as between Figure 5 and Figure 15. The number, codes and names of taxa in figures are explained in Table 8. A total of 446 bands were observed within 11 primers. The number of PCR products according to the primers varied from 32 to 46 and presented molecular weights between 300 and 3500 bp. (Table 9). The most common band was 1250 bp, found in 12 of 29 taxa in OPH-02 primer. The mean percentage of polymorphism for 11 RAPD primers was 84% (Table 9).

Random amplified polymorphic DNA fragments were scored for Jaccard's similarity coefficient calculations (Table 1C seen in Appendix C). According to this similarity table (Table 1C), the highest genetic similarity (0.33) was found between *T. hirtum* and *T. constantinapolitanum*, and the least similarity (0.06) was observed between *T. aureum* and *T. pallidum*. Those species with high genetic similarity belonged to the section *Trifolium*. In contrast, those species which had the least similarity, *T. aureum* and *T. pallidum*, were in the sections *Chronosemium* and *Trifolium*, respectively. A phenogram using the UPGMA algorithm based upon Jaccard's coefficient similarity was constructed (Figure 16).

Table 8- Numbers, codes and names of taxa for Figures 5-15

| TAXA NUMBERS | TAXA CODES | TAXA NAMES |
|---|---|---|
| 1 | RES | *T. resupinatum* |
| 2 | PHY | *T. physodes* |
| 3 | FRA | *T. fragiferum* |
| 4 | REPM | *T. repens* var. *macrorhizum* |
| 5 | REPR | *T. repens* var. *repens* |
| 6 | HYBA | *T. hybridum* var. *anatolicum* |
| 7 | HYBE | *T. hybridum* var. *elegans* |
| 8 | NIG | *T. nigrescens* subsp. *petrisavi* |
| 9 | RET | *T. retusum* |
| 10 | DUB | *T. dubium* |
| 11 | MIC | *T. micranthum* |
| 12 | SPA | *T. spadiceum* |
| 13 | AUR | *T. aureum* |
| 14 | CAM | *T. campestre* |
| 15 | PAT | *T. patens* |
| 1 | PRA | *T. pratense* var. *pratense* |
| 2 | PAN | *T.pannonicum subsp. elongatum* |
| 3 | SCA | *T. scabrum* |
| 4 | STR | *T. striatum* |
| 5 | HIR | *T. hirtum* |
| 6 | CON | *T. costantinapolitanum* |
| 7 | ECH | *T. echinatum* |
| 8 | PAL | *T. pallidum* |
| 9 | MEDE | *T. medium* var. *eriocalycinum* |
| 10 | MEDM | *T. medium* var. *medium* |
| 11 | OCH | *T. ochroleucum* |
| 12 | BOC | *T. bocconei* |
| 13 | DIF | *T. diffusum* |
| 14 | ARV | *T. arvense* |

Table 9- Percentage of polymorphic bands in RAPD primers

| **Primer** | **Sequence (5'-3')** | **Total number of bands** | **Percentage of polymorphic bands (PBS%)** |
|---|---|---|---|
| OPAL-12 | CCCAGGCTAC | 38 | 83.93 |
| OPAB-14 | CTGATCGCGG | 37 | 83.86 |
| OPAR-19 | AACCCTTCCC | 38 | 83.48 |
| OPH-02 | TCGGACGTGA | 45 | 86.13 |
| OPH-07 | CTGCATCGTG | 32 | 85.45 |
| OPH-12 | ACGCGCATGT | 45 | 82.92 |
| OPH-15 | AATGGCGCAG | 46 | 85.31 |
| OPAV-08 | AAGTGCGACC | 38 | 84.66 |
| OPBA-07 | GGGTCGCATC | 46 | 82.75 |
| OPAQ-09 | AGTCCCCCTC | 41 | 81.91 |
| OPB-14 | TCCGCTCTGG | 40 | 86.98 |
| **Total** | | 446 | **mean=** 84.30 |

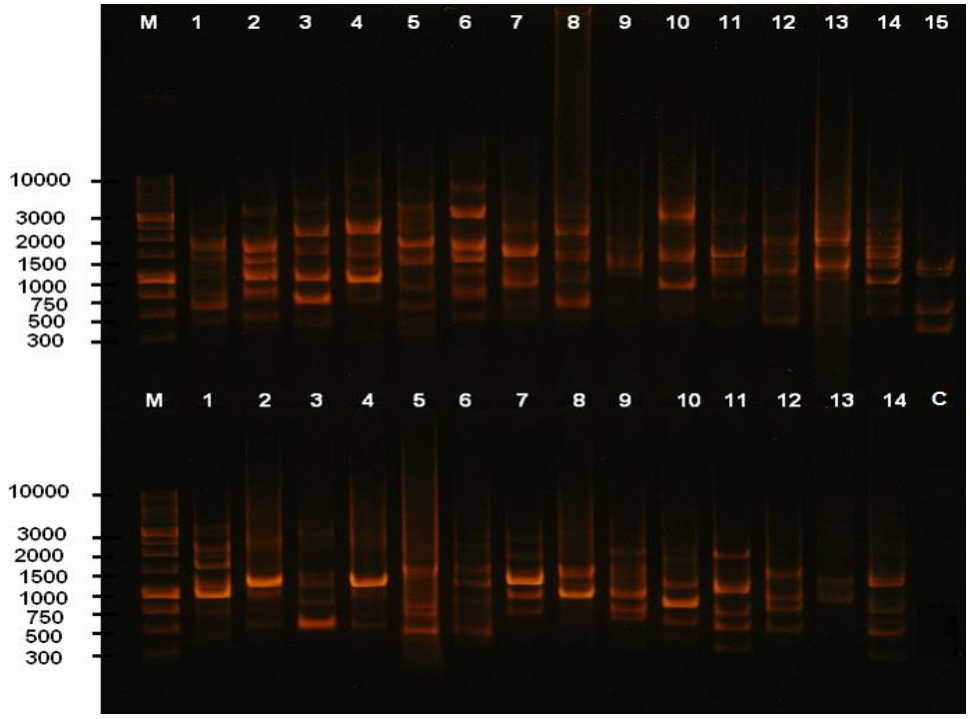


Figure 5- Banding pattern of the RAPD-PCR with OPH-02

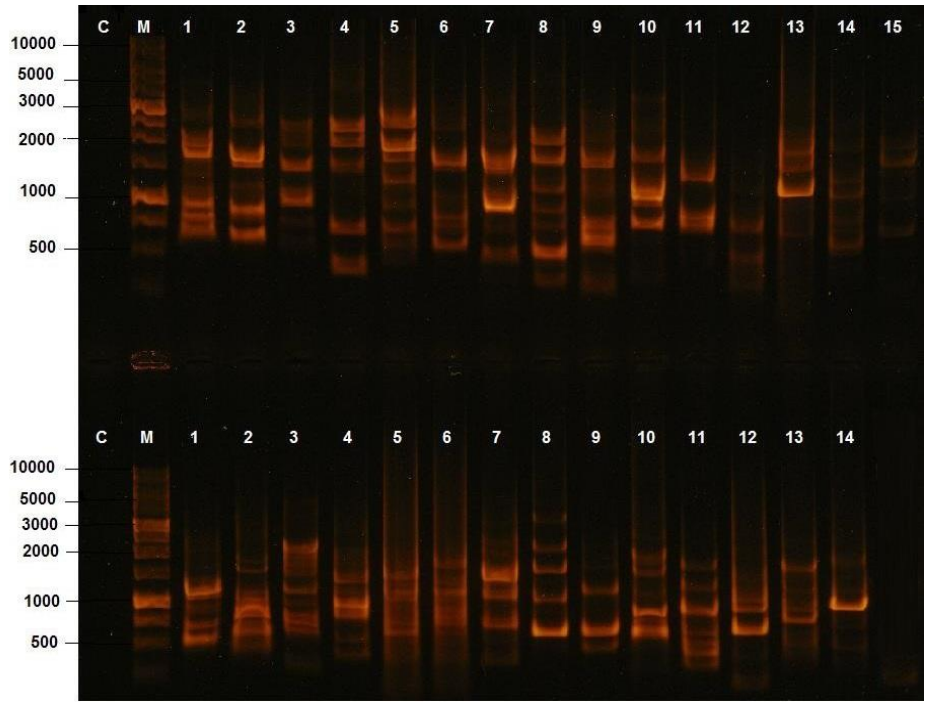


Figure 6- Banding pattern of the RAPD-PCR with OPH-07

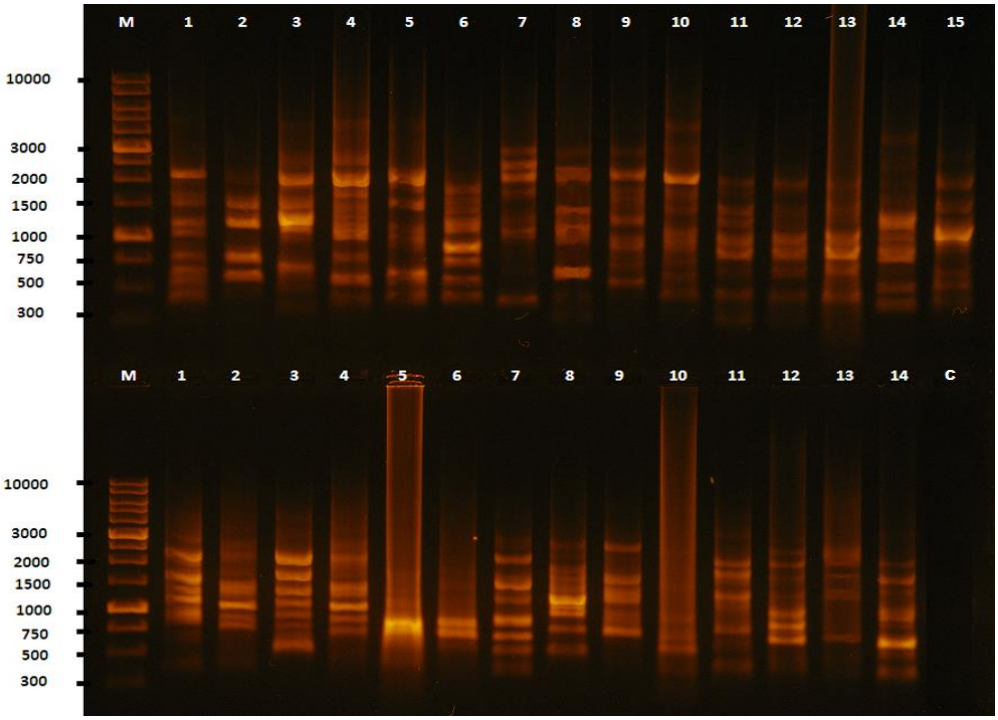


Figure 7- Banding pattern of the RAPD-PCR with OPH-12

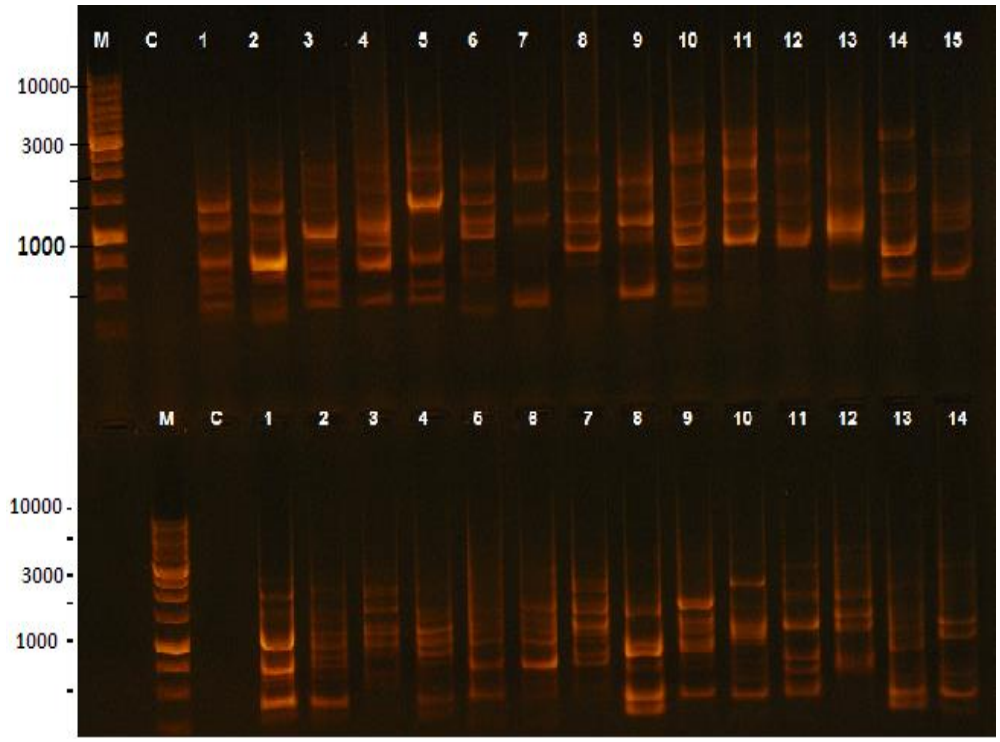


Figure 8- Banding pattern of the RAPD-PCR with OPH-15

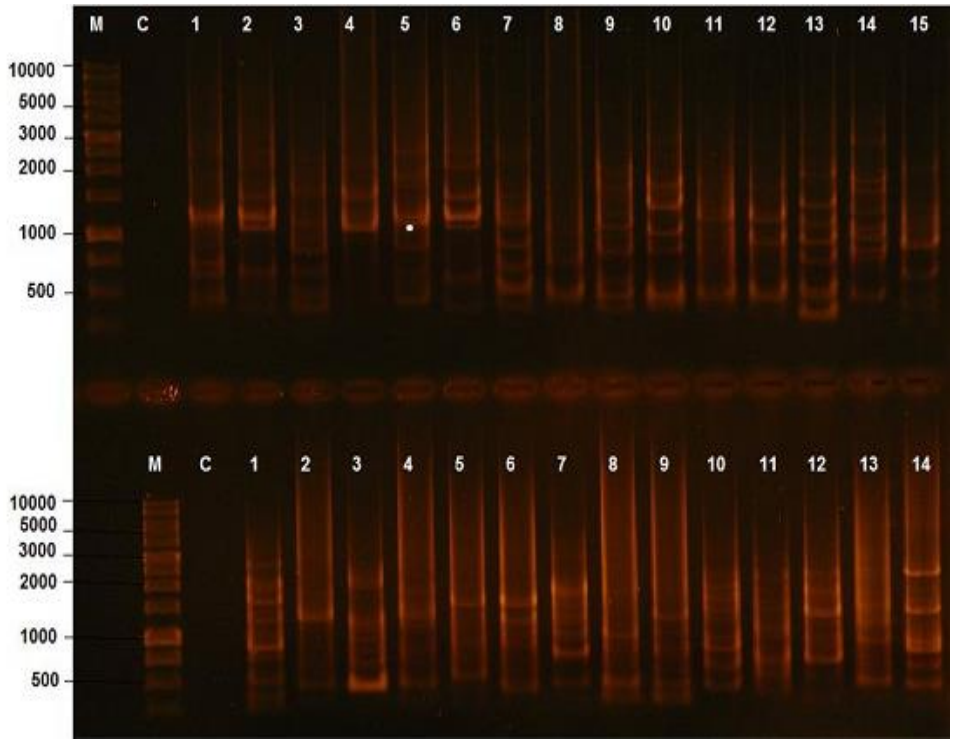


Figure 9- Banding pattern of the RAPD-PCR with OPAV-08

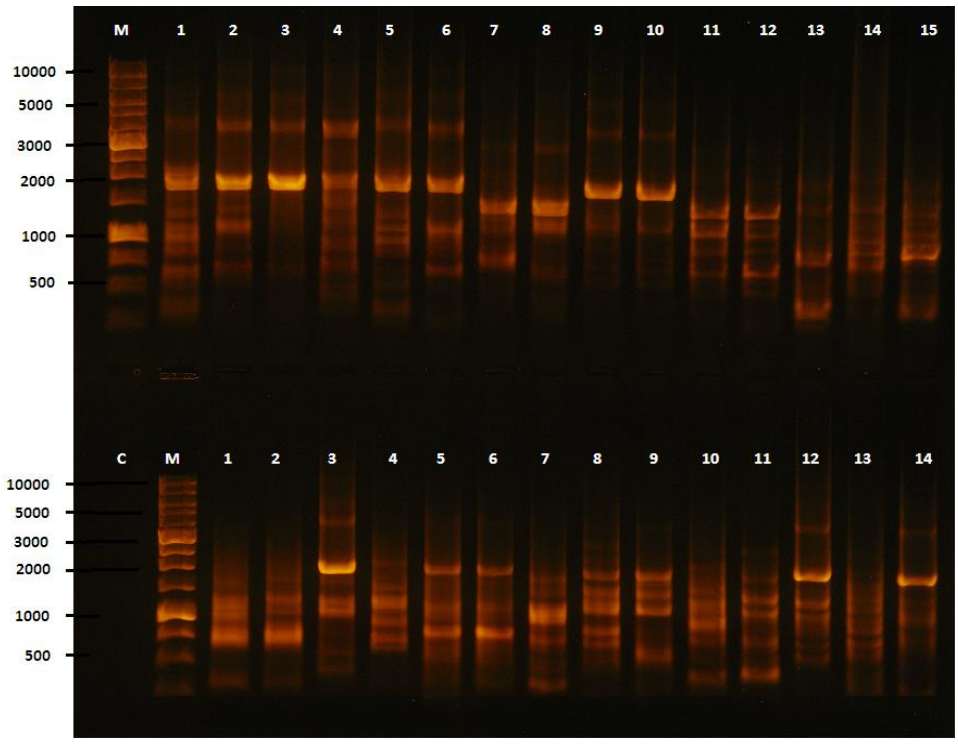


Figure 10- Banding pattern of the RAPD-PCR with OPAR-19

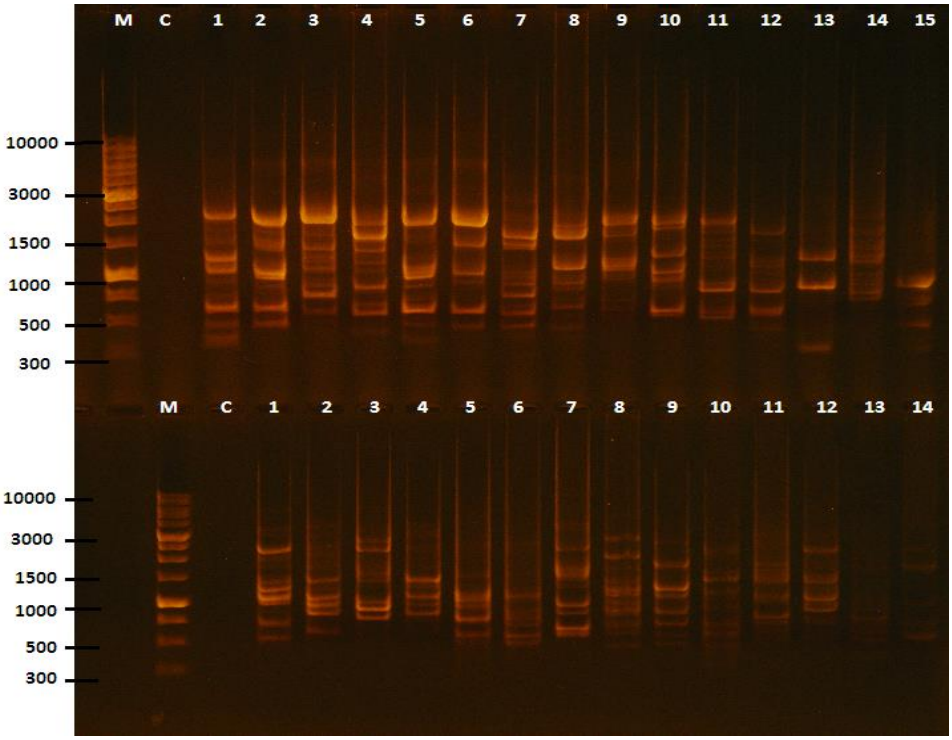


Figure 11- Banding pattern of the RAPD-PCR with OPAQ-09

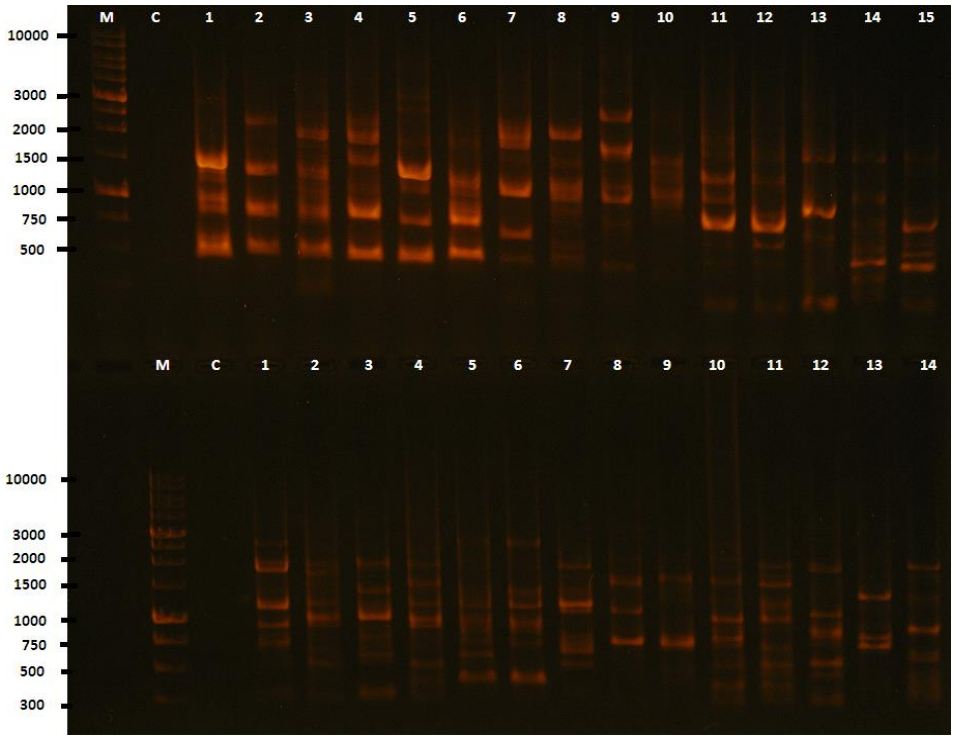


Figure 12- Banding pattern of the RAPD-PCR with OPAL-12

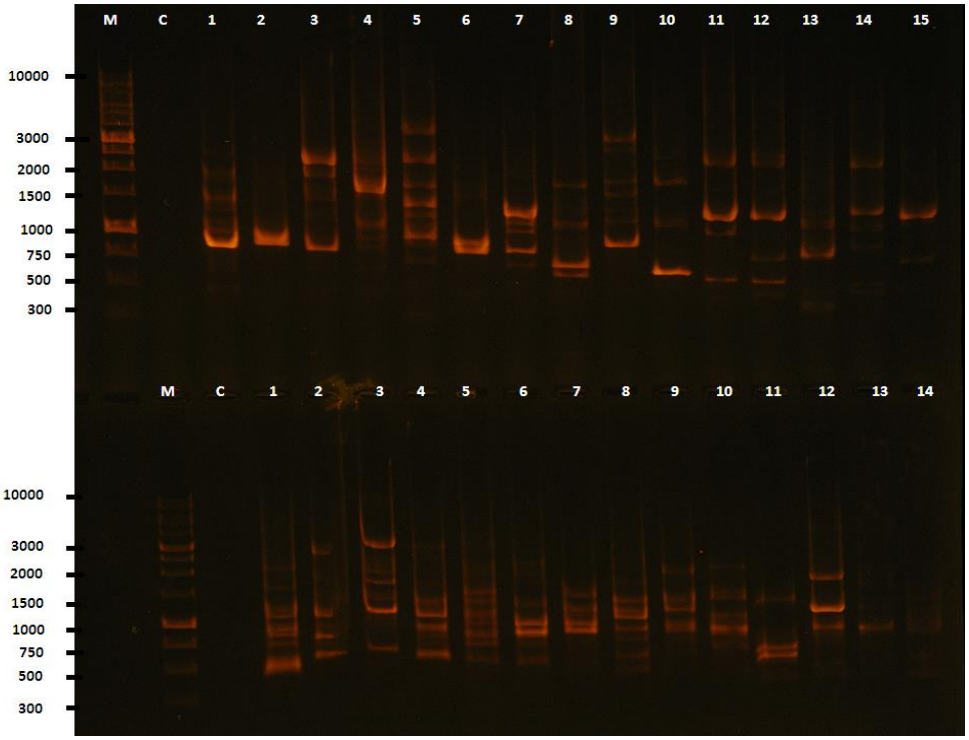


Figure 13- Banding pattern of the RAPD-PCR with OPB-14

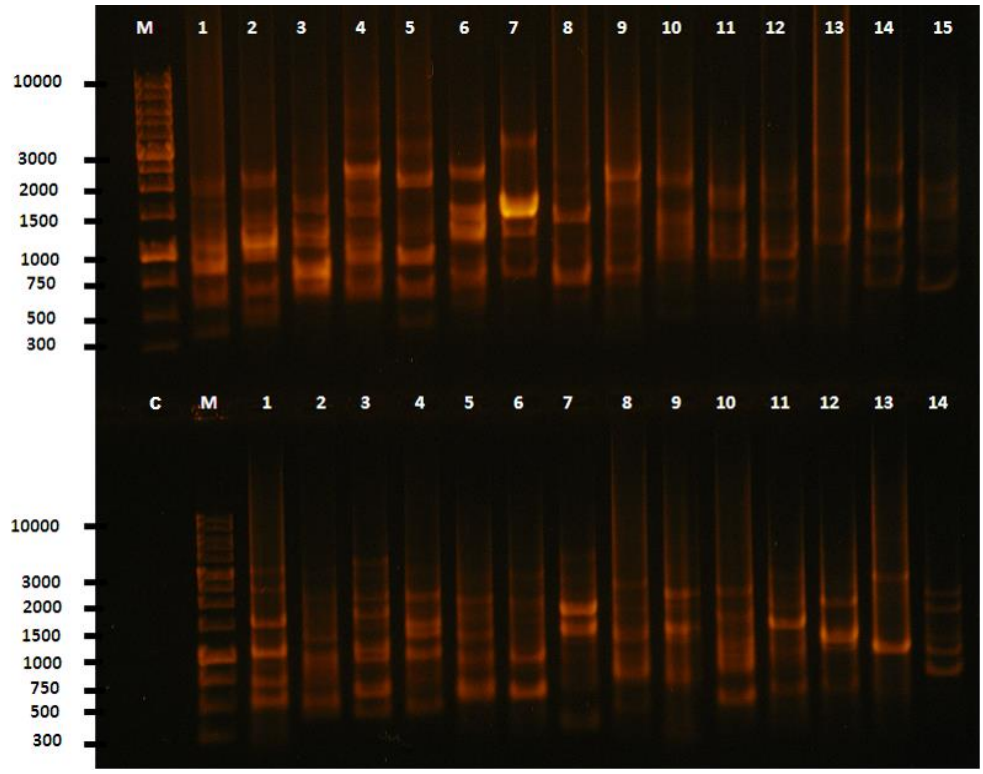


Figure 14- Banding pattern of the RAPD-PCR with OPAB-14

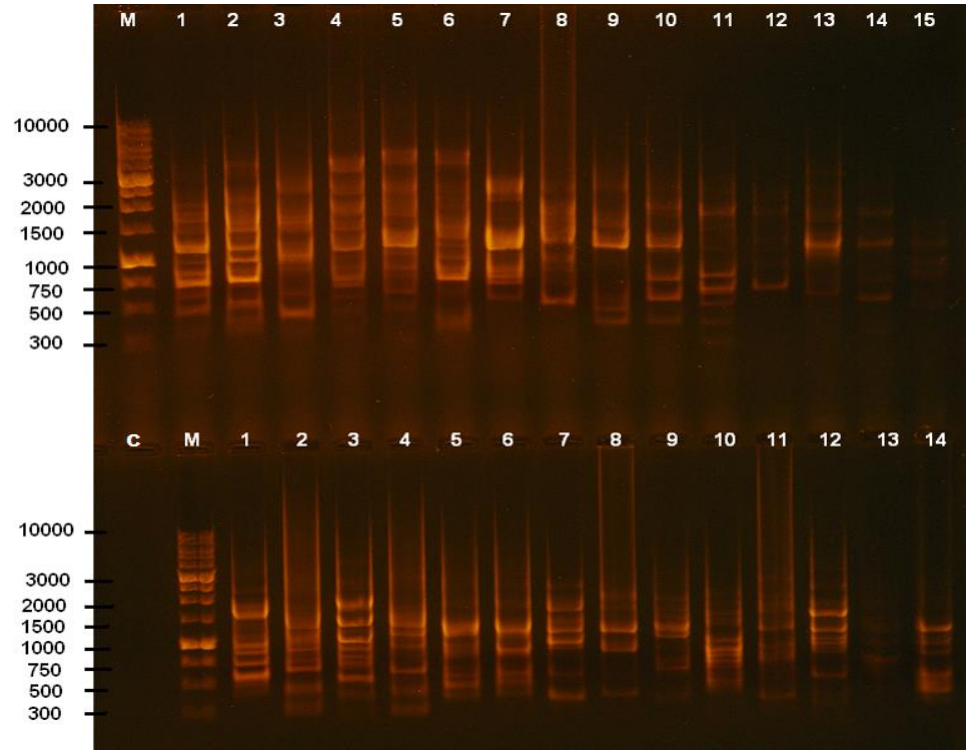


Figure 15- Banding pattern of the RAPD-PCR with OPBA-07

In the dendrogram the 29 *Trifolium* taxa formed two main clusters (Figure 16). One contained all members of the section *Trifolium* and the other contained members of three sections: *Chronosemium*, *Lotoidea*, and *Vesicaria*. However, all members of these sections were grouped as separate subclusters under the main cluster. Members of the section *Vesicaria* and the subcluster *Lotoidea* were the most similar groups. Section *Lotoidea* members formed two subgroups; the first one was *T. repens* varieties, *T. retusum*, *T. hybridum* var. *anatolicum* and the second one was *T. hybridum* var. *elegans*, *T. nigrescens*. In *Vesicaria* section *T. resupinatum* was bound to *T. fragiferum* and *T. resupinatum* as an outgroup. *Chronosemium* members were also separated as *T. patens*, *T. aureum*, and *T. campestre* in one small group and *T. dubium*, *T. micranthum*, and *T. spadiceum* in another small group. Members of the section *Trifolium* were first separated into two clusters and then these clusters were divided into small clusters.

The first main cluster was formed by *T. medium* varieties, *T. pallidum, T. echinatum, T. diffusum, T. arvense, T. bocconei*. In this cluster, *T. medium* var. *eriocalycinum* and *T. pallidum* were bounded to the others from outside. The second main cluster was formed by the remaining members of section *Trifolium* and divided into two subgroup as *T. ochroleucum, T. scabrum, T. pannonicum* subsp. *elongatum, T. patense* var. *pratense* in one group and *T. striatum, T. costantinapolitanum, T. hirtum* in another group (Figure 16).

### 3.1.2 SSR marker results

Initially, 7 SSR markers were selected for the testing of polymorphism with 29 *Trifolium* taxa. Two of them, TSSRA02B08 and TSSRA01H11, did not produce any fragments in the studied taxa. The images of the 5 SSR primer patterns are seen in between Figures 17 and 26. The number, codes and names of taxa in figures are explained in Table 10. These SSR markers gave a total of 331 fragments among 29 *Trifolium* taxa (Table 11). Between 56 and 83 fragments were amplified in each marker and their sizes varied from 125 to 3000 bp (Table 11). The most common bands were 370 and 400 bp fragments, found in 19 taxa of 29. The mean percentage of polymorphism with SSR markers was 81%.

The similarity matrix of SSR scored bands was calculated by Jaccard's similarity coefficient (Table 2C shown in Appendix C). According to the SSR banding scored results, the highest similarity (0.49) was found between *T. medium* var. *eriocalycinum* and *T. medium* var. *medium,* and the least similarity (0.03) was shared between *T. ochroleucum* and *T. aureum*, and between *T. aureum* and *T. resupinatum*. An UPGMA dendrogram was drawn using Jaccard's similarity coefficient (Figure 27).

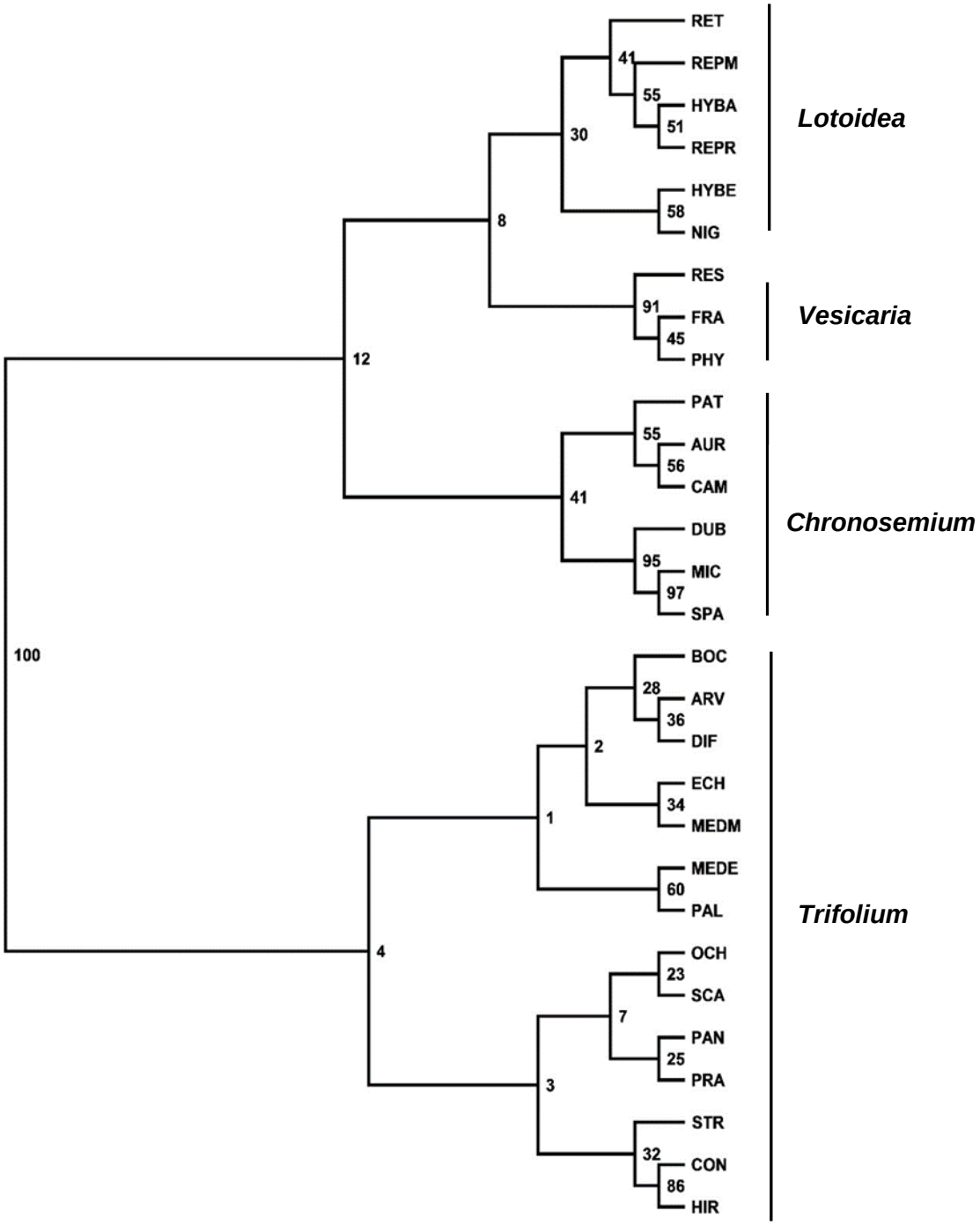


Figure 16- UPGMA dendrogram based on RAPD

Table 10- Numbers, codes and names of taxa for Figures 17-26

| TAXA NUMBERS | TAXA CODES | TAXA NAMES |
|---|---|---|
| 1 | RES | *T. resupinatum* |
| 2 | PHY | *T. physodes* |
| 3 | FRA | *T. fragiferum* |
| 4 | REPM | *T. repens* var. *macrorhizum* |
| 5 | REPR | *T. repens* var. *repens* |
| 6 | HYBA | *T. hybridum* var. *anatolicum* |
| 7 | HYBE | *T. hybridum* var. *elegans* |
| 8 | NIG | *T. nigrescens* subsp. *petrisavi* |
| 9 | RET | *T. retusum* |
| 10 | DUB | *T. dubium* |
| 11 | MIC | *T. micranthum* |
| 12 | SPA | *T. spadiceum* |
| 13 | AUR | *T. aureum* |
| 14 | CAM | *T. campestre* |
| 15 | PAT | *T. patens* |
| 16 | PRA | *T. pratense* var. *pratense* |
| 17 | PAN | *T. pannonicum subsp. elongatum* |
| 18 | SCA | *T. scabrum* |
| 19 | STR | *T. striatum* |
| 20 | HIR | *T. hirtum* |
| 21 | CON | *T. costantinapolitanum* |
| 22 | ECH | *T. echinatum* |
| 23 | PAL | *T. pallidum* |
| 24 | MEDE | *T. medium* var. *eriocalycinum* |
| 25 | MEDM | *T. medium* var. *medium* |
| 26 | OCH | *T. ochroleucum* |
| 27 | BOC | *T. bocconei* |
| 28 | DIF | *T. diffusum* |
| 29 | ARV | *T. arvense* |

Table 11- Percentage of polymorphic bands in SSR primers

| **Primer** | **Forward primers (F) (5'-3')** | **Total number of bands** | **PBS%** |
|---|---|---|---|
| TRSSRATS054 | (F) GACACCGATTATGTGCAAGA | 83 | 83.42 |
| | (R) AATCACGACGAGCGACAAC | | |
| TRSSRATS055 | (F) CAATACAATCACCGCACCAG | 62 | 75.80 |
| | (R) TCTCTGCTTCGCGTCTTCTC | | |
| TRSSRAXX31 | (F) TCTGTTTTGTTGGCCATGC | 56 | 83.50 |
| | (R) TTGCAAAGTGTTTGGAAGGA | | |
| TRSSRA02C03 | (F) TATGCTGGTAGATAAACTTAAA | 67 | 83.47 |
| | (R) TGCTCTGGAGATTGATGG | | |
| TRSSRA02C02 | (F) AAATAAAAACCACAAGTAACTAG | 63 | 83.02 |
| | (R) TATAGGTGATTTGAAATGGC | | |
| **Total** | | 331 | **mean** = 81.84 |

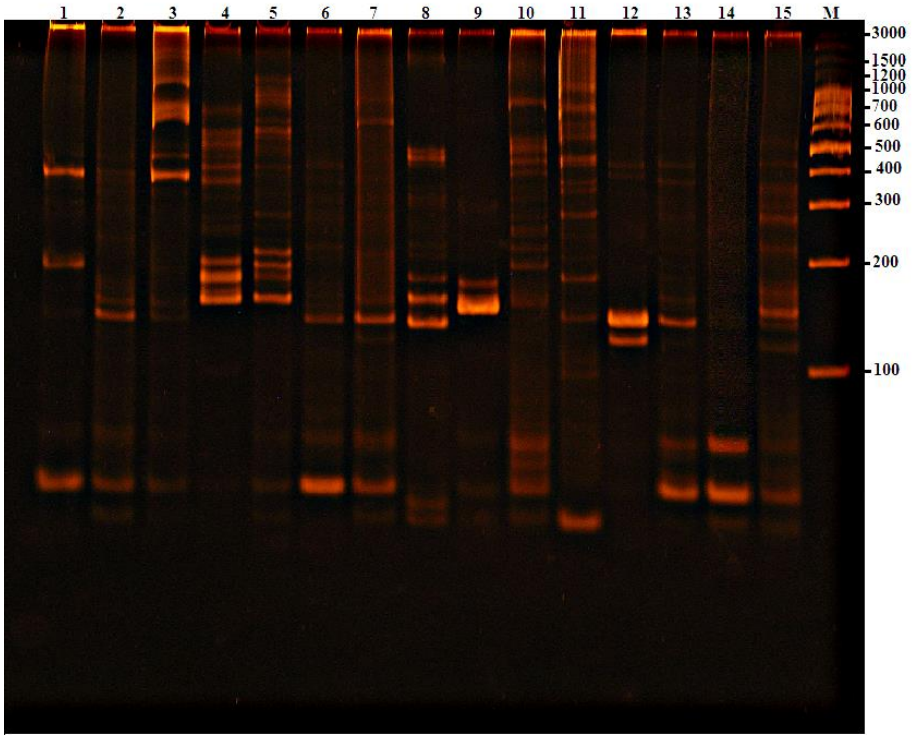


Figure 17- Banding pattern of the SSR-PCR with 02C02 part1

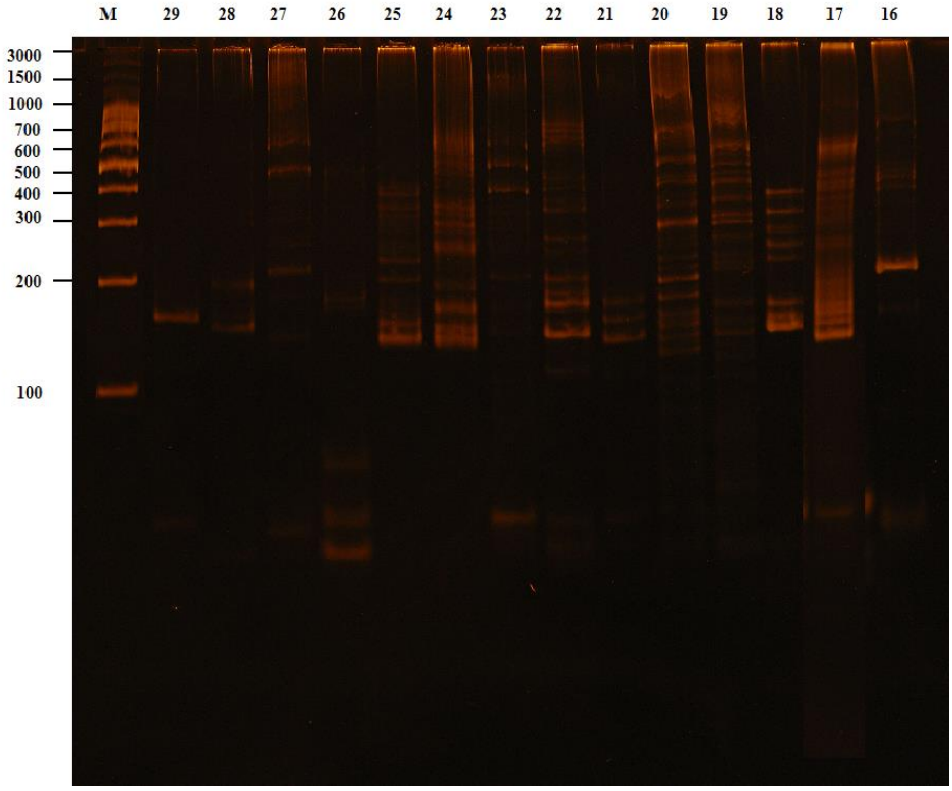


Figure 18- Banding pattern of the SSR-PCR with 02C02 part 2

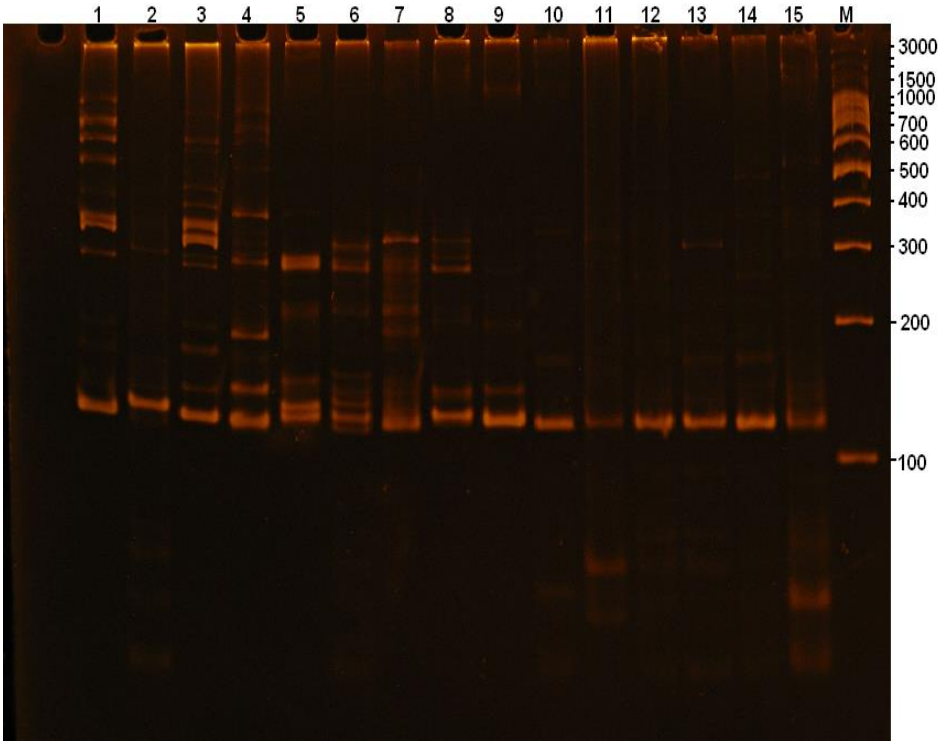


Figure 19- Banding pattern of the SSR-PCR with 02C03 part 1

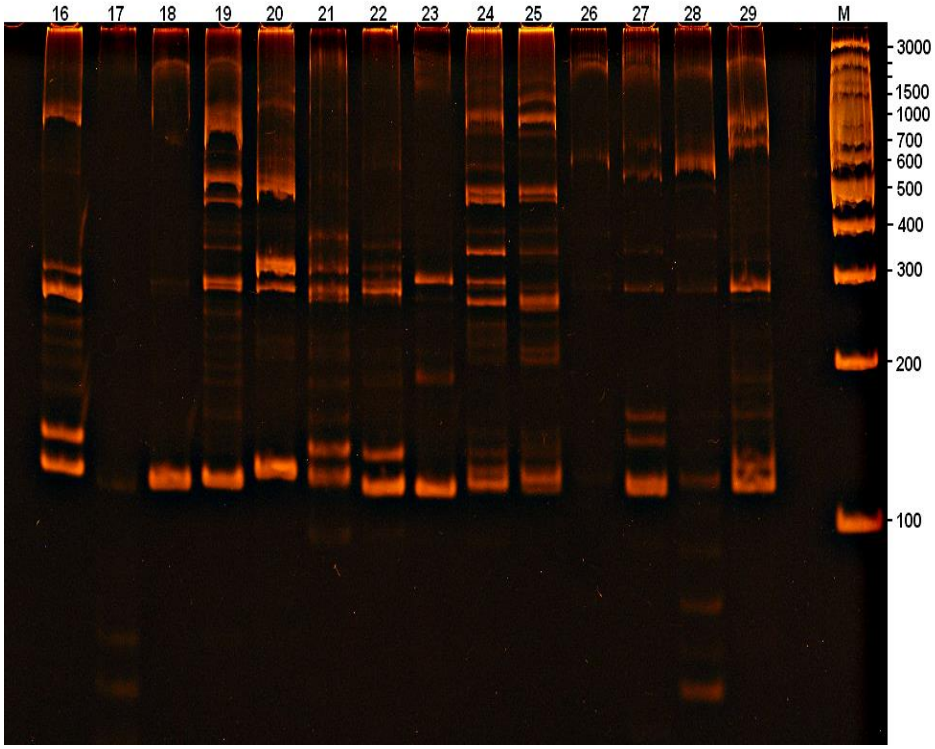


Figure 20- Banding pattern of the SSR-PCR with 02C03 part 2

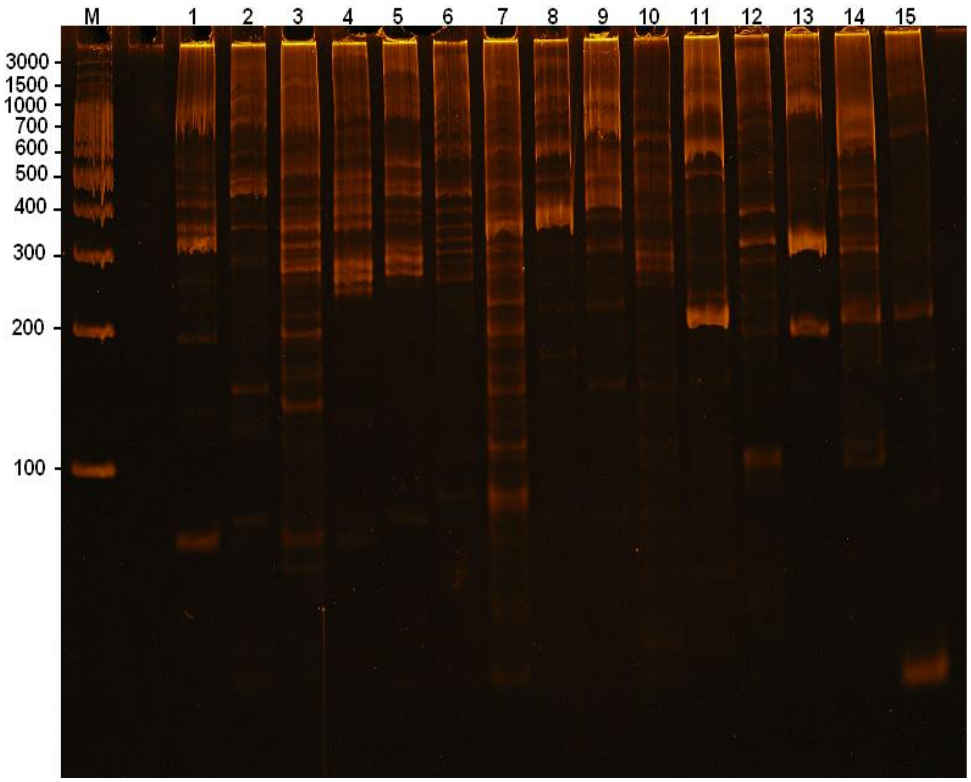


Figure 21- Banding pattern of the SSR-PCR with TS054 part 1

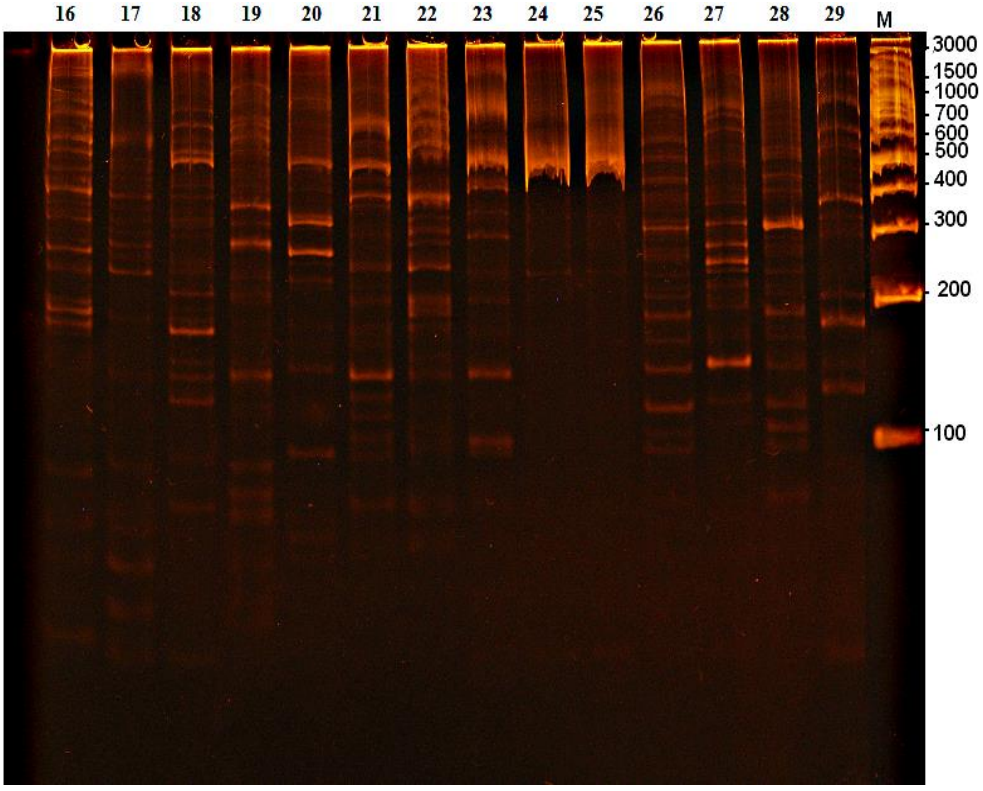


Figure 22- Banding pattern of the SSR-PCR with TS054 part 2

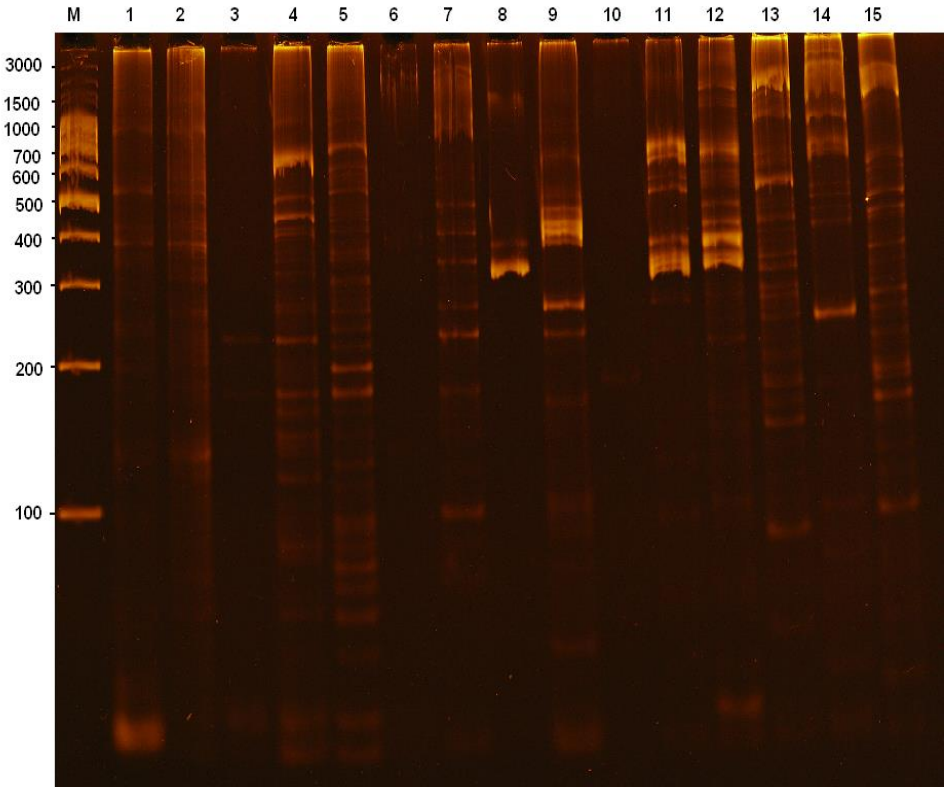


Figure 23- Banding pattern of the SSR-PCR with TS055 part 1

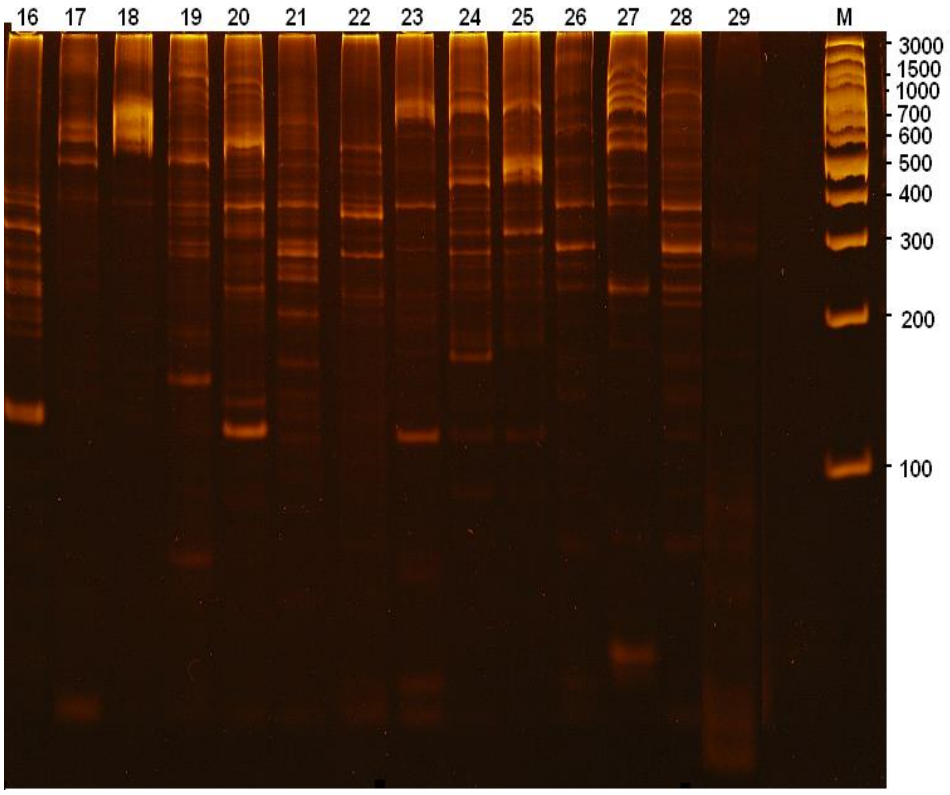


Figure 24- Banding pattern of the SSR-PCR with TS055 part 2

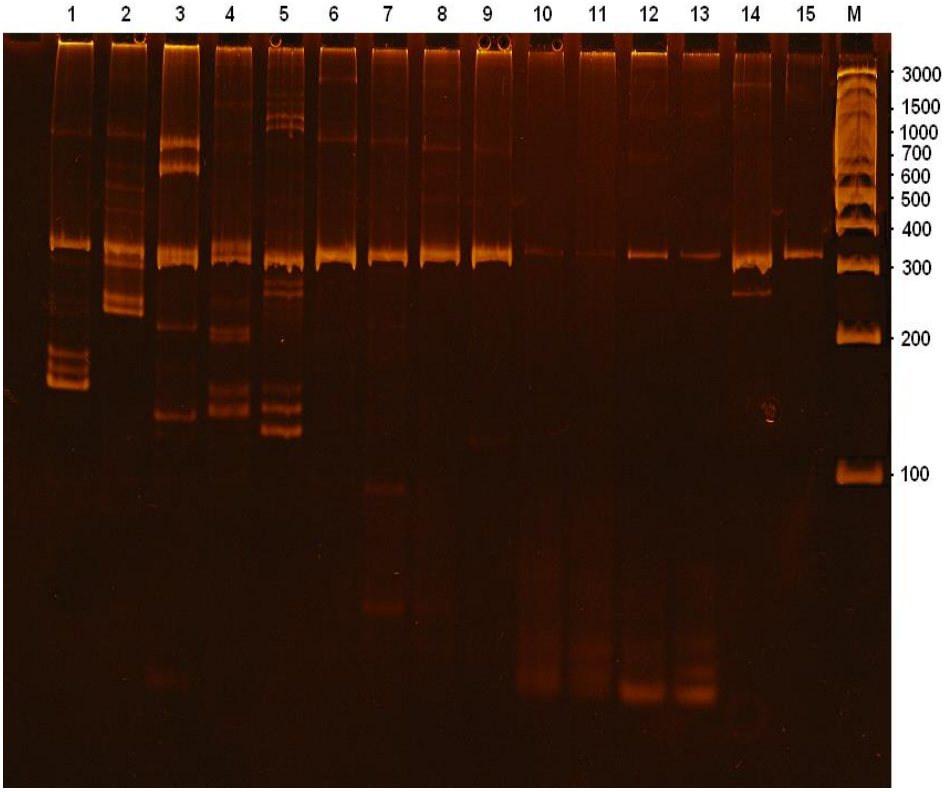


Figure 25- Banding pattern of the SSR-PCR with XX31 part 1

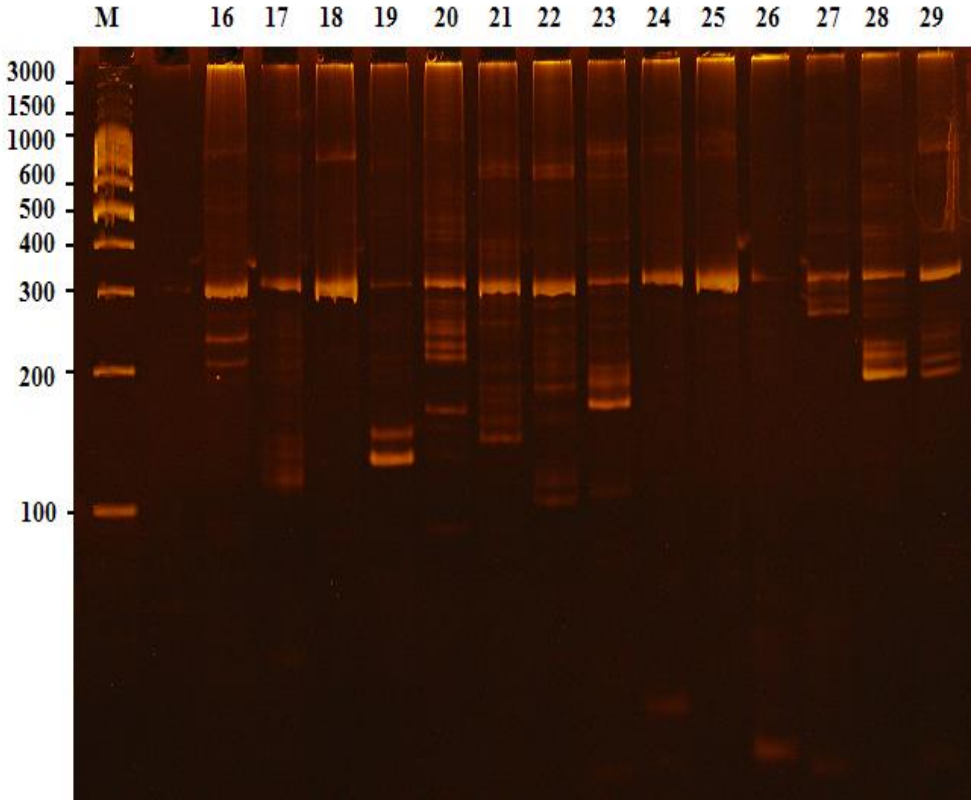


Figure 26- Banding pattern of the SSR-PCR with XX31 part 2

There were two main clusters, with one containing all members of the section *Trifolium*, and the other presenting the remaining members of the three sections (Figure 27). In this regard, the SSR marker results showed similar results with those of RAPD markers (Figures 16 and 27). *Trifolium* section was separated into two main clusters; one of them was a small group included *T. arvense*, *T. bocconei*, *T. diffusum*, *T. ochroleucum* and the other was a big group contained the remaining taxa of *Trifolium* members. In the big cluster, *T. scabrum*, *T. pannonicum* subsp. *elongatum*, *T. pratense* var. *pratense* and *T. striatum* formed an outgroup of the remaining members. Then, *T. medium* varieties were seperated from the other species and formed a small group alone. *T. hirtum*, *T. pallidum*, *T. costantinapolitanum* and *T. echinatum* joined with these *T. medium* group. As mentioned previously, *Vesicaria*, *Lotoide*a and *Chronosemium* sections were formed in the same main group. First, the members of *Vesicaria* section; *T. physodes*, *T. fragiferum*, *T. resupinatum* were seperated from the other two sections sharply and formed a group alone. *T. repens* varieties, which are belongs to the section *Lotoidea*, was seperated from its group and attended members of the section *Chronosemium* as an outgroup. Thus, a perfect distinction was not observed between *Lotoidea* and *Chronosemium*. The rests of *Lotoidea* members were formed two small groups in itself; first one was *T. hybridum* varieties and second one was *T. nigrescens*, *T. retusum*. As seen in figure 27, these groups were added to section *Chronosemium* members and *T. repens* varieties. *Chronosemium* members were divided in two subclasses contained *T. aureum*, *T. dubium* in a small group and *T. patens*, *T. campestre*, *T. micranthum*, *T. spadiceum* in the other group.

### 3.1.3 RAPD and SSR markers combined results

RAPD and SSR data were combined to observe the genetic relationships of *Trifolium* taxa. A phenogram was generated using Jaccard's similarity coefficients (Figure 28; Table 3C seen in Appendix C). According to this result, the highest genetic similarity (0.40) was found between *T. micranthum* and *T. spadiceum* (Table 3C). Both taxa were members of the section *Chronosemium*. On the other hand, the least similarity (0.07) was detected between *T. patens* (a member of the section *Chronosemium*) and *T. bocconei* (from section *Trifolium*).

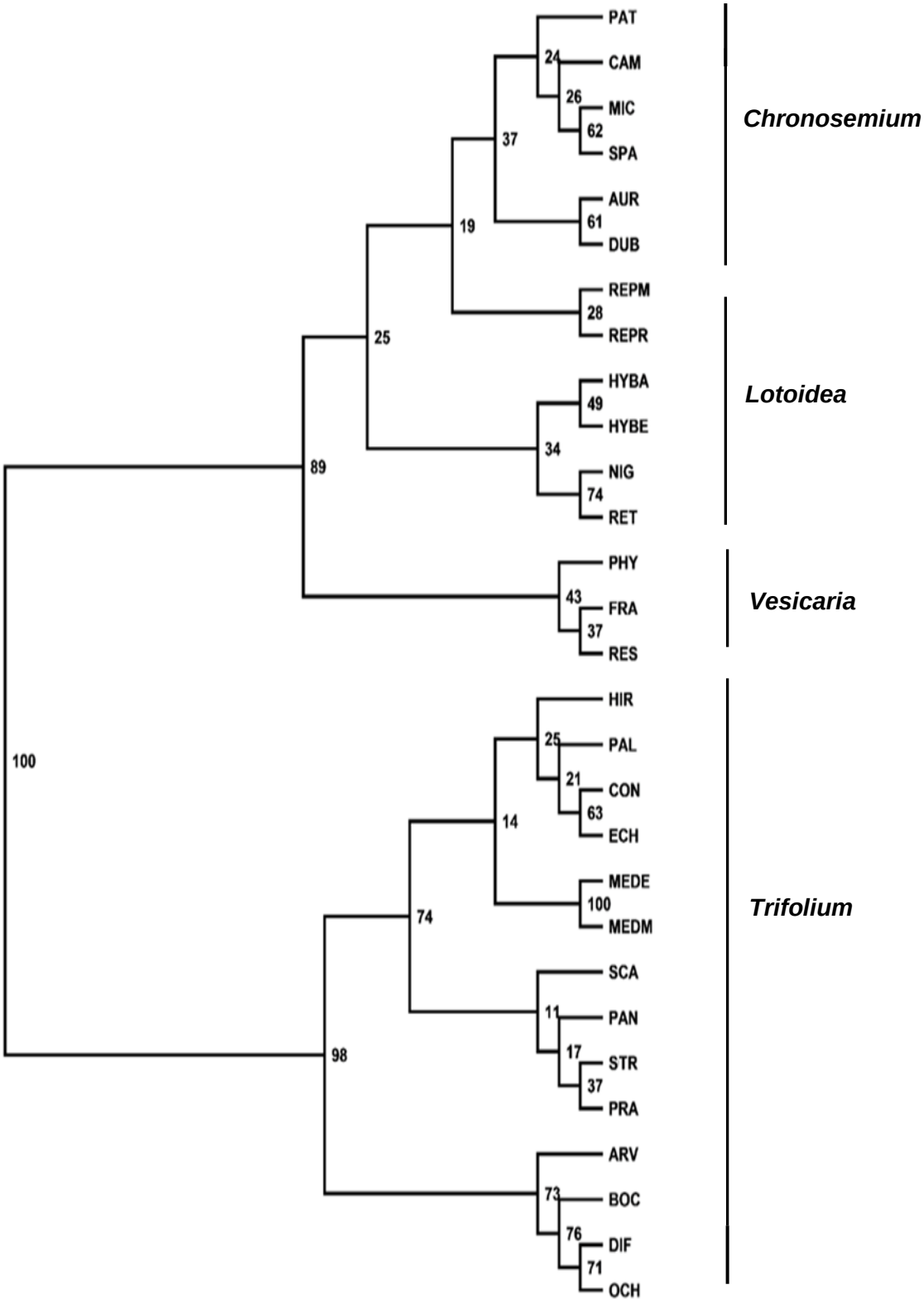


Figure 27- UPGMA dendrogram based on SSR

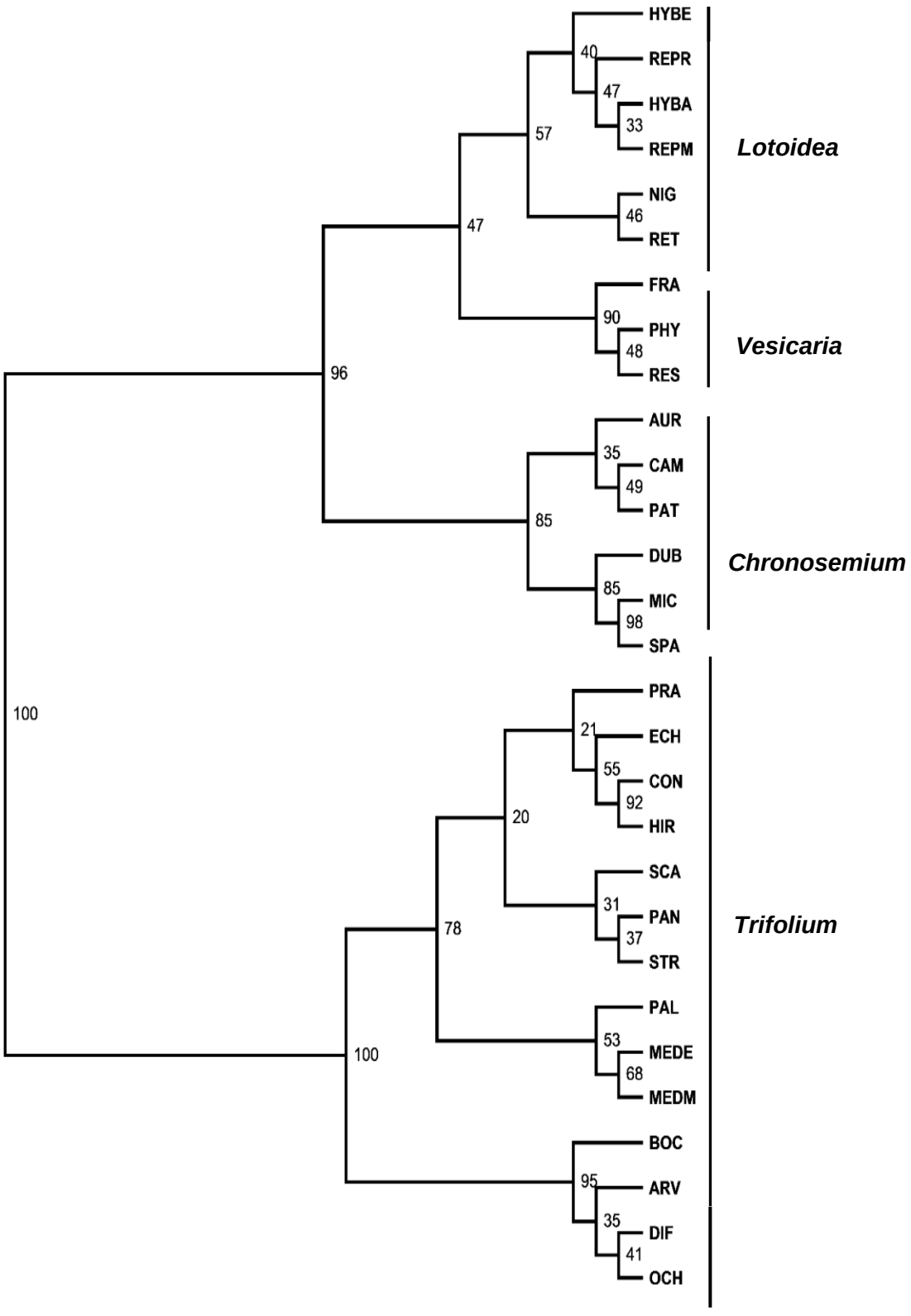


Figure 28- UPGMA dendrogram based on combined RAPD and SSR

The integrated results (Figure 28) resembled to the independent results of the two markers system (Figure 16, Figure 27). The combined data also supported with the higher percentage probabilities (the number presented in the branching of dendrogram) of taxa groupings than those independent ones. All of them could separate *Trifolium* from the other three sections. However, the three dendrogram results showed different connections in subclassifications of the main groups. The integrated result was found to be so successful in separating sections from each other. It provided perfect separation in this respect. Section *Trifolium* was divided into two clusters; *T. bocconei, T. arvense, T. diffusum, T. ochroleucum* formed to give first cluster and joined the rest of the members. This classification structure was alike that of the dendrogram based on SSR data (Figure 27). The remaining *Trifolium* section members grouped under the second main cluster. *T. pallidum* was found closer to *T. medium* varieties than the others and they attached as outgroup of the remaining species. *T. scabrum, T. striatum, T. pannonicum* subsp. *elongatum* formed a small group and *T. pratense* var. *pratense, T. echinatum, T. constantinapolitanum, T. hirtum* formed an another group. *Vesicaria* and *Lotoidea* sections were found to be closer to each other and *Chronosemium* section members were joined them from outside. In section *Chronosemium, T. dubium, T. micranthum* and *T. spadiceum* were found to be different from *T. aureum, T. campestre* and *T. patens*. In section *Vesicaria T. fragiferum* was linked to *T. physodes, T. resupinatum* from outside. In section *Lotoidea, T. nigrescens* subsp. *petrisavii* and *T. retusum* were linked to the other species of section distantly.

### 3.2 Morphological Results

The phenogram was constructed as seen in Figure 29. In this dendrogram members of the four sections (*Trifolium, Chronosemium, Lotoidea, Vesicaria*) were found to be entered into one another.

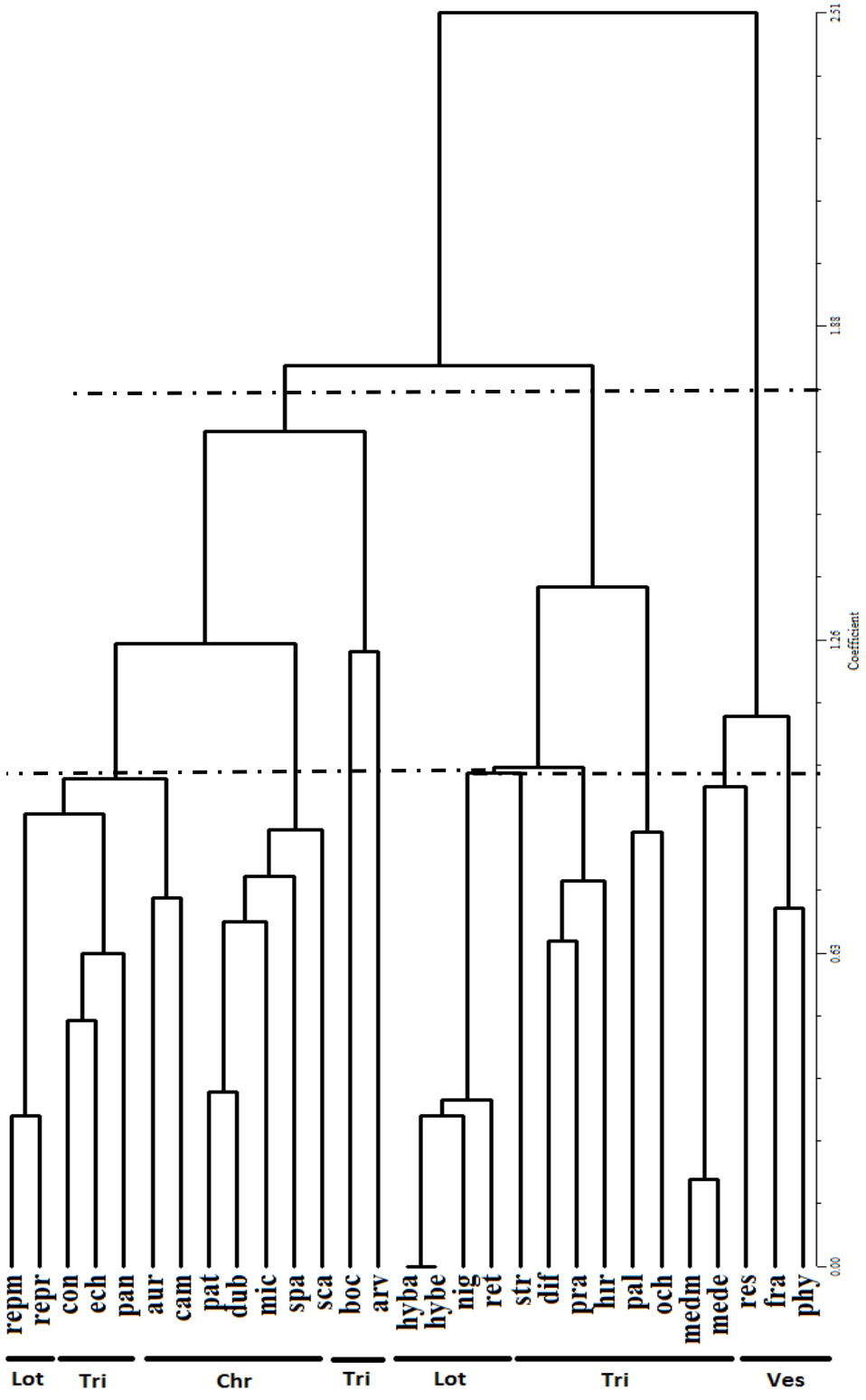


Figure 29- UPGMA dendrogram based on morphological characters

According to the similarity level 175, there were 3 main clusters. Although the dendrogram succeeded to collect the members of section *Vesicaria* together, as a first cluster *T. medium* varieties, which belongs to section *Trifolium*, joined in this cluster. Then, the rest of species were divided into two subclusters. The big one contained members of section *Chronosemium* and some those of sections *Trifolium* and *Lotoidea*. The smaller one contained the other *Trifolium* and *Lotoidea* members. In the small cluster, at similarity level 98, *T. hybridum* varieties *T. nigrescens* subsp. *petrisavii* and *T. retusum* formed small group with *T. striatum* under this small cluster. Then, *T. diffusum* and *T. pratense* var. *pratense* joined to *T. hirtum* as an another small group. Finally, *T. pallidum* and *T. ochroleucum* together formed the last small group under the main cluster. In the big cluster, *T. bocconei* and *T. arvense* were separated from the others. Then, rest of the species were divided in two groups; one contained sect. *Chronosemium* members alone. The other one had the members of the three sections (*Trifolium*, *Chronosemium*, *Lotoidea*) with coefficient 98. The complex group had *T. campestre*, *T. aureum* in a subgroup and *T. constantinapolitanum*, *T. echinatum*, *T. pannonicum* subsp. *elongatum* were clustered together with *T. repens* varieties in a second subgroup.

# 4. DISCUSSION AND CONCLUSION

Molecular (RAPD and SSR) and morphometric analyses were carried out to investigate the relationships among 29 *Trifolium* taxa from Bolu province. This is the first comprehensive study on *Trifolium* genus by combining molecular and morphologic methods in Bolu.

Results of the molecular studies demonstrated that RAPD and SSR primers are highly polymorphic on *Trifolium* taxa. The mean percentages of polymorphism for 11 RAPD primers and 5 SSR marker were 84% (Table 9) and 81%, respectively (Table 11). In similar studies with RAPD primers, the polymorphic rates were found to be 0.74 among red clover (*T. pratense*) (Ulloa et al. 2003) and 0.80 among clover genotypes in Iran (Arzani and Samei, 2004). The average polymorphism for 6 SSR markers was 0.88 among white clover (*T. repens*) plants (Kölliker et al. 2001a). In this study, results revealed that the genetic diversity of RAPD markers was slightly higher than those of SSR markers. The polymorphic bands percentage of RAPD primers ranged between 82% and 87%. This demonstrated that the selected primers were efficient for all 29 *Trifolium* taxa. The highest separation was belonged to OPB-14 RAPD primer with 87% level. The polymorphic band percentages of SSR primers changed between 76% and 83.5%. This result also proved that the selected primers may be prefered for studying *Trifolium* taxa. The most efficient SSR primer was found as TRSSRAXX31 (Table 11).

The phenograms of these two molecular markers (RAPD and SSR) showed concurrency to separate 29 *Trifolium* taxa into two main clusters (Figures 16 and 27). One of the clusters contained all members of the section *Trifolium* and the other cluster included three different sections: *Vesicaria*, *Lotoidea*, and *Chronosemium* members. According to molecular phylogenetic studies (Ellison et al. 2006), *Trifolium* taxa were grouped under the two subgenera: *Trifolium* and *Chronosemium*. The subgenus *Chronosemium* contained all the members of the section

*Chronosemium* (Zohary and Heller, 1984) and the subgenus *Trifolium* covered all members of the remained sections, such as *Paramesus*, *Lupinaster*, *Trifolium*, *Trichocephalum*, *Vesicastrum*, *Trifoliastrum* and *Involucrarium*.

As mentioned above, results of this study showed similarities to findings of Ellison et al. (2006) to some extent. Although the results presented two main groups, but only one of them showed congruency with the subgenus *Trifolium*. The other group contained the members of the subgenus *Chronosemium* and the taxa of the sections *Vesicaria* and *Lotoidea*. Then, *Lotoidea* and *Vesicaria* sections together joined to the section *Chronosemium* instead of the section *Trifolium*. In other words, it could be concluded that section *Trifolium* taxa were distantly related to those of the remained sections in the genus. When the results of RAPD and SSR were compared, the main groups (two clusters) were the same in both dendrograms (Figures 16 and 27), but there were some differences in the subgroups of the sections *Chronosemium*, *Lotoidea*, and *Trifolium*. In the RAPD marker results, *Chronosemium* members *T. patens*, *T. aureum* and *T. campestre* separated as a first subcluster and *T. dubium*, *T. micranthum*, and *T. spadiceum* were in the second subcluster (Figure 16). On the other hand, the SSR formed two subclusters. One contained four taxa which were *T. patens*, *T. campestre*, *T. micranthum*, *T. spadiceum* and the other one contained only *T. aureum* and *T. dubium* (Figure 27). The combined data result in Figure 28 also produced the same clusters as those of RAPD marker with a high genetic similarity rate (86%). *T. campestre* and *T. dubium* from the section *Chronosemium* were also found to be closer genetically in another study (Ellison et al. 2006). In the study of Vizintin et al. (2006), *T. aureum*, *T. campestre*, and *T. dubium* were found to be very close to each other as well. This study results supported their findings. A subcluster of *Lotoidea* members also showed differences between the two marker results (Figures 16 and 27), but the dendrogram based on the integrated data presented the highest genetic similarity rate (56%) in comparison to the results of those in separate markers (Figures 16 and 27). In the combined data dendrogram, there were two subclusters containing both varieties of *T. hybridum* and *T. repens*. The second subcluster consisted of only *T. nigrescens* and *T. retusum* (Figure 28). Similarly,

Watson et al. (2000) also found that *T. hybridum* and *T. nigrescens* were rather distant from each other.

In the study, similar results to those of Ellison et al. (2006) were obtained. Although section *Trifolium* members presented a separate main clusters in both marker dendrograms (Figures 16 and 27) and both contained more or less same subgroups. However their members in these subgroups were different according to RAPD and SSR results. The dendrogram based on the integrated data was closer to those of SSR markers. *T. bocconei, T. arvense, T. diffusum,* and *T. ochroleucum* were distantly joined to the other subclusters of the section *Trifolium* in both SSR and the integrated data dendrograms. In the SSR dendrogram, both varieties of *T. medium* were separated from the second subcluster, but in the dendrogram of the integrated data, *T. pallidum* was also found in the same subcluster with both varieties of *T. medium*. The third subcluster of section *Trifolium* members contained *T. scabrum, T. pannonicum,* and *T. striatum* and the last subcluster covered *T. hirtum, T. constantinapolitanum, T. echinatum,* and *T. pratense* as the remaining taxa of the section *Trifolium* (Figure 28).

There were some similarities between the results of Ellison et al. (2006) and the groups obtained in this study, such as *T. echinatum* and *T. constantinapolitanum* were found closer to each other. Moreover, *T. arvense* and *T. bocconei* similarities were also supported by both results. The remaining subgroups of the section *Trifolium* showed some different relationships than those of Ellison et al. (2006)'s groups. However, all the members of the section *Trifolium* remained under the main cluster in this study. Vizintin et al. (2006), obtained the groups of the section *Trifolium,* also supported this study results.

In the last section *Vesicaria, T. resupinatum, T. physodes,* and *T. fragiferum* were clustered together and they were placed as a separate subgroup between the *Lotoidea* and *Chronosemium* section subclusters under the main cluster (Figure 28). However, in the other study (Ellison et al. 2006), these taxa were found to be close to each other and were grouped under the section *Vesicastrum* of the subgenus

*Trifolium*. Similar results were also found concerning members of the section *Vesicaria* in the study of Vizintin et al. (2006).

In the comparison between RAPD and SSR results, there were different subgroup patterns obtained from the RAPD and SSR data in the present study. This could be attributed to the different genomic constitution of the two markers (Ravi et al. 2003; Zhang et al. 2010). RAPDs can randomly detect DNA fragments from both coding and non-coding regions (Williams et al. 1990; Li et al. 2000), but SSR markers can only detect the tandem repeated area of simple sequences, which are widely dispersed in non-coding regions in all eukaryotic genomes (Kashi et al. 1997; Li et al. 2000). An another explanation can be related with the source of polymorphisms in both markers (SSR and RAPD). SSR polymorphism is created by the shifting of the DNA polymerase (Tautz et al. 1986). Therefore, types of genomic variations do not influence the polymorphisms (Mengoni et al. 2000). On the other hand, RAPD variations are included in many different types of mutational events, with different evolutionary implications that ocur in annealing site of the primer and between the two adjacent site responsible for the amplification (Mengoni et al. 2000). All of these can be accepted as reasons for indicating higher polymorphism of RAPD markers than that of SSR markers and also for explanation of findings to different subgroups in both markers.

The study of morphological results indicated no significant correlation with the molecular results. Such kinds of results were observed in other studies as well (Cholastova and Knotova, 2012; Zhang et al. 2010; Dias et al. 2008; Greene et al. 2004). Molecular methods were successful in section separation, whereas morphological ones could not differentiate them each other. Morphological results divided section *Trifolium* and *Lotoidea* into different groups where they showed homogenously dispersion. Section *Trifolium* were divided into three and sect. *Lotoidea* were divided into two groups and they were entered into between each other (Figure 29).

The differences between the results of molecular and morphologic markers may be explained with some reasons. Dias et al. (2008) explained that morphological

traits are strongly related with environmental variations and the morphological similarities can result from different combinations of alleles producing similar phenotypes (Johns et al. 1997). It means that different species can show the same characters by different genetic origin. Also they added that specific traits with adaptive values may have accumulated in similar ecological conditions independently of the genetic origins (Steiner and los Santos, 2001). These can explain the revealing closer relationships of different plant species in morphological methods. In addition to these, neutral genes can be shown as a possible reason for differences of two methods (Dias et al. 2008). Beside of these, the origins of molecular markers and morphological markers may be given as an another reason. Molecular markers can cover either a larger genomic segments or whole genome, while morphological traits are controlled by a relatively small number of loci (Bruschi et al. 2003; Zhang et al. 2010).

As a conclusion, ecological structure and conditions of Bolu were suitable for naturally growing *Trifolium* species. Hence the collected numbers of *Trifolium* species were twice that which had been reported in the Turkish Flora. This finding gives some opportunities for plant breeding in regional agriculture. Therefore, in this study, it has been tried to characterize the 29 *Trifolium* taxa and showed their genetical relations. In the molecular part of the study, 26 species and 3 infraspecific taxa could be separated and characterized clearly in terms of section level. Selected molecular markers (RAPD and SSR markers) were able to differentiate *Trifolium* section from the other officially acepted sections (Zohary and Heller, 1984). Thus, these markers are found to beconsiderably important for the classification of these taxa. They can be used in similar taxonomic and genetic studies efficiently. However, they showed some disagreements in systematic positions of the sections *Lotoidea*, *Chronosemium* and *Vesicaria*. In addition, some disparity were revealed between the two marker systems in terms of infrasectional taxa. In order to study taxa within and below the sections, additional marker systems should be used for further studies. Similarly, morphological markers could not provide clear separations among the studied taxa. Therefore, different morphological characters and/or molecular markers together should be tried for future studies.

To sum up, this study reached to one of its aims on the basis of molecular part. The markers gave enough polymorphism and could separate the taxa in sections distinctly. At the same time, the study showed a reliable result, since this genetic relationship was supported by both RAPD and SSR markers. In addition, some of the varieties such as *T. medium* var. *eriocalycinum*, *T. repens* var. *macrorhizum* and *T. hybridum* var. *anatolicum* and *T. hybridum* var. *elegans* were used for the first time in this molecular study.

In morphological part, the characters which were used in most studies, were not found to be very useful in interspecific groupings. A large numbers of morphological characters on a high numbers of taxa were used for characterization of some Turkish *Trifolium* for the first time in this study.

According to this study, the following conclusions and suggestions may be proposed;

1) This preliminary study performed in Bolu province revealed that both type of molecular markers (RAPD, and SSR) taxonomically have separated *Trifolium* taxa in sectional base. However, interspecies studies should be supported with additional molecular markers especially for infrasectional members.
2) The similar results of RAPD and SSR markers prooved that they are both supporting the sectional separations. Therefore, using two or more marker sytems in such studies are more reliable than using just single marker system. This study emphasized the necessity of the use high numbers of markers and taxa for obtaining more reliable results.
3) Molecular and morphological parts of the study provided different results. The disparity of our morphological and molecular results can be explained by adaptive characters that are accumulated in similar ecological conditions and different combinations of alleles producing similar phenotypes. In addition to these, the deficiency of effective morphological characters can be another reason.
4) The results showed that the relationships of *Trifolium* taxa from Bolu province support more or less Zohary and Heller (1984) classification system

and partially some phylogenetic classifications (Ellison et al. 2006, Vizintin et al. 2006, Watson et al. 2000).

## 5. REFERENCES


Abberton, M. T. 2007. Interspecific hybridization in the genus *Trifolium*. Plant Breeding. 126: 337-342.

Algan, G. and Büyükkartal, H. N. B. 1999. Apomictic development in tetraploid red clover (*Trifolium pratense* L.). Turkish Journal of Agriculture and Forestry. 23: 519-525.

Arzani, A., Samei, K. 2004. Assessment of genetic diversity among persian clover cultivars as revealed by RAPD markers. In: Vollmann, J., Grausgruber, H. and Ruckenbauer, P. (eds.) Genetic Variation for Plant Breeding. EUCARPIA and BOKU- University of Natural Resources and Applied Life Sciences, Vienna, Austria, 85-88.

Başaran, U., Acar, Z., Mut H., Aşçı, Ö. Ö. 2006. Some morphological and agricultural characters of some forage legumes naturally grown. Journal of the Faculty of Agriculture, OMU. 21: 314-317.

Bennett, S. J. 2000. Genetic variation of five species of *Trifolium* L. from south-west Turkey. Genetic Resources and Crop Evolution. 47: 81-91.

Bennett, S. J. and Mathews, A. 2003. Assessment of genetic diversity in clover species from sardinia, Italy, using AFLP analysis. Plant Breeding. 122: 362-367.

Blakesmith, S. J., Lyons–Wall, P. M., George, C., Joannou, G. E., Petocz, P., Samman, S. 2003. Effects of supplementation with purified red clover (*Trifolium pratense*) isoflavones on plasma lipids and insulin resistance in healthy premenopausal women. British Journal of Nutrition. 89: 467-474.

Booth, N. L., Overk, C. R.,Yao, P., Totura, S., Deng, Y., Hedayat, A. S.,Bolton, J. L., Pauli, G. F., Farnsworth, N. R. 2006. Seasonal variation of red clover (*Trifolium pratense* L., Fabaceae) isoflavones and estrogenic activity. Journal of Agricultural and Food Chemistry. 54: 1277-1282.

Bortolini, F., Dall'Agnol, M., Wittmann, M T. S. 2006. Molecular characterization of the USDA white clover (*Trifolium repens* L.) core collection by RAPD markers. Genetic Resources and Crop Evolution. 53: 1081-1087.

Bruschi, P., Vendramin, F., Bussotti, P., Grossoni, G. G. 2003. Morphological and molecular diversity among Italian populations of *Quercus petraea* (Fagaceae). Annals of Botany. 91: 707-716.

Bulinska-Radomska, Z. B. 2000. Morphological relationships among 15 pecies of *Trifolium* occuring in Poland. Genetic Resources and Crop Evolution. 47: 267-172.

Choi, H., Mun, J., Kim, D., Zhu, H., Baek, J., Mudge, J., Roe, B., Ellis, N., Doyle, J., Kiss, G. B., Young, N. D., Cook, D. R. 2004. Estimating genome conservation between crop and model legume species. PNAS 101: 15289-15294.

Cholastova, T. and Knotova, D. 2012. Using morphological and microsatellite (SSR) markers to assess the genetic diversity in alfalfa (*Medicago sativa* L.). World Academy of Science, Engineering and Technology. 69: 856-862.

Cocks, P. S. 1993. Legumes from mediterranean basin: a continuing source of agricultural wealth for southern Australia. Technical paper No. 1. CLIMA, Perth, Australia.

Coombe, D. E. 1968. *Trifolium* L. In: Tutin, T. G., Heywood, V. H., Burges, N. A.,Moore, D. M., Valentine, D. H., Walters, S. M. & Webb, D. A. (eds.), Cambridge University Press, Cambridge. *Flora Europaea*, Vol. 2: 157-172.

Coon, J. T., Pittler, M. H., Ernst, E. 2007. *Trifolium pratense* isoflavones in the treatment of menopausal hot flushes: a systematic review and meta-analysis. Phytomedicine. 14: 153-159.

Crawford, D. J., Esselman, E. J., Windus, J. L., Pabin, C. S. 1998. Genetic variation in running buffalo clover (*Trifolium stoloniferum*: Fabaceae) using random amplified polymorphic DNA markers (RAPDs). 85: 81-89.

Çakmakçı, S. and Çeçen, S. 1999. The possibilities at entering crop rotation system of certain annual legume plants in Antalya. Turkish Journal of Agriculture and Forestry. 23: 119-123.

Davis, P. H. 1970. Flora of Turkey and the East Aegean Islands. Edinburgh University Press, Edinburgh, 3: 384-448.

Demirel, R., Saruhan, V., Baran, M. S., Andiç, N., Demirel, D. Ş. 2010. Determination of different ratios of *Trifolium repens* and *Hordeum vulgare* mixtures on silage quality. YYU The Journal of Agricultural Science. 20: 26-31.

Demiroğlu, G. and Avcıoğlu, R. 2010. An investigation on the performances of some new Leguminous forage crops cultivars under Mediterranean climatic conditions. Ege Üniversitesi Ziraat Fakültesi Dergisi. 47: 151-159.

De Rijkea, E., Zafra-Gómezb, A., Ariesea, F., Brinkmana, U. A. T., Gooijer, C. 2001. Determination of isoflavone glucoside malonates in *Trifolium pratense* L. (red clover) extracts: quantification and stability studies. Journal of Chromatography A. 932: 55-64.

Dias, P. M. B., Julier, B., Sampoux, J. P., Barre, P., Dall'Agnol, M. 2008. Genetic diversity in red clover (*Trifolium pratense* L.) revealed by morphological and microsatellite (SSR) markers. Euphytica. 160: 189-205.

Diggs, G. M., Lipscomb, B. L., O'Kennon, R. J. 1999. Shinners & Mahler's Illustrated Flora of North Central Texas. Botanical Research Institute of Texas and Austin College, Fort Worth, Texas, USA, 618-623.

Dolanska, L., Curn, V. 2004. Identification of white clover (*Trifolium repens* L.) cultivars using molecular markers. Plant Soil Environment. 50 (3): 95-100.

Drenin, A. A., Botirov, E. K., Turov, Y. P. 2011. A new isoflavone glycoside from *T. pratense*. Russian Journal of Bioorganic Chemistry. 37: 862-865.

Ellison, N. W., Liston, A., Steiner, J. J., Williams, W. M., Taylor, N. L. 2006. Molecular phylogenetics of the clover genus (*Trifolium- Leguminosae*). Molecular Phylogenetics and Evolution. 39: 688-705.

Endo, Y. and Ohashi, H. 1997. Cladistic analysis of phylogenetic relationships among tribes Cicereae, Trifolieae and Viceae (Leguminosae). American Journal of Botany. 84: 523-529.

Erdemli, S., Çolak, E., Kendir, H. 2007. Determination of some plant and agricultural characteristics in persian clover (*Trifolium resupinatum* L.). Tarım Bilimleri Dergisi, Ankara Üniversitesi Ziraat Fakültesi. 13: 240-245.

Fahima, T., Röder, M. S., Wendehake, K., Kirzhner, V. M., Nevo, E. 2002. Microsatellite polymorphism in natural populations of wild emmer wheat, *Triticum dicoccoides*, in Israel. Theoretical and Applied Genetics. 104: 17-29.

Foo, L. Y., Lu, Y., Molan, A. L., Woodfield, D. R., McNabb, W. C. 2000. The phenols and prodelphinidins of white clover flowers. Phytochemistry. 54: 539-548.

Fotiadis, G., Vrahnakis, M. S., Merou, T. H., Vidakis, K. 2010. Ecology, chorology and commonness of the *Trifolium* taxa in Greece. Feddes Repertorium. 121: 66-80.

Gazara, M., Kamel, W., Haider, A. 2001. Cladistic analysis of the genera: *Trifolium*, *Trigonella*, *Melilotus* (Fabaceae: Papilionaceae) in Egypt. Egyptian Journal of Biology. 3: 161-170.

George, J., Dobrowolski, M. P., De Jong, E. V. Z., Cogan, N. O. I., Smith, K. F., Foster, J. W. 2006. Assessment of genetic diversity in cultivars of white clover (*Trifolium repens* L.) detected by SSR polymorphisms. Genome. 49: 919-930.

Graham, P. H. and Vance C. P. 2003. Legumes: importance and constraints to greater use. Plant phsiology. 131: 872-877.

Greene, S. L., Gritsenko, M., Vandemark, G. 2004. Relating morphologic and RAPD marker variation to collection site environment in wild populations of red clover (*Trifolium pratense* L.). Genetic Ressources and Crop Evolution. 51: 643-653.

Gustine, D. L., Voigt, P. W., Brummer, E. C., Papadopoulos, Y. A. 2002. Genetic variation of RAPD markers for North American white clover collections and cultivars. Crop Science. 42: 343-347.

Hakyemez, B. H. and Sancak, C. 2005. Adaptation of some berseem clover (*Trifolium alexandrinum* L.) varieties to the irrigated conditions of Ankara and changes in yield according to the cutting orders. Tarım Bilimleri Dergisi. 11: 406-410.

Hampl V, Pavlícek A, Flegr J. 2001. Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with a freeware program FreeTree: application to trichomonad parasites. International Journal of Systematic and Evolutionary Microbiology. 51: 731-735.

Herrmann, D. 2006. Characterisation of Genetic Diversity and Molecular Dissection of Seed Yield and Persistence in Swiss Mattenklee (*Trifolium pratense* L.) Zürich University, Ph.D Thesis.

Herrmann D, Boller B, Studer B, Widmer F, Kölliker R. 2008. Improving persistence in red clover - insights form QTL analysis and comparative phenotypic evaluation. Crop Science. 48: 269-277.

Herrmann, D., Boller, B., Studer, B., Widmer, F., Kölliker, R. 2006. QTL analysis of seed yield components in red clover (*Trifolium pratense* L.) Theoretical and Applied Genetics. 112: 536-545.

Hollingsworth, P. M., Ennos, R. A. 2004. Neighbour joining trees, dominant markers and population genetic structure. Heredity. 92: 490-498.

Işık, F. E. 2005. Edirne Bölgesinde Yetişen *Trifolium resupinatum* L. var. *microcephalum* Bitkisinin Fitokimyasal İncelenmesi. Trakya University, Ph.D Thesis.

Jaccard, P. 1908. Nouvelles recherches sur la distribution florale. Bull Soc Vaud Nat. 44: 223-270.

Jones, W. T., Broadhurst, R. B., Lyttleton, J. W. 1976. The condensed tannins of pasture legume species. Phytochemistry. 15: 1407-1409.

Jones, E. S., Hughes, L. J., Drayton, M. C., Abberton, M. T., Yeates, T. P. T. M., Bowen, C., Forster, J. W. 2003. An SSR and AFLP molecular marker based genetic map of white clover (*Trifolium repens* L.). Plant Science. 165: 531-539.

Johns, M., Skroch, P., Nienhuis, P., Bascur, G., MunozSchick, C. 1997. Gene pool classification of common bean landraces from Chile based on RAPD and morphological data. Crop Science. 37: 605-613.

Joyce, . A., Abberton, M. T., Yeates, T. P. T. M., Foster, J. W. 1999. relationships between genetic distance measured by RAPD-PCR and heterosis in inbred lines of white clover (*Trifoliumrepens* L.). Euphytica. 107: 159-165.

Judd, W. S., Campbell, C. S., Kellogg, E. A., Stevens, P. F., Donoghue, M. J. 2002. Plant systematics a phylogenetic approach. Sinauer Associates, 2. Edition, Sunderland, UK, 356-357.

Kadıoğlu, O. 2008. *T. pratense* L.'nin Fitoterapideki Önemi. Gazi University, M.Sc Thesis.

Kashi, Y., King, D. G., Soller, M. 1997. Simple sequence repeats as a source of quantitative genetic variation. Trends in Genetics. 13: 74-78.

Kaufman, P. B., Carlson, T. F., Dayanandan, P., Evans M. L., Fisher, J. B., Parks, C., Wells, J. R. 1989. Plants their biology and importance. Harper and Row, Newyork, USA, 652-653.

Khan, S. W. and Khatoon, S. 2008. Ethnobotanical studies on some useful herbs of Haramosh and Bugrote Valleys in Gilgit, Northern Areas of Pakistan. Pakistan Journal of Botany. 40: 43-58.

Khanlou, K. M., Vandepitte, K., Asl, L. K., Bockstaele, E. V. 2011. Towards an optimal sampling strategy for assessing genetic variation within and among white clover (*Trifolium repens* L.) cultivars using AFLP. Genetic and Molecular Biology. 34: 252-258.

Kıran, Y., Sahin, A., Turkoglu, İ., Kursat, M., Emre, İ. 2010. Karyology of seven *Trifolium* L. taxa growing in Turkey. Acta Biologica Cracoviensia. 2: 93-97.

Kjaergaard, T. 2003. A Plant that changed the world: the rise and fall of clover 1000-2000. Landscape Research. 28: 41-49.

Koçyiğit, M., Keskin, M., Daştan, T. 2013. Pollen morphology of some *Trifolium* species which are favorite plants of honey bees in Istanbul. Journal of Faculty Pharmacy of Istanbul University. 43: 85-94.

Kolodziejczyk-Czepas, J. 2012. *Trifolium* species-derived substances and extracts-biological activity and prospects for medicinal applications. Journal of Ethnopharmacology. 143: 14-23.

Kölliker, R., Herrmann, D., Boller, B., Widmer, F. 2003. Swiss Mattenklee landraces, a distinct and diverse genetic resource of red clover. Theoretical and Applied Genetics. 107: 306-315.

Kölliker, R., Jones, E. S., Drayton, M. C., Dupal, M. P., Forster, J. W. 2001a. Development and characterisation of simple sequence repeat (SSR) markers for white clover (*Trifolium repens* L.). Theoretical and Applied Genetics. 102: 416-424.

Kölliker, R., Jones, E. S., Jahufer, M. Z. Z., Foster, J. W. 2001b. Bulked AFLP analysis for the asessment of genetic diversity in white clover (*Trifolium repens* L.). Euphytica. 121: 305-315.

Leistner, O. A. 2005 Seed plants of southern tropical africa: families and genera. In Mössmer, M., Gibson, L., van Heerden, H. (Eds). Southern African Botanical Diversity Network Report No. 26. SABONET, Pretoria, Africa,12 (27): 349-1802.

Li, Y., Fahima, T., Krugman, T., Beiles, A., Röder, M. S., Korol, A. B., Nevo, E. 2000. Parallel microgeographic patterns of genetic diversity and divergence revealed by allozyme, RAPD, and microsatellites in *Triticum dicoccoides* at Ammiad, Israel. Conservation Genetics. 1: 191-207.

Linnaeus, C. 1753. Species plantarum, exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas. Impensis Laurentii Salvii, Holmiae (Stockholm), Vol 1.

Liu, Z. and Wang, L. 2014. A plant species (*Trifolium repens*) with strong enrichment ability for mercury. Ecological Engineering. 70: 349-350.

Lojacono, M. 1883a. Revisione dei Trifolgi dell' America settentionale. Nuov. Giorn. Bot. Ital. 15: 113-198.

Lojacono, M. 1883b. Clavis specierum Trifoliorum. Nuov. Giorn. Bot. Ital. 3: 15-278.

Majumdar, S., Banerjee, S., Kumar De, K. 2004. Meiotic behaviour of chromosomes in PMCs and karyotype of *Trifolium repens* L. from Darjeeling Himalaya. Acta Biologica Cracoviensia. 46: 217-220.

Mengoni, A., Gori, A., Bazzicalupo, M. 2000. Use of RAPD and microsatellite (SSR) variation to assess genetic relationships among populations of tetraploid alfalfa, *Medicago sativa.* Plant Breeding.119: 311-317.

Mustafa, B., Hajdari, A., Krasniqi, F., Hoxha, E., Ademi, H., Quave, C. L., Pieroni, A. 2012. Medical ethnobotany of the Albanian Alps in Kosovo. Journal of Ethnobiology and Ethnomedicine. 8: 1-14.

Occhiuto, F., De Pasquale, R., Guglielmo, G., Palumbo, D. R.,Zangla, G., Samperi, S., Renzo, A., Circosta, C. 2007. Effects of phytoestrogenic isoflavones from red clover (*Trifolium pratense* L.) on experimental osteoporosis. Phytotherapy Research. 21: 130-134.

Oleszek, W., Stochmal, A., Janda, B. 2007. Concentration of isoflavones and other phenolics in the aerial parts of *Trifolium* species. Journal of Agricultural and Food Chemistry. 55: 8095-8100.

Olmos, F., Wilman, D., Hamilton, R. S. 2003. Variation between and within *Trifolium repens* L. populations collected from sown swards in Uruguay. Euphytica. 130:131-141.

Örsdöven, A. and Kendir, H. 2006. Determination of seed yield and yield components in some persian clover (*Trifolum resupinatum* L.) lines. Tarım Bilimleri Dergisi Ankara Üniversitesi Ziraat Fakültesi. 12: 210-215.

Peterson, T. A. and Russelle, M. P. 1991. Alfalfa and the nitrogen cycle in the corn belt. Journal of Soil Water Conservation. 46: 229-235.

Presl C. B. 1832. '*Trifolium*', in: Symbolae Botanicae. Sumpt. Auctoris, Vol 1: 44-50, Pragae.

Quiroz, H. C. and Klose, F. O. 2001. Genetic variability among elite red clover (*Trifolium pratense* L.) parents used in Chile as revealed by RAPD markers. Euphytica. 122: 61-67.

Ravagnani, A., Abberton, M. T., Skot, L. 2012. Development of genomic resources in the species of *Trifolium* L. and its application in forage legume breeding. Agronomy. 2: 116-131.

Ravi, M., Geethanjali, S., Sameeyafarheen, F., Maheswaran, M. 2003. Molecular marker based genetic diversity analysis in rice (*Oryza sativa* L.) using RAPD, SSR markers. Euphytica. 133: 243-252.

Savi, G. 1808-1810. Observationes in varias *Trifolium* species, Piatti Florentinae, p.p 118.

Seringe, N. 1825. '*Trifolium*' in : De Candolle A.P., Prodromus Systematis Naturalis regni Vegetabilis Vol 2: 189-207, Parisiis.

Sheokand, S., Dudeja, S. S., Swaraj, K. 2012. Nitrogen fixation in tropical environments-adaptive responses and benefits. Research on Crops. 13: 743-753.

Smil, V. 1999. Nitrogen in crop production: an account of global flows. Global Biogeochemical Cycles. 13: 647-662.

Soldanova, M. 2007. New microsatellite loci for red clover (*Trifolium pratense* L.) and study its polymorphism. Book of Abstracts of the 27th EUCARPIA Symposium on improvement of forage crops and amenity grasses. Copenhagen, Denmark, on 19th –23rd August 2007: 85.

Steele, K. P. and Wojciechowski, M. F. 2003. Phylogenetic analysis of tribes Trifolieae and Vicieae, based on sequences of the plastid gene *mat*K

(Papilionoideae: Leguminosae). In: Klitgaard, B. B. and Bruneau, A. (eds). Advanced in Legume Systematics, part 10, Higher Level Systematics, Royal Botanic Gardens, Kew, U.K 355-370.

Steiner, J. J., los Santos, G. G. 2001. Adaptive ecology of *Lotus corniculatus* L. genotypes: I. Plant morphology and RAPD marker characterization. Crop Science. 41: 552-563.

Stern, K. R., Jansky, S., Bidlack, J. E. 2003. Introductory plant biology. McGraw-Hill Higher Education, New York, USA, 471.

Sürmen, M., Yavuz, T., Albayrak, S. 2013. Yield and forage quality of red clover (*Trifolium pratense* L.) varieties in Black Sea Coastal Area of Turkey. Iğdır University Journal of the Institute of Science and Technology. 3: 87-92.

Tangpu, V., Temjenmongla, K., Yadav, A. K. 2004. Anticestodal activity of *Trifolium repens* extract. Pharmaceutical Biology. 42: 656-658.

Tautz, D., Trick, M., Dover, G. A. 1986. Cryptic simplicity in DNA is a major source of variation. Nature. 322: 652-656.

Taylor, N. L. 2008. A century of clover breeding developments in the United States. Crop Science. 48: 1-13.

Taylor, N. L., Quesenberry, K. H., Anderson, M. K. 1979. Genetic system relationships in *Trifolium*. Economic Botany. 33: 431-441.

Tham, D. M., Gardner, C. D., Haskell, W. L. 1998. Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. The Journal of Clinical Endocrinology and Metabolism. 83: 2223-2235.

Ulloa, O., Ortega, F., Campos, H. 2003. Analysis of genetic diversity in red clover (*Trifolium pratense* L.) breeding populations as revealed by RAPD genetic markers. Genome. 46: 529-535.

Uslu, E. 2012. Karyology of nine *Trifolium* L. taxa from Turkey. Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics. 65 (4): 304-310.

Vasiljević, S., Ćupina, B., Krstic, D., Pataki, I., Katanski, S., Milošević, B. 2011. Seasonal changes of proteins, structural carbohydrates, fats and minerals in herbage dry matter of red clover (*Trifolium pratense* L.). Biotechnology in Animal Husbandry. 27: 1543-1550.

Vizintin, L., Javornik, B., Bohanec, B. 2006. Genetic characterization of selected *Trifolium* species as revealed by nuclear DNA content and ITS rDNA region analysis. Plant Science. 170: 859-866.

Vymyslicky, T., Smarda, P., Pelikan, J., Cholastova, T., Nedelnik, J., Moravcova, H., Pokorny, R., Soldanova, M., Polakova, M. 2012. Evaluation of the Czech core collection of *Trifolium pratense*, including morphological, molecular and phytopathological data. African Journal of Biotechnology. 11: 3583-3595.

Wang, J., Drayton, M., George, J., Cogan, N. O. L., Baillie, R. C., Hand, M. L., Kearney, G. A., Erb, S., Wilkinson, T.,Bannan, N. R.,Forster, J. W., Smith, K. F. 2010. Identification of genetic factors influencing salt stress tolerance in white clover (*Trifolium repens* L.) by QTL analysis. Theoretical and Applied Genetics. 120: 607-619.

Watson, L. E., Ahmed, H. S., Badr, A. 2000. Molecular phylogeny of old world *Trifolium* (Fabaceae), based on plastid and nuclear markers. Plant Systematics and Evolution. 224: 153-171.

Williams J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey S. V. 1990. DNA polymorhisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Research. 18: 6531-6535.

Williams, W. M., Ansari, H. A., Hussain, S. W., Ellison, N. W., Hussain, S. W. 2001. Annuals of Botany. 87: 683-691.

Williams, W. M., Ansari, H. A., Hussain, S. W., Ellison, N. W., Williamson, M. L., Verry, I. M. 2008. Hybridization and introgression between two diploid wild relatives of white clover, *Trifolium nigrescens* Viv. and *T. occidentale* Coombe. Crop Science. 48: 139-148.

Yücel, E. 2011. Tıbbi Bitki Islahı ve Yetiştiriciliği. Bitkilerle Tedavi Sempozyumu. Zeytinburnu, İstanbul, 5-6 Haziran. 2011: 97.

Zhang, Y., Sledge, M. K., Bouton, J. H. 2007. Genome mapping of white clover (*Trifolium repens* L.) and comparative analysis within the Trifolieae using cross-species SSR markers. Theoretical and Applied Genetics. 114: 1367-1378.

Zhang, Y., He, J., Zhao, P. X., Bouton, J. H., Monteros, M. J. 2008. Genome-wide identification of microsatellite in white clover (*Trifolium repens* L.) using FIASCO and phpSSRMiner. Plant Methods. 4: 19.

Zhang, X., Zhang, Y., Yan, R., Han, J., Hong, F., Wang, J., Coa, K. 2010. Genetic variation of white clover (*Trifolium repens* L.) collections from China detected by morphological traits, RAPD and SSR. African Journal of Biotechnology. 9: 3032-3041.

Zohary, M. 1972. Origins and evolution in the genus *Trifolium*. *Botaniska Notiser*. 125: 501-511.

Zohary, M. and Heller, D. 1984. The Genus *Trifolium*. Israel Academy of Sciences and Huminities. Jerusalem, Israel.

Zoric, L., Merkulov, L., Lukovic, J., Boza, P. 2012. Comparative analysis of qualitative anatomical characters of *Trifolium* L. (Fabaceae) and their taxonomic implications: preliminary results. Plant Systematic Evolution. 298: 205-219.

Zoric, L., Merkulov, L., Lukovic, J., Boza, P. 2010. Comparative seed morphology of *Trifolium* L. species (Fabaceae). Periodicum Biologorum. 112: 263-272.

Zoric, L., Merkulov, L., Lukovic, J., Boza, P., Polic, D. 2009. Leaf epidermal characteristics of *Trifolium* L. species from Serbia and Montenegro. Flora. 204: 198-209.

**URL1** ILDIS online database.

[ http://www.ildis.org/Leguminosae]

**URL2** Diagnosis key program DELTA

[www.intkey.com]

**URL3** Illustration of *T. repens*

[http://pl.wikipedia.org/wiki/Koniczyna_bia%C5%82a#mediaviewer/File:Illustration_Trifolium_repens0.jpg]

Table 1A Arithmetic means with standart errors of quantitative data

| TAXA | PDL | PTL | SL | SW | TLL | TLW | ID | IL | TPL | FCL |
|---|---|---|---|---|---|---|---|---|---|---|
| ARV | 20.47±2.26 | 12.33±0.80 | 7.32±0.18 | 3.10±0.07 | 16.46±0.30 | 3.18±0.08 | 11.03±0.28 | 13.70±0.51 | 0.57±0.02 | 6.13±0.10 |
| AUR | 19.70±1.26 | 17.11±1.08 | 9.98±0.19 | 3.35±0.09 | 16.19±0.34 | 7.77±0.20 | 14.61±0.19 | 15.35±0.49 | 0.41±0.03 | 4.17±0.16 |
| BOC | 20.55±0.86 | 16.98±2.00 | 7.72±0.25 | 2.77±0.12 | 10.20±0.37 | 3.24±0.14 | 8.12±0.33 | 11.24±0.40 | 0.46±0.02 | 4.07±0.06 |
| CAM | 15.02±1.45 | 9.96±0.90 | 5.11±0.07 | 3.60±0.20 | 9.53±0.34 | 5.10±0.21 | 8.67±0.35 | 8.26±0.43 | 2.19±0.12 | 1.97±0.10 |
| CON | 36.09±3.85 | 34.28±2.99 | 10.05±0.46 | 2.85±0.14 | 20.17±0.73 | 9.23±0.33 | 15.12±0.54 | 17.01±0.65 | 0.74±0.02 | 6.30±0.20 |
| DIF | 0.96±0.13 | 49.28±3.99 | 13.04±0.44 | 6.24±0.13 | 12.56±0.52 | 8.64±0.35 | 27.21±0.74 | 27.07±0.45 | 0.77±0.03 | 7.60±0.29 |
| DUB | 11.10±0.96 | 5.06±0.40 | 2.76±0.19 | 2.15±0.11 | 6.33±0.36 | 3.28±0.10 | 6.88±0.23 | 7.25±0.36 | 0.94±0.10 | 1.36±0.04 |
| ECH | 27.83±3.13 | 26.91±1.88 | 9.88±0.40 | 2.98±0.19 | 13.28±0.60 | 6.12±0.42 | 11.56±0.39 | 12.13±0.72 | 0.77±0.03 | 5.31±0.22 |
| FRA | 99.80±11.10 | 55.73±5.59 | 14.24±0.62 | 4.55±0.18 | 11.42±0.48 | 7.40±0.28 | 10.65±0.34 | 10.37±0.42 | 0.91±0.03 | 3.75±0.11 |
| HIR | 0.29±0.07 | 29.52±2.90 | 9.13±0.25 | 5.30±0.26 | 13.94±0.31 | 8.09±0.17 | 21.04±0.95 | 21.65±0.88 | 0.56±0.04 | 9.65±0.40 |
| HYBA | 52.96±3.50 | 39.49±3.56 | 9.86±0.96 | 5.48±0.37 | 14.46±1.03 | 10.76±0.78 | 14.81±0.29 | 13.45±0.48 | 0.82±0.04 | 3.28±0.15 |
| HYBE | 47.25±3.95 | 60.32±3.26 | 10.23±0.39 | 5.28±0.22 | 13.91±0.70 | 10.01±0.54 | 15.30±0.35 | 15.12±0.51 | 0.74±0.03 | 3.24±0.08 |
| MEDE | 17.01±1.64 | 46.53±3.13 | 16.33±0.47 | 7.59±0.35 | 36.69±1.08 | 14.47±0.44 | 23.61±1.43 | 27.93±1.65 | 0.88±0.05 | 10.06±0.24 |
| MEDM | 5.25±0.49 | 39.74±1.80 | 15.63±0.37 | 7.37±0.31 | 33.95±0.63 | 14.21±0.23 | 24.31±0.78 | 23.98±0.77 | 0.78±0.03 | 9.45±0.14 |
| MIC | 7.88±0.71 | 1.73±0.13 | 2.44±0.10 | 1.83±0.08 | 6.01±0.45 | 3.19±0.19 | 4.93±0.23 | 4.53±0.25 | 0.50±0.08 | 1.58±0.03 |
| NIG | 35.44±3.02 | 34.14±4.72 | 4.86±0.34 | 4.00±0.24 | 9.48±0.81 | 7.16±0.67 | 15.56±0.42 | 14.52±0.33 | 0.68±0.06 | 4.19±0.10 |
| OCH | 1.40±0.32 | 52.41±5.81 | 14.69±0.60 | 7.24±0.26 | 15.29±0.75 | 10.03±0.34 | 25.98±1.30 | 25.36±0.69 | 0.84±0.04 | 9.33±0.49 |
| PAL | 5.62±0.70 | 11.65±0.91 | 5.78±0.11 | 2.39±0.12 | 7.19±0.20 | 4.37±0.14 | 10.39±0.37 | 8.73±0.39 | 0.50±0.02 | 6.04±0.12 |
| PAN | 36.55±5.46 | 36.62±3.85 | 24.59±2.03 | 4.61±0.34 | 22.60±0.70 | 4.90±0.27 | 19.38±1.94 | 28.52±2.95 | 0.96±0.03 | 11.05±0.66 |
| PAT | 16.12±0.10 | 7.23±1.15 | 4.45±0.20 | 2.90±0.23 | 8.23±0.31 | 5.22±0.12 | 8.10±0.22 | 7.40±0.21 | 1.77±0.17 | 2.18±0.06 |
| PHY | 35.60±3.06 | 51.96±6.03 | 18.10±0.62 | 5.26±0.22 | 16.42±0.48 | 10.94±0.28 | 20.30±0.46 | 18.90±0.65 | 0.85±0.03 | 6.10±0.13 |
| PRA | 2.30±0.39 | 60.23±4.50 | 13.48±0.52 | 8.12±0.34 | 24.36±1.01 | 12.46±0.41 | 20.74±0.71 | 22.14±0.95 | 1.00±0.06 | 7.74±0.26 |
| REPM | 101.88±7.54 | 65.22±4.76 | 11.32±0.43 | 3.41±0.14 | 15.15±0.48 | 13.86±0.40 | 24.62±0.43 | 23.46±0.75 | 1.09±0.06 | 5.53±0.09 |
| REPR | 202.80±30.10 | 94.53±6.82 | 11.75±0.73 | 4.75±0.50 | 19.84±0.54 | 17.20±0.85 | 25.99±1.25 | 24.55±1.40 | 1.21±0.06 | 5.49±0.15 |
| RES | 27.81±4.19 | 36.36±3.52 | 7.55±0.37 | 4.37±0.17 | 14.16±0.54 | 9.20±0.43 | 10.82±0.44 | 7.69±0.36 | 0.59±0.04 | 2.76±0.10 |
| RET | 18.87±1.45 | 48.27±2.87 | 10.03±0.26 | 3.71±0.11 | 14.34±0.69 | 6.68±0.30 | 11.91±0.09 | 11.34±0.33 | 0.64±0.03 | 5.30±0.12 |
| SCA | 1.30±0.18 | 23.62±2.81 | 4.84±0.18 | 3.45±0.23 | 8.38±0.34 | 4.92±0.15 | 7.65±0.51 | 8.82±0.32 | 0.44±0.02 | 4.90±0.19 |
| SPA | 27.88±2.54 | 24.04±1.57 | 10.58±0.51 | 3.56±0.12 | 13.43±0.62 | 6.57±0.24 | 10.64±0.42 | 11.59±0.78 | 0.39±0.01 | 2.63±0.12 |
| STR | 1.62±0.30 | 39.54±3.32 | 7.31±0.34 | 5.13±0.45 | 13.85±0.63 | 8.86±0.41 | 7.24±0.53 | 12.35±0.72 | 0.74±0.04 | 5.31±0.12 |

Table 1A Arithmetic means with standart errors of quantitative data (Continued)

| TAXA | LTCL | STCL | CTL | NCV | PCL | SDL | SDW | IFL | LTCL/STCL | LTCL/CTL |
|---|---|---|---|---|---|---|---|---|---|---|
| **ARV** | 4.22±0.08 | 3.82±0.07 | 1.92±0.05 | 10.00±0.00 | 0.43±0.01 | 2.54±0.08 | 1.13±0.03 | 6.14±0.10 | 1.10±0.01 | 2.21±0.05 |
| **AUR** | 3.24±0.18 | 1.79±0.06 | 0.84±0.02 | 5.00±0.00 | 0.59±0.02 | 6.15±0.10 | 4.92±0.09 | 7.28±0.09 | 1.84±0.06 | 3.96±0.27 |
| **BOC** | 2.33±0.06 | 2.02±0.05 | 1.85±0.03 | 10.00±0.00 | 0.39±0.02 | 1.98±0.04 | 0.89±0.03 | 4.07±0.06 | 1.16±0.01 | 1.27±0.04 |
| **CAM** | 1.37±0.09 | 0.40±0.03 | 0.59±0.01 | 5.00±0.00 | 0.57±0.04 | 3.47±0.15 | 3.41±0.12 | 4.40±0.14 | 3.49±0.10 | 2.34±0.13 |
| **CON** | 4.14±0.14 | 2.15±0.09 | 2.24±0.05 | 10.00±0.00 | 0.35±0.01 | 6.43±0.19 | 3.07±0.08 | 10.25±0.29 | 1.94±0.04 | 1.86±0.05 |
| **DIF** | 4.15±0.22 | 2.43±0.11 | 3.54±0.10 | 10.00±0.00 | 0.60±0.02 | 11.12±0.14 | 3.95±0.06 | 20.62±0.30 | 1.71±0.05 | 1.18±0.05 |
| **DUB** | 0.80±0.03 | 0.29±0.02 | 0.50±0.02 | 5.00±0.00 | 0.55±0.03 | 3.06±0.11 | 2.70±0.17 | 3.75±0.12 | 2.85±0.05 | 1.67±0.07 |
| **ECH** | 3.70±0.20 | 2.11±0.09 | 1.74±0.05 | 10.00±0.00 | 0.24±0.01 | 6.40±0.13 | 2.34±0.06 | 9.93±0.30 | 1.85±0.14 | 2.17±0.13 |
| **FRA** | 2.01±0.08 | 1.72±0.06 | 1.69±0.06 | 9.00±0.00 | 0.71±0.03 | 5.01±0.07 | 2.06±0.08 | 6.67±0.11 | 1.18±0.02 | 1.21±0.06 |
| **HIR** | 6.87±0.38 | 5.67±0.34 | 3.14±0.12 | 20.73±0.20 | 0.16±0.01 | 8.47±0.30 | 2.47±0.07 | 14.82±0.42 | 1.22±0.02 | 2.30±0.09 |
| **HYBA** | 1.94±0.13 | 1.48±0.07 | 1.41±0.06 | 10.00±0.00 | 1.63±0.12 | 5.16±0.20 | 3.18±0.12 | 6.10±0.26 | 1.31±0.03 | 1.40±0.10 |
| **HYBE** | 2.10±0.08 | 1.78±0.06 | 1.24±0.02 | 10.00±0.00 | 1.56±0.12 | 5.02±0.08 | 3.18±0.10 | 6.19±0.12 | 1.19±0.02 | 1.70±0.05 |
| **MEDE** | 6.70±0.29 | 3.32±0.21 | 3.48±0.12 | 10.00±0.00 | 0.53±0.04 | 8.39±0.22 | 4.33±0.19 | 15.93±0.38 | 2.06±0.11 | 1.97±0.12 |
| **MEDM** | 5.99±0.12 | 3.75±0.13 | 3.72±0.04 | 10.44±0.23 | 0.56±0.02 | 7.83±0.10 | 4.49±0.14 | 15.05±0.29 | 1.63±0.09 | 1.61±0.02 |
| **MIC** | 0.98±0.02 | 0.53±0.02 | 0.54±0.02 | 5.00±0.00 | 0.98±0.06 | 2.12±0.08 | 1.36±0.07 | 2.72±0.10 | 1.90±0.05 | 1.87±0.07 |
| **NIG** | 2.46±0.10 | 1.24±0.12 | 1.66±0.08 | 10.00±0.00 | 2.16±0.09 | 5.70±0.09 | 3.16±0.02 | 6.93±0.09 | 2.10±0.12 | 1.50±0.06 |
| **OCH** | 5.39±0.30 | 2.83±0.14 | 4.08±0.17 | 10.00±0.00 | 0.71±0.04 | 12.57±0.64 | 4.49±0.21 | 22.09±1.37 | 1.92±0.05 | 1.33±0.05 |
| **PAL** | 4.05±0.10 | 3.67±0.10 | 2.02±0.05 | 17.70±0.43 | 0.00±0.00 | 3.23±0.09 | 2.05±0.05 | 7.08±0.15 | 1.11±0.01 | 2.02±0.04 |
| **PAN** | 8.05±0.48 | 5.15±0.39 | 3.50±0.22 | 10.00±0.00 | 0.55±0.03 | 11.64±1.19 | 3.30±0.22 | 18.01±1.53 | 1.61±0.05 | 2.34±0.07 |
| **PAT** | 1.40±0.05 | 0.62±0.02 | 0.73±0.03 | 5.00±0.00 | 0.85±0.01 | 3.42±0.07 | 2.50±0.09 | 4.66±0.06 | 2.30±0.06 | 1.95±0.07 |
| **PHY** | 3.82±0.10 | 3.13±0.10 | 2.85±0.10 | 20.00±0.00 | 0.45±0.02 | 8.14±0.17 | 2.24±0.06 | 11.58±0.19 | 1.24±0.02 | 1.37±0.05 |
| **PRA** | 4.86±0.19 | 2.51±0.12 | 3.04±0.10 | 10.00±0.00 | 0.32±0.02 | 6.72±0.15 | 3.51±0.10 | 13.04±0.41 | 1.99±0.11 | 1.61±0.07 |
| **REPM** | 2.83±0.06 | 2.03±0.05 | 2.64±0.07 | 10.00±0.00 | 3.12±0.15 | 7.76±0.14 | 3.90±0.09 | 9.99±0.21 | 1.41±0.03 | 1.08±0.03 |
| **REPR** | 2.79±0.11 | 1.97±0.09 | 2.68±0.05 | 10.00±0.00 | 3.20±0.45 | 7.79±0.29 | 4.57±0.19 | 10.16±0.39 | 1.44±0.04 | 1.04±0.03 |
| **RES** | 1.57±0.06 | 1.14±0.09 | 1.13±0.03 | 10.00±0.00 | 0.44±0.03 | 3.86±0.11 | 1.96±0.06 | 5.66±0.21 | 1.51±0.12 | 1.41±0.05 |
| **RET** | 3.82±0.10 | 1.34±0.03 | 1.68±0.03 | 10.00±0.00 | 0.51±0.02 | 3.07±0.07 | 2.21±0.05 | 5.24±0.06 | 2.88±0.06 | 2.31±0.07 |
| **SCA** | 2.75±0.12 | 1.64±0.07 | 2.06±0.07 | 10.00±0.00 | 0.30±0.02 | 3.45±0.14 | 1.77±0.07 | 5.36±0.20 | 1.70±0.06 | 1.34±0.04 |
| **SPA** | 1.97±0.10 | 0.68±0.07 | 0.59±0.02 | 5.00±0.00 | 0.40±0.02 | 4.06±0.14 | 3.08±0.05 | 5.05±0.14 | 3.14±0.25 | 3.44±0.18 |
| **STR** | 2.68±0.08 | 2.06±0.08 | 2.59±0.06 | 10.00±0.00 | 0.47±0.02 | 2.93±0.10 | 1.20±0.05 | 5.47±0.09 | 1.31±0.03 | 1.04±0.03 |

## APPENDIX B BOXPLOTS AND CHARACTER CODING

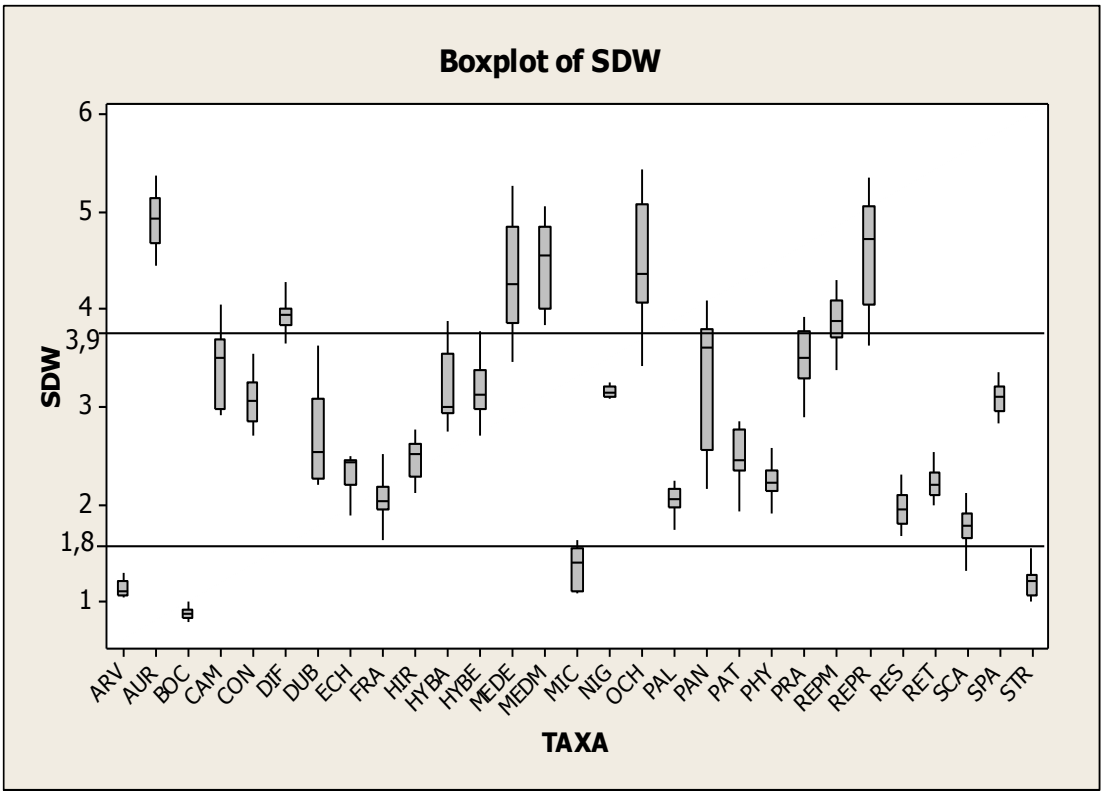


Figure 1B- Boxplot of standard width (SDW)

Table 1B- Scoring character states for standard width (SDW)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <1.80 | 0 |
| 2 | 1.80-3.90 | 1 |
| 3 | 3.90< | 2 |

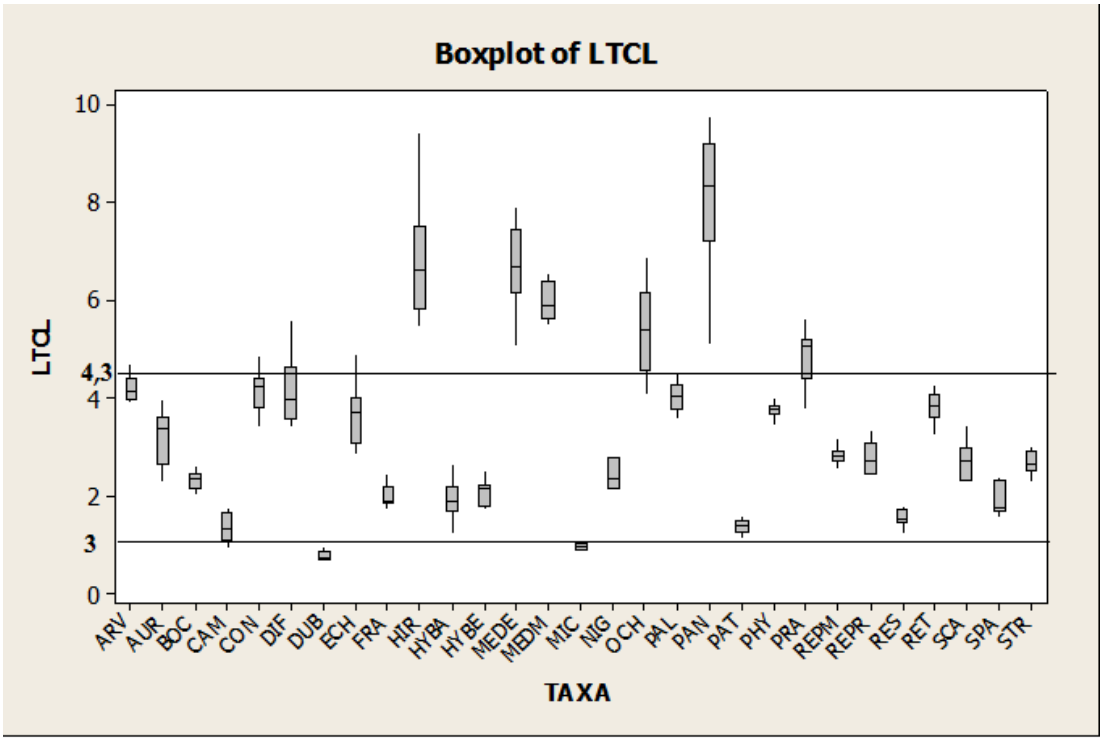


Figure 2B- Boxplot of long teeth calyx length (LTCL)

Table 2B- Scoring character states for long teeth calyx length (LTCL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <3.00 | 0 |
| 2 | 3.00-4.30 | 1 |
| 3 | 4.30< | 2 |

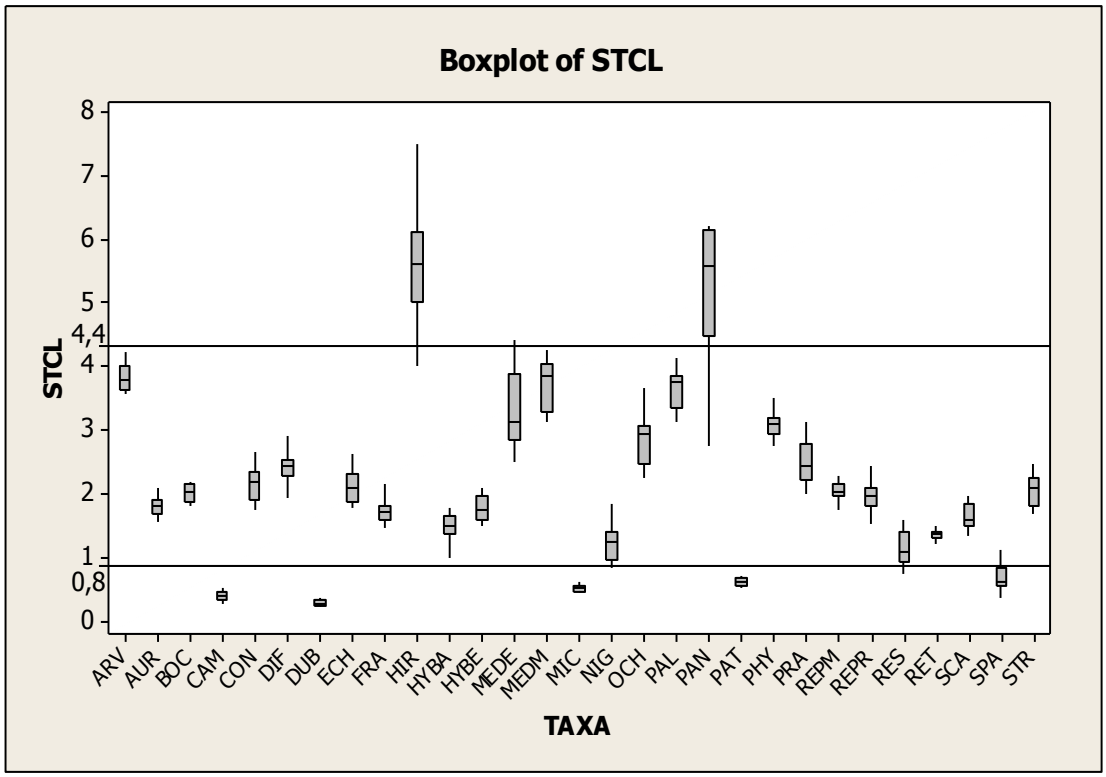


Figure 3B- Boxplot of short teeth calyx length (STCL)

Table 3B- Scoring character states for short teeth calyx length (STCL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <0.80 | 0 |
| 2 | 0.80-4.40 | 1 |
| 3 | 4.40< | 2 |

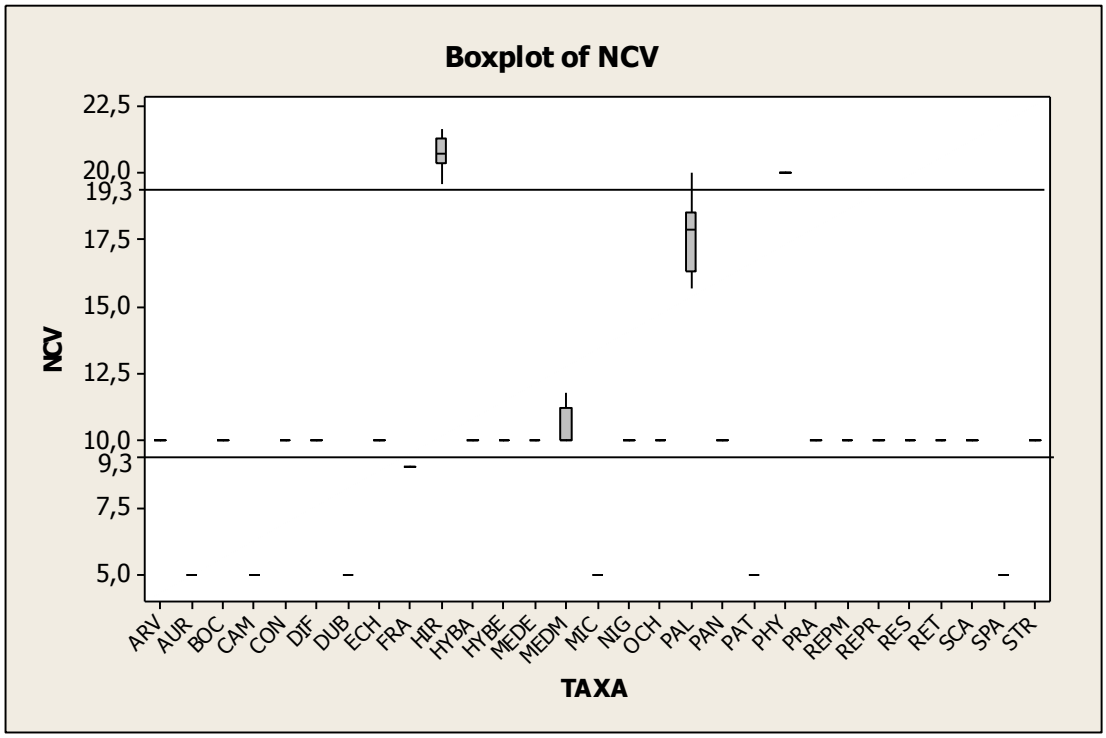


Figure 4B- Boxplot of number of calyx veins (NCV)

Table 4B- Scoring character states for number of calyx veins (NCV)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <9.30 | 0 |
| 2 | 9.30-19.30 | 1 |
| 3 | 19.30< | 2 |

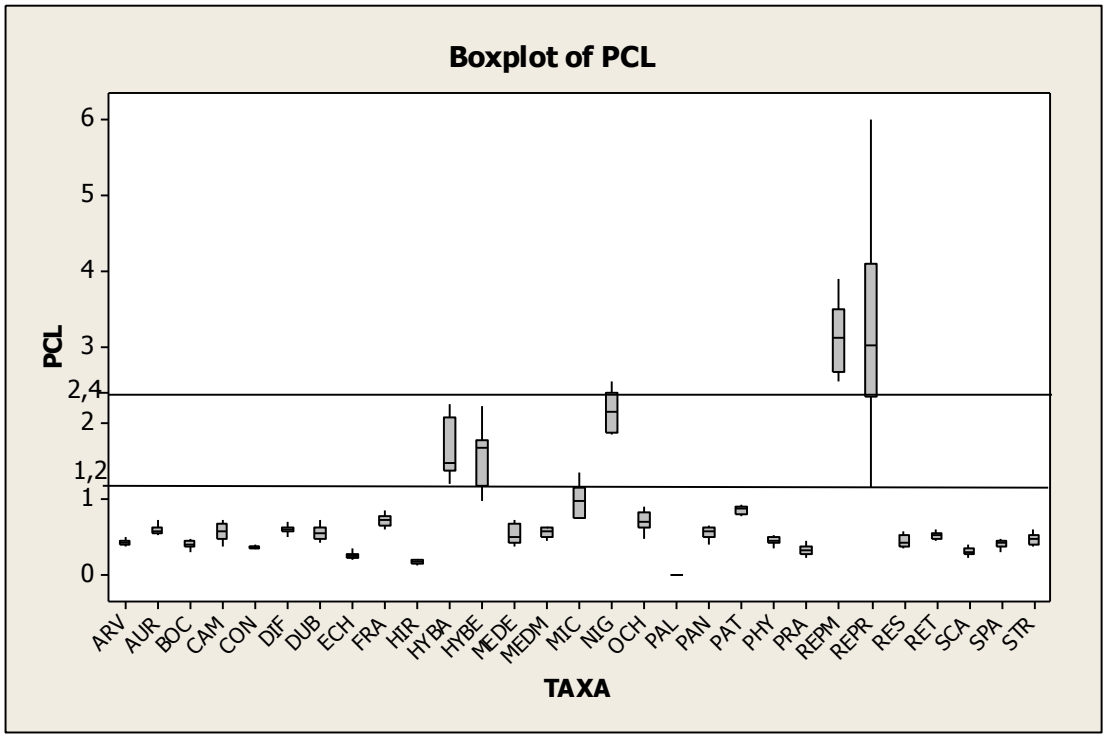


Figure 5B- Boxplot of number of pedicel length (PCL)

Table 5B- Scoring character states for pedicel length (PCL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <1.20 | 0 |
| 2 | 1.20-2.40 | 1 |
| 3 | 2.40< | 2 |

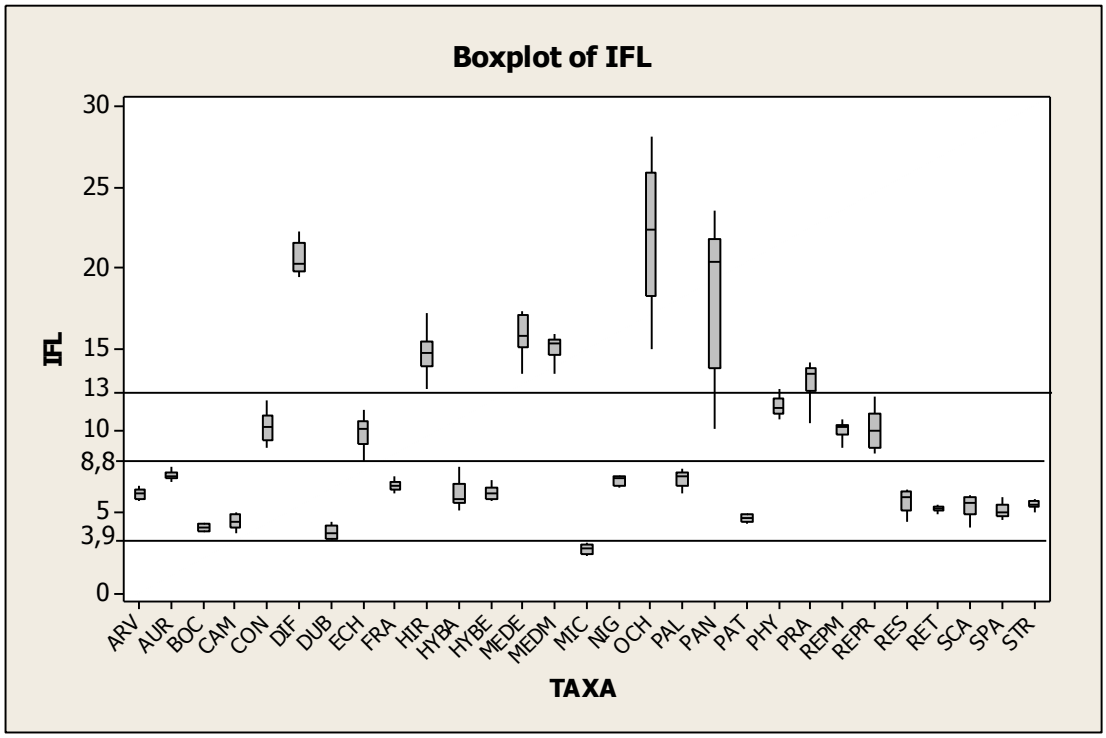


Figure 6B- Boxplot of individual flowering length (IFL)

Table 6B- Scoring character states for individual flowering length (IFL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <3.90 | 0 |
| 2 | 3.90-8.80 | 1 |
| 3 | 8.80-13.00 | 2 |
| 4 | 13.00< | 3 |

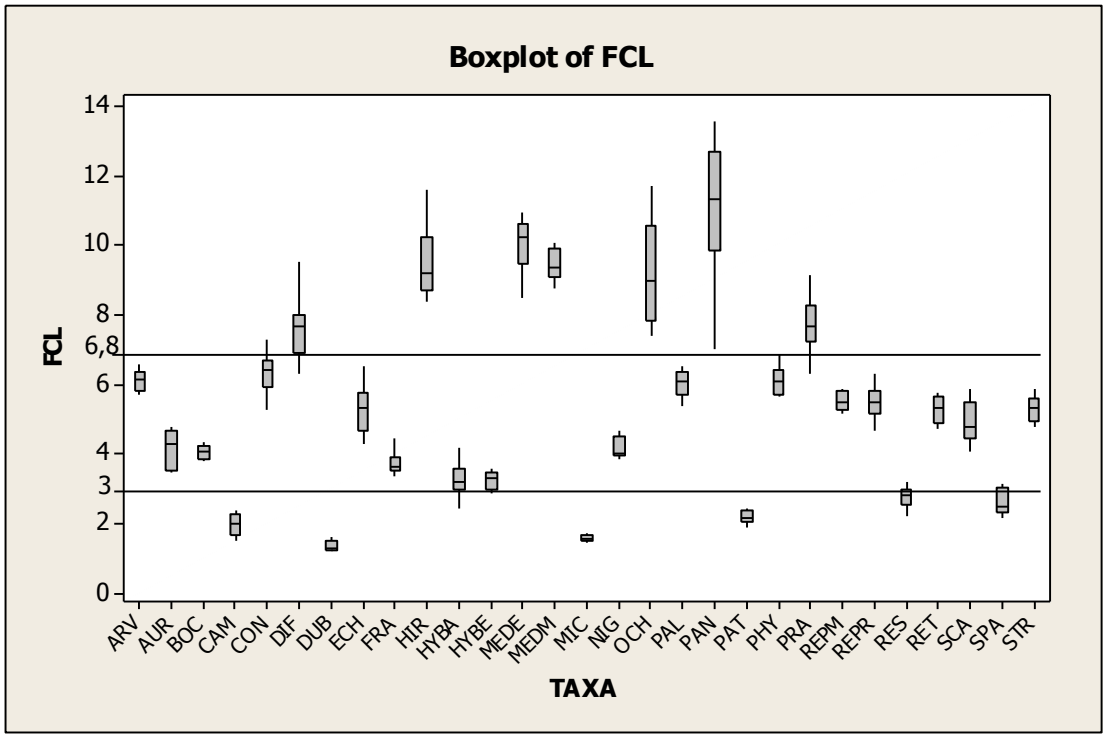


Figure 7B- Boxplot of flowering calyx length (FCL)

Table 7B- Scoring character states for flowering calyx length (FCL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <3.00 | 0 |
| 2 | 3.00-6.80 | 1 |
| 3 | 6.80< | 2 |

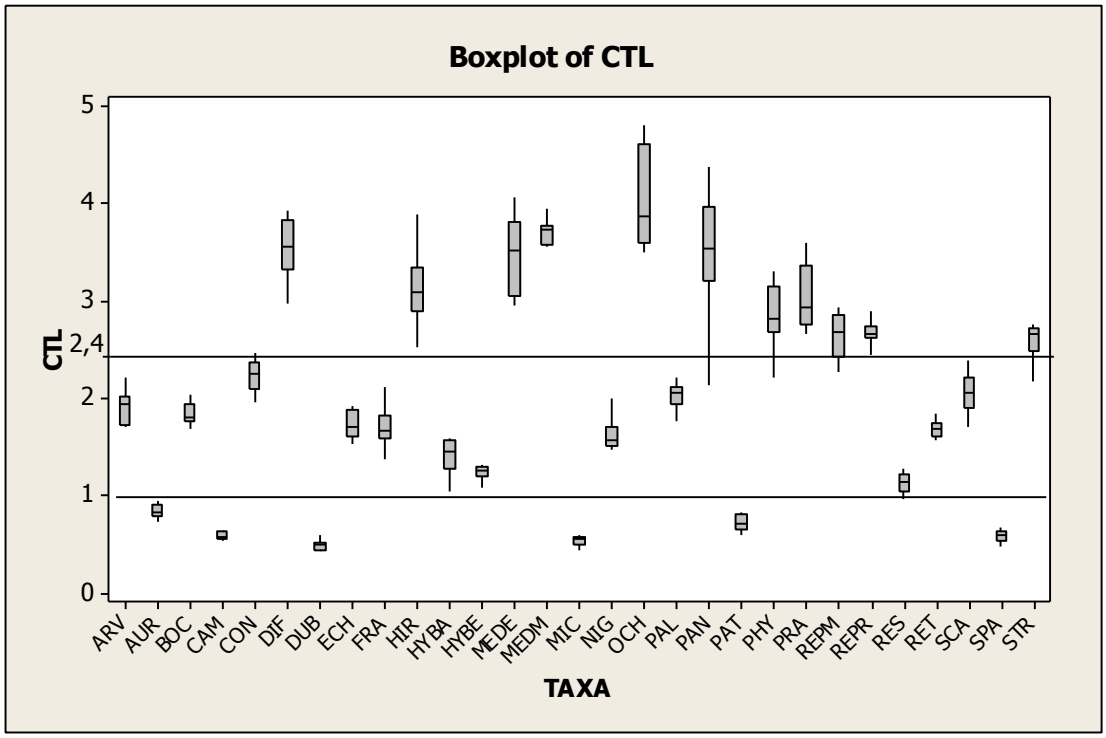


Figure 8B- Boxplot of calyx tube length (CTL)

Table 8B- Scoring character states for calyx tube length (CTL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <1.00 | 0 |
| 2 | 1.00-2.40 | 1 |
| 3 | 2.40< | 2 |

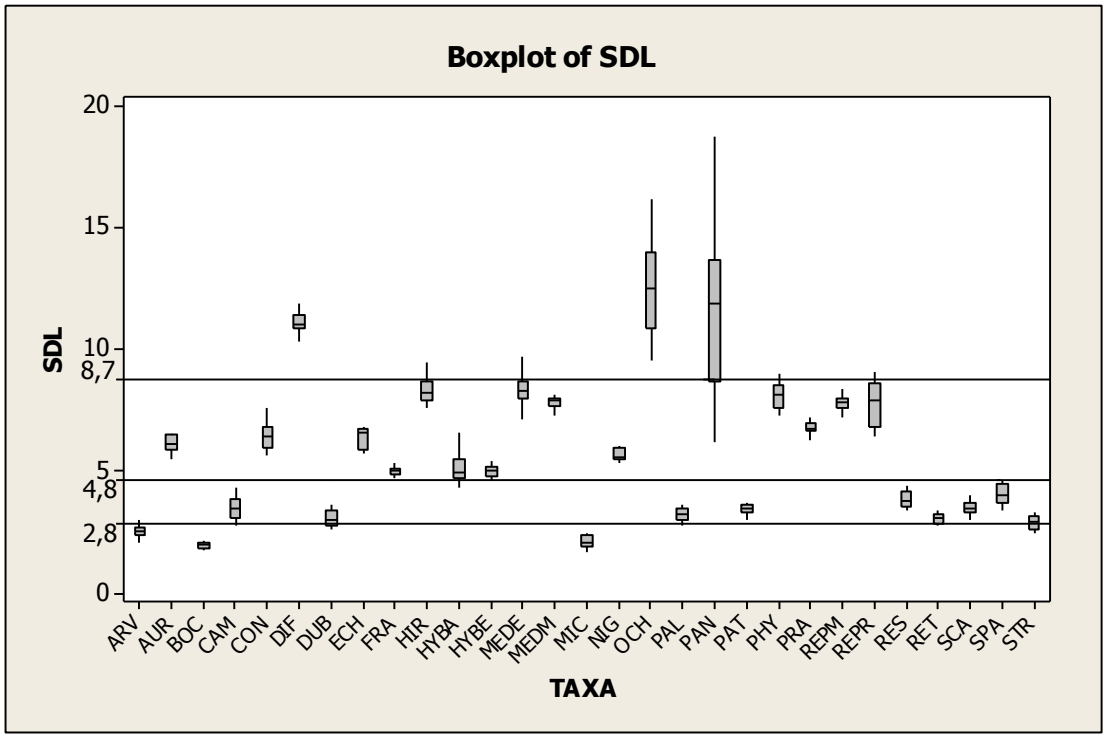


Figure 9B- Boxplot of standard length (SDL)

Table 9B- Scoring character states for standard length (SDL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <2.80 | 0 |
| 2 | 2.80-4.80 | 1 |
| 3 | 4.80-8.70 | 2 |
| 4 | 8.70< | 3 |

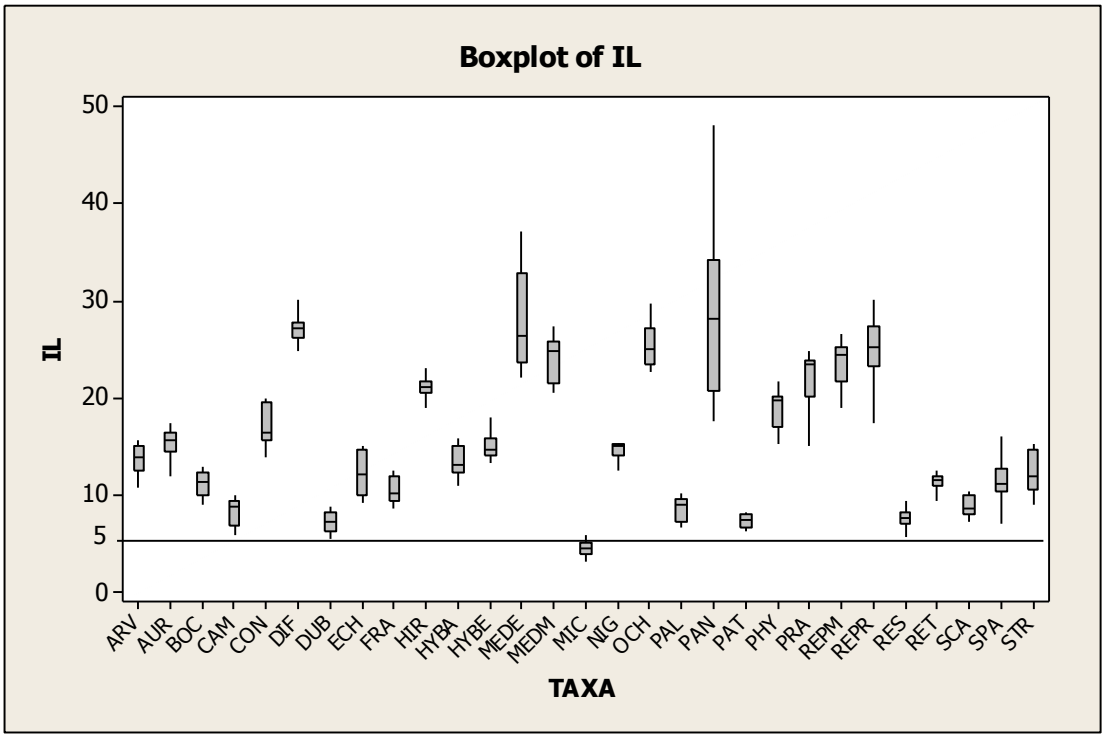


Figure 10B- Boxplot of inflorescence length (IL)

Table 10B- Scoring character states for inflorescence length (IL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <5.00 | 0 |
| 2 | 5.00< | 1 |

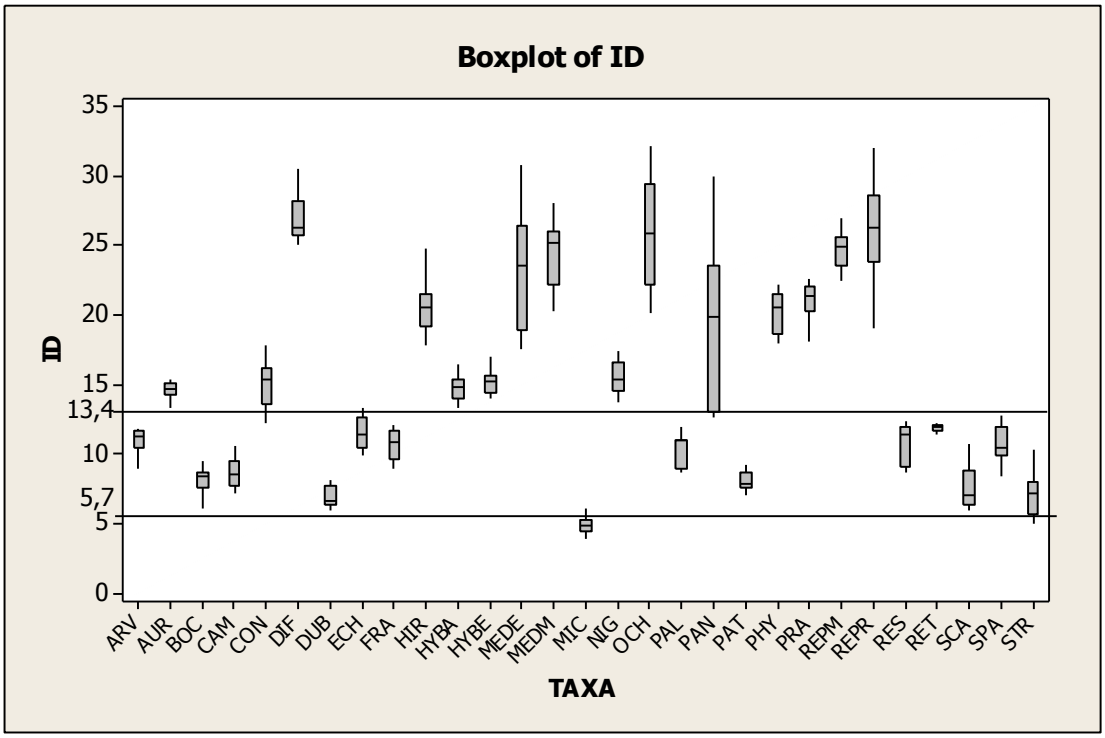


Figure 11B- Boxplot of inflorescence diameter (ID)

Table 11B- Scoring character states for inflorescence diameter (ID)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <5.70 | 0 |
| 2 | 5.70-13.40 | 1 |
| 3 | 13.40< | 2 |

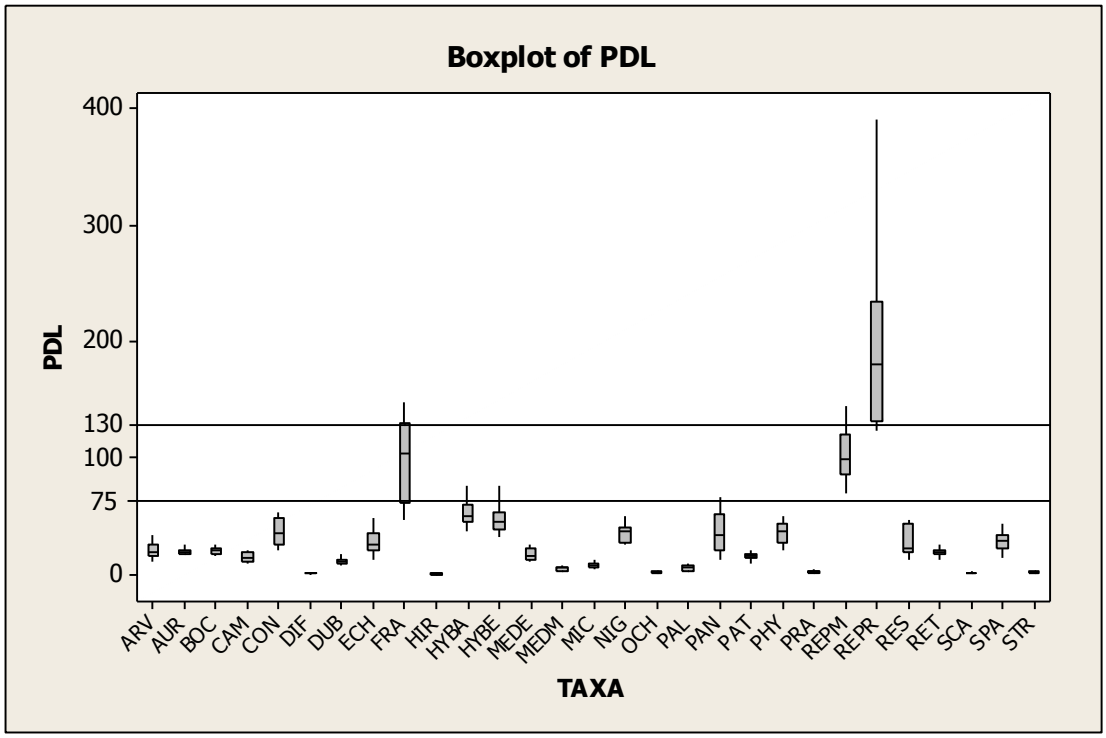


Figure 12B- Boxplot of peduncle length (PDL)

Table 12B- Scoring character states for peduncle length (PDL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <75 | 0 |
| 2 | 75-130 | 1 |
| 3 | 130< | 2 |

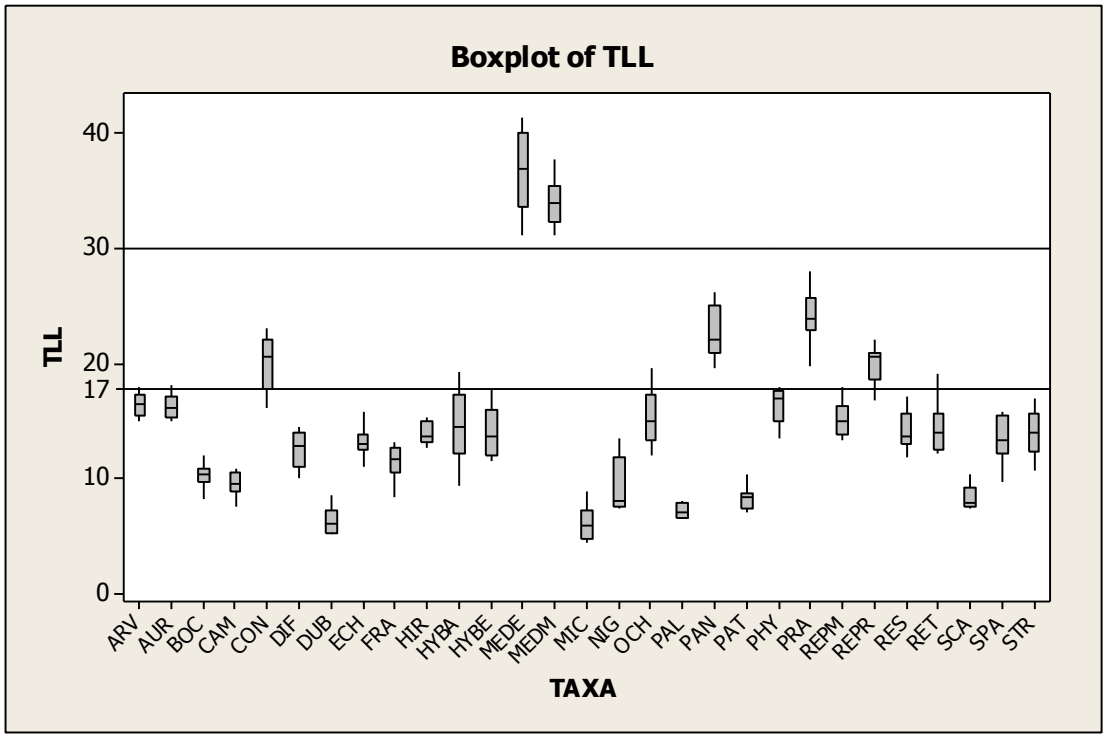


Figure 13B- Boxplot of terminal leaflet length (TLL)

Table 13B- Scoring character states for terminal leaflet length (TLL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <17 | 0 |
| 2 | 17-30 | 1 |
| 3 | 30< | 2 |

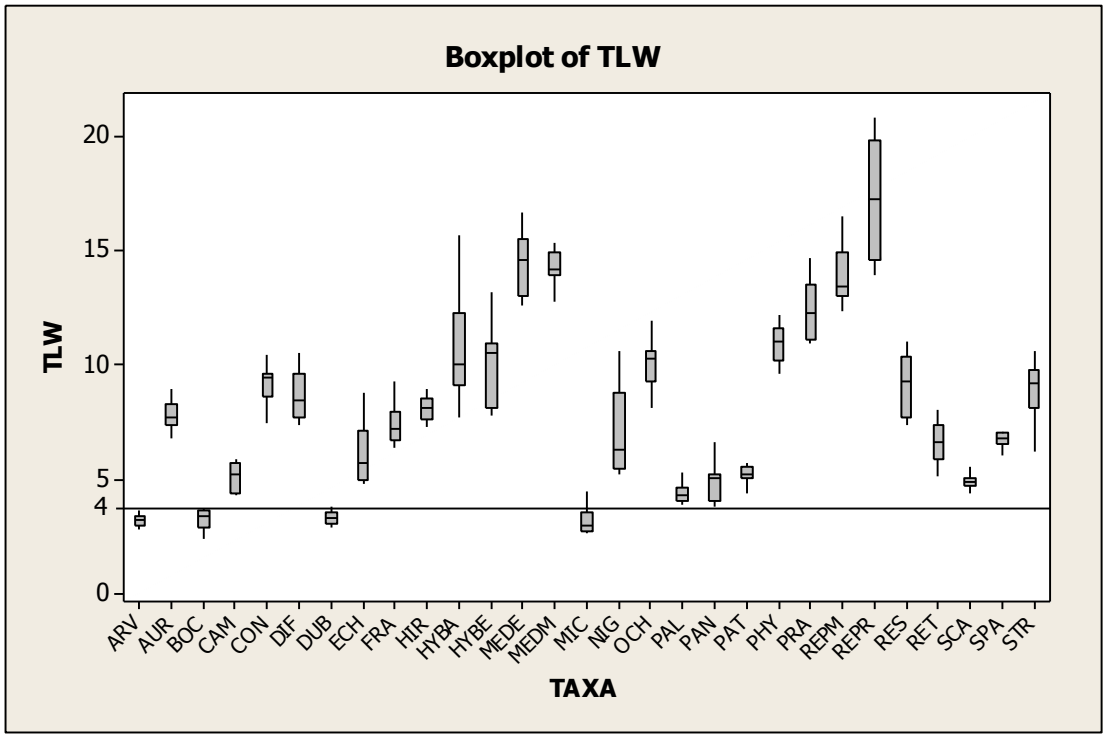


Figure 14B- Boxplot of terminal leaflet width (TLW)

Table 14B- Scoring character states for terminal leaflet width (TLW)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <4 | 0 |
| 2 | 4< | 1 |

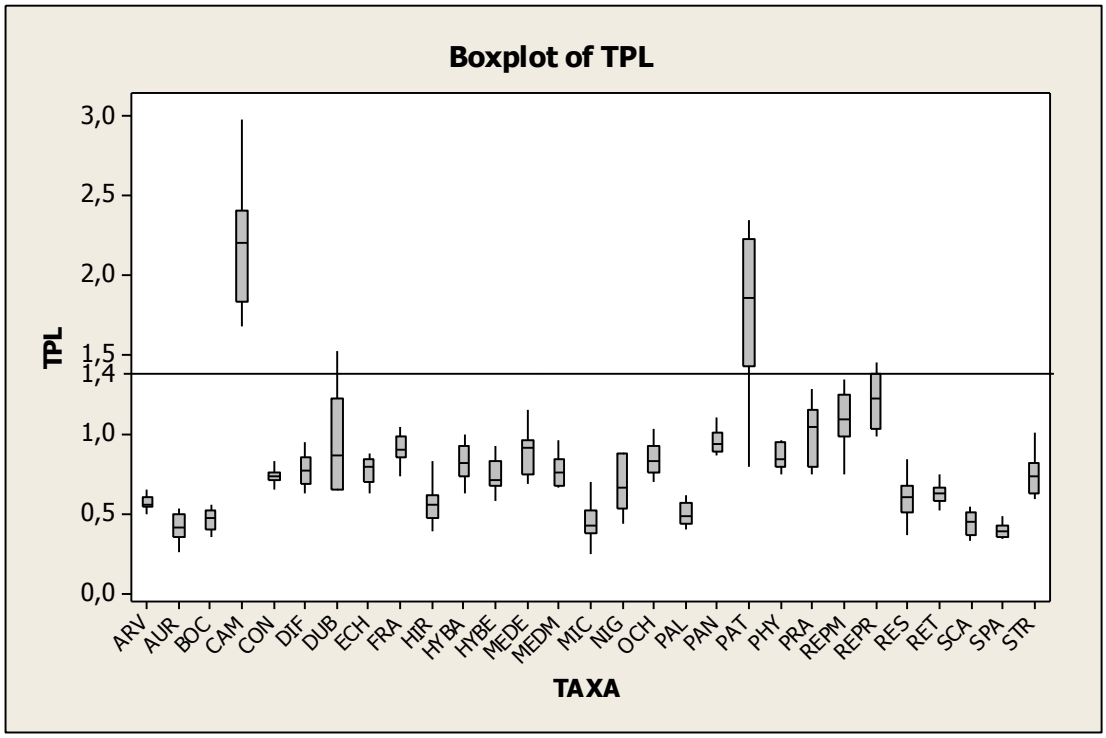


Figure 15B- Boxplot of terminal petiolule length (TPL)

Table 15B- Scoring character states for terminal petiolule length (TPL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <1.4 | 0 |
| 2 | 1.4< | 1 |

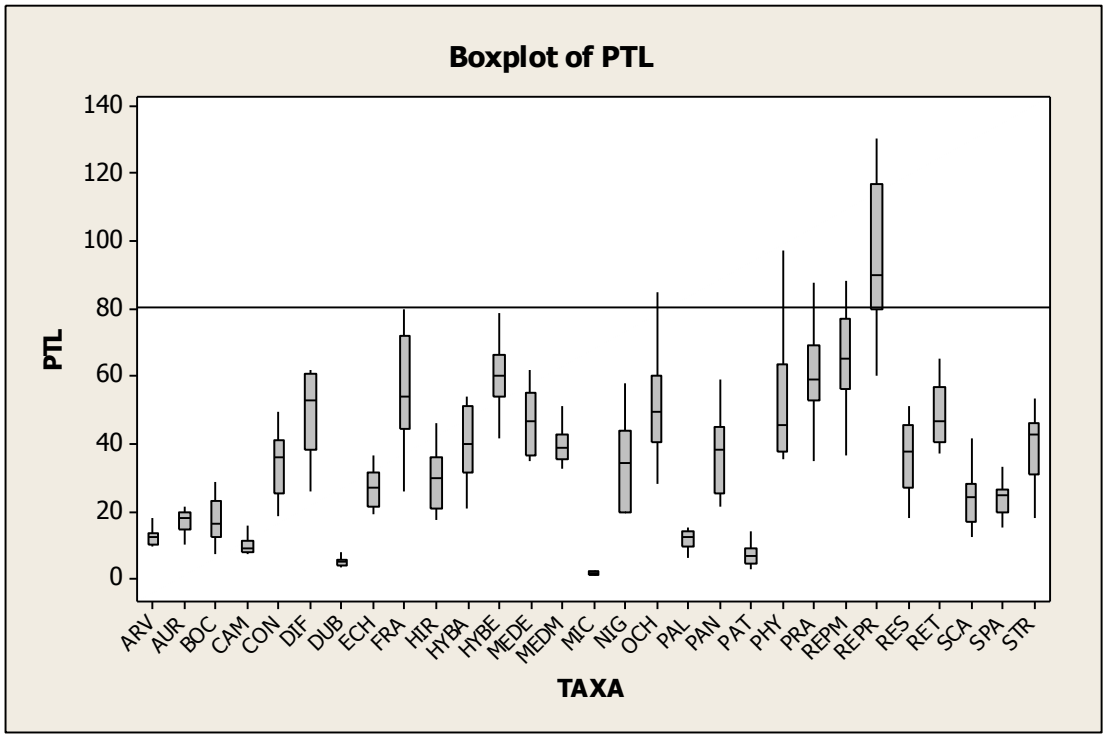


Figure 16B- Boxplot of petiole length (PTL)

Table 16B- Scoring character states for petiole length (PTL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <80 | 0 |
| 2 | 80< | 1 |

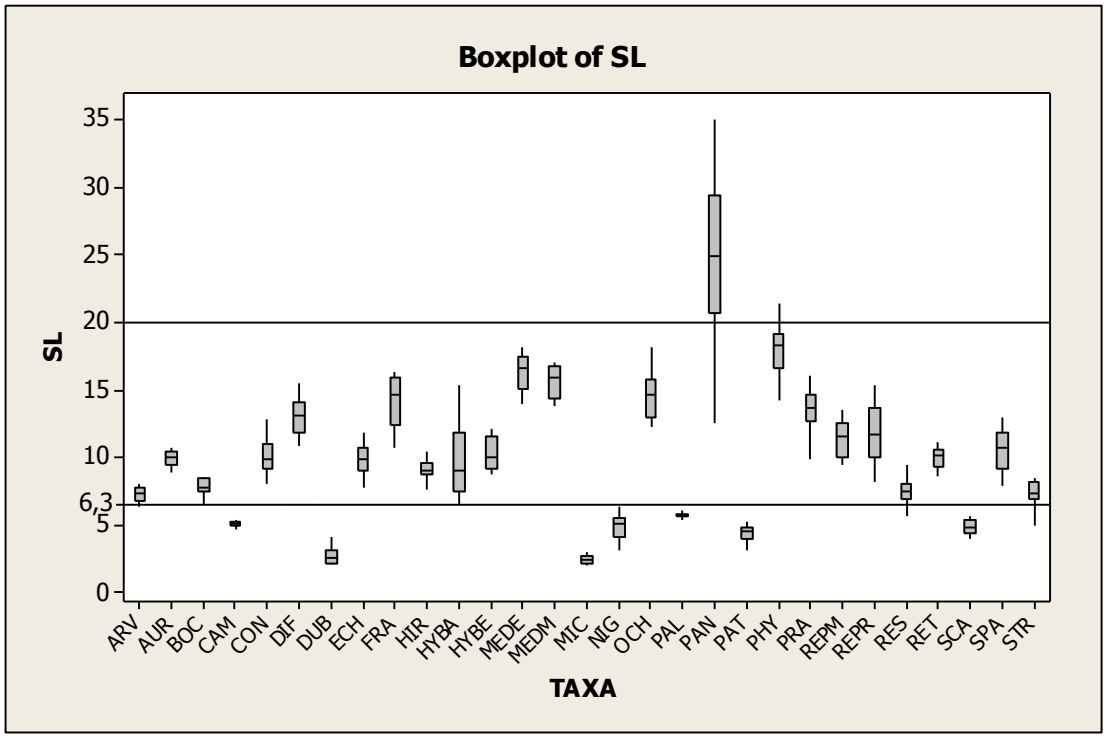


Figure 17B- Boxplot of stipule length (SL)

Table 17B- Scoring character states for stipule length (SL)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <6.30 | 0 |
| 2 | 6.30-20 | 1 |
| 3 | 20< | 2 |

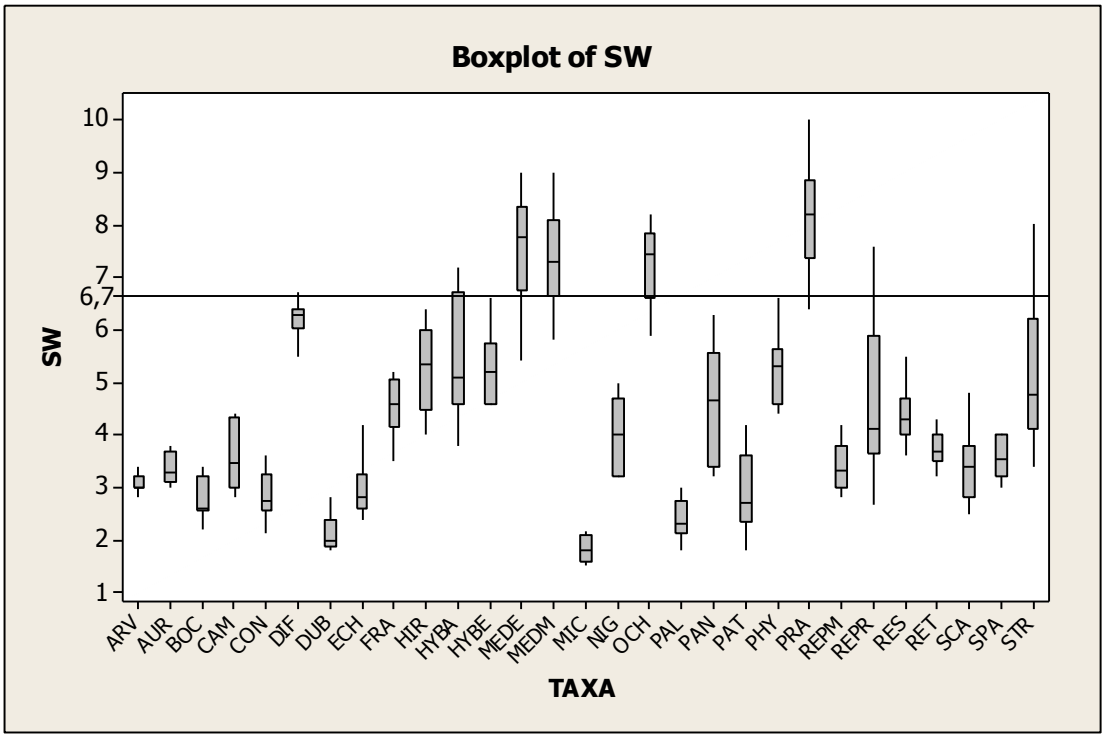


Figure 18B- Boxplot of stipule width (SW)

Table 18B- Scoring character states for stipule width (SW)

| GROUP NUMBER | RANGE | CODES |
|---|---|---|
| 1 | <6.70 | 0 |
| 2 | 6.70< | 1 |

Table 1C- Jaccard's coefficient of similarity matrix, based on RAPD data

| TAXA | RES | PHY | FRA | REPM | REPR | HYBA | HYBE | NIG | RET | DUB | MIC | SPA | AUR | CAM | PAT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **RES** | | | | | | | | | | | | | | | |
| **PHY** | 0,293 | | | | | | | | | | | | | | |
| **FRA** | 0,279 | 0,295 | | | | | | | | | | | | | |
| **REPM** | 0,206 | 0,180 | 0,220 | | | | | | | | | | | | |
| **REPR** | 0,199 | 0,131 | 0,193 | 0,243 | | | | | | | | | | | |
| **HYBA** | 0,194 | 0,207 | 0,157 | 0,298 | 0,303 | | | | | | | | | | |
| **HYBE** | 0,129 | 0,084 | 0,154 | 0,182 | 0,232 | 0,231 | | | | | | | | | |
| **NIG** | 0,134 | 0,109 | 0,104 | 0,187 | 0,138 | 0,172 | 0,224 | | | | | | | | |
| **RET** | 0,147 | 0,133 | 0,127 | 0,240 | 0,229 | 0,239 | 0,174 | 0,190 | | | | | | | |
| **DUB** | 0,180 | 0,161 | 0,202 | 0,135 | 0,182 | 0,156 | 0,106 | 0,139 | 0,212 | | | | | | |
| **MIC** | 0,180 | 0,149 | 0,171 | 0,160 | 0,191 | 0,205 | 0,130 | 0,137 | 0,234 | 0,290 | | | | | |
| **SPA** | 0,169 | 0,116 | 0,129 | 0,139 | 0,154 | 0,142 | 0,117 | 0,155 | 0,191 | 0,307 | 0,416 | | | | |
| **AUR** | 0,115 | 0,106 | 0,149 | 0,149 | 0,128 | 0,112 | 0,127 | 0,085 | 0,180 | 0,200 | 0,167 | 0,176 | | | |
| **CAM** | 0,129 | 0,115 | 0,134 | 0,127 | 0,140 | 0,128 | 0,142 | 0,104 | 0,169 | 0,205 | 0,215 | 0,175 | 0,248 | | |
| **PAT** | 0,159 | 0,145 | 0,211 | 0,120 | 0,119 | 0,130 | 0,116 | 0,076 | 0,128 | 0,167 | 0,187 | 0,198 | 0,210 | 0,224 | |
| **PRA** | 0,166 | 0,122 | 0,157 | 0,104 | 0,160 | 0,111 | 0,099 | 0,082 | 0,125 | 0,156 | 0,154 | 0,119 | 0,110 | 0,178 | 0,109 |
| **PAN** | 0,105 | 0,141 | 0,116 | 0,070 | 0,065 | 0,101 | 0,096 | 0,077 | 0,083 | 0,074 | 0,094 | 0,081 | 0,073 | 0,124 | 0,136 |
| **SCA** | 0,131 | 0,161 | 0,145 | 0,170 | 0,150 | 0,156 | 0,163 | 0,150 | 0,172 | 0,135 | 0,160 | 0,149 | 0,112 | 0,127 | 0,094 |
| **STR** | 0,142 | 0,137 | 0,130 | 0,148 | 0,129 | 0,132 | 0,128 | 0,089 | 0,112 | 0,138 | 0,175 | 0,133 | 0,133 | 0,164 | 0,112 |
| **HIR** | 0,180 | 0,170 | 0,132 | 0,179 | 0,147 | 0,174 | 0,102 | 0,099 | 0,142 | 0,113 | 0,158 | 0,125 | 0,135 | 0,185 | 0,124 |
| **CON** | 0,152 | 0,091 | 0,122 | 0,168 | 0,154 | 0,133 | 0,083 | 0,136 | 0,160 | 0,103 | 0,127 | 0,124 | 0,176 | 0,156 | 0,133 |
| **ECH** | 0,158 | 0,110 | 0,114 | 0,165 | 0,099 | 0,142 | 0,120 | 0,135 | 0,140 | 0,138 | 0,164 | 0,153 | 0,124 | 0,130 | 0,096 |
| **PAL** | 0,168 | 0,118 | 0,130 | 0,157 | 0,162 | 0,161 | 0,168 | 0,144 | 0,178 | 0,111 | 0,136 | 0,103 | **0,067** | 0,146 | 0,103 |
| **MEDE** | 0,178 | 0,119 | 0,113 | 0,123 | 0,113 | 0,115 | 0,101 | 0,107 | 0,179 | 0,130 | 0,127 | 0,155 | 0,124 | 0,138 | 0,143 |
| **MEDM** | 0,139 | 0,134 | 0,154 | 0,129 | 0,112 | 0,138 | 0,109 | 0,081 | 0,190 | 0,119 | 0,142 | 0,103 | 0,086 | 0,111 | 0,129 |
| **OCH** | 0,126 | 0,110 | 0,114 | 0,157 | 0,129 | 0,160 | 0,148 | 0,144 | 0,176 | 0,113 | 0,193 | 0,106 | 0,106 | 0,130 | 0,132 |
| **BOC** | 0,176 | 0,155 | 0,102 | 0,130 | 0,120 | 0,160 | 0,118 | 0,124 | 0,157 | 0,128 | 0,116 | 0,084 | 0,093 | 0,136 | 0,074 |
| **DIF** | 0,105 | 0,093 | 0,108 | 0,120 | 0,101 | 0,142 | 0,126 | 0,102 | 0,183 | 0,117 | 0,093 | 0,120 | 0,131 | 0,127 | 0,108 |
| **ARV** | 0,168 | 0,099 | 0,121 | 0,131 | 0,162 | 0,181 | 0,100 | 0,071 | 0,168 | 0,197 | 0,186 | 0,143 | 0,133 | 0,164 | 0,152 |

Table 1C- Jaccard's coefficient of similarity matrix, based on RAPD data (Continued)

| TAXA | PRA | PAN | SCA | STR | HIR | CON | ECH | PAL | MEDE | MEDM | OCH | BOC | DIF | ARV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PRA** | | | | | | | | | | | | | | |
| **PAN** | 0,188 | | | | | | | | | | | | | |
| **SCA** | 0,156 | 0,178 | | | | | | | | | | | | |
| **STR** | 0,128 | 0,195 | 0,185 | | | | | | | | | | | |
| **HIR** | 0,172 | 0,130 | 0,179 | 0,252 | | | | | | | | | | |
| **CON** | 0,188 | 0,129 | 0,132 | 0,144 | **0,330** | | | | | | | | | |
| **ECH** | 0,144 | 0,120 | 0,157 | 0,161 | 0,183 | 0,153 | | | | | | | | |
| **PAL** | 0,178 | 0,128 | 0,167 | 0,153 | 0,145 | 0,125 | 0,134 | | | | | | | |
| **MEDE** | 0,121 | 0,085 | 0,168 | 0,125 | 0,108 | 0,145 | 0,092 | 0,216 | | | | | | |
| **MEDM** | 0,111 | 0,109 | 0,153 | 0,131 | 0,098 | 0,106 | 0,183 | 0,167 | 0,159 | | | | | |
| **OCH** | 0,136 | 0,173 | 0,192 | 0,125 | 0,118 | 0,153 | 0,094 | 0,125 | 0,144 | 0,148 | | | | |
| **BOC** | 0,143 | 0,118 | 0,165 | 0,151 | 0,184 | 0,124 | 0,133 | 0,161 | 0,124 | 0,156 | 0,142 | | | |
| **DIF** | 0,144 | 0,157 | 0,120 | 0,132 | 0,073 | 0,167 | 0,123 | 0,154 | 0,133 | 0,120 | 0,153 | 0,163 | | |
| **ARV** | 0,144 | 0,128 | 0,122 | 0,106 | 0,165 | 0,164 | 0,152 | 0,162 | 0,116 | 0,185 | 0,210 | 0,212 | 0,224 | |

Table 2C- Jaccard's coefficient of similarity matrix, based on SSR data

| TAXA | RES | PHY | FRA | REPM | REPR | HYBA | HYBE | NIG | RET | DUB | MIC | SPA | AUR | CAM | PAT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **RES** | | | | | | | | | | | | | | | |
| **PHY** | 0,270 | | | | | | | | | | | | | | |
| **FRA** | 0,282 | 0,231 | | | | | | | | | | | | | |
| **REPM** | 0,165 | 0,219 | 0,278 | | | | | | | | | | | | |
| **REPR** | 0,157 | 0,123 | 0,190 | 0,267 | | | | | | | | | | | |
| **HYBA** | 0,087 | 0,161 | 0,165 | 0,211 | 0,165 | | | | | | | | | | |
| **HYBE** | 0,162 | 0,196 | 0,250 | 0,238 | 0,210 | 0,272 | | | | | | | | | |
| **NIG** | 0,065 | 0,136 | 0,167 | 0,152 | 0,129 | 0,225 | 0,214 | | | | | | | | |
| **RET** | 0,186 | 0,144 | 0,196 | 0,206 | 0,174 | 0,184 | 0,235 | 0,290 | | | | | | | |
| **DUB** | 0,087 | 0,098 | 0,111 | 0,162 | 0,218 | 0,222 | 0,226 | 0,145 | 0,154 | | | | | | |
| **MIC** | 0,175 | 0,173 | 0,196 | 0,303 | 0,250 | 0,186 | 0,219 | 0,122 | 0,268 | 0,291 | | | | | |
| **SPA** | 0,157 | 0,118 | 0,194 | 0,229 | 0,160 | 0,182 | 0,165 | 0,125 | 0,208 | 0,247 | 0,382 | | | | |
| **AUR** | **0,037** | 0,104 | 0,095 | 0,138 | 0,200 | 0,220 | 0,130 | 0,092 | 0,121 | 0,333 | 0,229 | 0,316 | | | |
| **CAM** | 0,149 | 0,086 | 0,154 | 0,186 | 0,191 | 0,086 | 0,198 | 0,101 | 0,216 | 0,189 | 0,291 | 0,300 | 0,180 | | |
| **PAT** | 0,155 | 0,077 | 0,136 | 0,179 | 0,272 | 0,164 | 0,190 | 0,105 | 0,130 | 0,232 | 0,320 | 0,257 | 0,278 | 0,288 | |
| **PRA** | 0,170 | 0,138 | 0,189 | 0,187 | 0,132 | 0,133 | 0,207 | 0,180 | 0,198 | 0,155 | 0,188 | 0,162 | 0,084 | 0,167 | 0,149 |
| **PAN** | 0,094 | 0,104 | 0,106 | 0,139 | 0,173 | 0,106 | 0,167 | 0,096 | 0,140 | 0,143 | 0,146 | 0,103 | 0,086 | 0,130 | 0,122 |
| **SCA** | 0,088 | 0,120 | 0,120 | 0,167 | 0,114 | 0,138 | 0,140 | 0,126 | 0,135 | 0,165 | 0,177 | 0,109 | 0,153 | 0,138 | 0,157 |
| **STR** | 0,116 | 0,133 | 0,131 | 0,112 | 0,119 | 0,119 | 0,131 | 0,129 | 0,136 | 0,109 | 0,141 | 0,116 | 0,074 | 0,109 | 0,132 |
| **HIR** | 0,103 | 0,102 | 0,172 | 0,187 | 0,114 | 0,087 | 0,109 | 0,123 | 0,167 | 0,104 | 0,188 | 0,146 | 0,080 | 0,141 | 0,116 |
| **CON** | 0,120 | 0,100 | 0,119 | 0,159 | 0,079 | 0,063 | 0,107 | 0,103 | 0,152 | 0,082 | 0,137 | 0,140 | 0,073 | 0,082 | 0,126 |
| **ECH** | 0,165 | 0,089 | 0,167 | 0,164 | 0,102 | 0,151 | 0,161 | 0,142 | 0,181 | 0,140 | 0,172 | 0,202 | 0,104 | 0,119 | 0,144 |
| **PAL** | 0,123 | 0,093 | 0,130 | 0,144 | 0,098 | 0,084 | 0,074 | 0,075 | 0,113 | 0,094 | 0,150 | 0,155 | 0,099 | 0,149 | 0,130 |
| **MEDE** | 0,081 | 0,090 | 0,102 | 0,144 | 0,077 | 0,091 | 0,089 | 0,115 | 0,146 | 0,102 | 0,140 | 0,133 | 0,095 | 0,091 | 0,129 |
| **MEDM** | 0,130 | 0,098 | 0,109 | 0,114 | 0,058 | 0,058 | 0,050 | 0,069 | 0,109 | 0,038 | 0,088 | 0,108 | 0,068 | 0,068 | 0,138 |
| **OCH** | 0,143 | 0,119 | 0,109 | 0,095 | 0,083 | 0,064 | 0,106 | 0,125 | 0,121 | 0,064 | 0,107 | 0,096 | **0,037** | 0,064 | 0,090 |
| **BOC** | 0,085 | 0,115 | 0,107 | 0,121 | 0,071 | 0,108 | 0,124 | 0,097 | 0,105 | 0,096 | 0,125 | 0,082 | 0,048 | 0,084 | 0,064 |
| **DIF** | 0,138 | 0,096 | 0,116 | 0,086 | 0,066 | 0,098 | 0,134 | 0,088 | 0,096 | 0,077 | 0,115 | 0,117 | 0,055 | 0,067 | 0,112 |
| **ARV** | 0,072 | 0,105 | 0,107 | 0,112 | 0,100 | 0,122 | 0,126 | 0,123 | 0,068 | 0,095 | 0,082 | 0,092 | 0,086 | 0,070 | 0,099 |

Table 2C- Jaccard's coefficient of similarity matrix, based on SSR data (Continued)

| TAXA | PRA | PAN | SCA | STR | HIR | CON | ECH | PAL | MEDE | MEDM | OCH | BOC | DIF | ARV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PRA** | | | | | | | | | | | | | | |
| **PAN** | 0,286 | | | | | | | | | | | | | |
| **SCA** | 0,250 | 0,292 | | | | | | | | | | | | |
| **STR** | 0,336 | 0,310 | 0,288 | | | | | | | | | | | |
| **HIR** | 0,280 | 0,232 | 0,221 | 0,273 | | | | | | | | | | |
| **CON** | 0,271 | 0,174 | 0,275 | 0,235 | 0,331 | | | | | | | | | |
| **ECH** | 0,319 | 0,243 | 0,243 | 0,339 | 0,313 | 0,404 | | | | | | | | |
| **PAL** | 0,336 | 0,257 | 0,323 | 0,311 | 0,328 | 0,313 | 0,376 | | | | | | | |
| **MEDE** | 0,230 | 0,240 | 0,202 | 0,267 | 0,295 | 0,230 | 0,268 | 0,333 | | | | | | |
| **MEDM** | 0,237 | 0,178 | 0,222 | 0,273 | 0,280 | 0,248 | 0,220 | 0,340 | **0,494** | | | | | |
| **OCH** | 0,224 | 0,184 | 0,181 | 0,252 | 0,230 | 0,272 | 0,229 | 0,208 | 0,154 | 0,208 | | | | |
| **BOC** | 0,229 | 0,178 | 0,200 | 0,167 | 0,205 | 0,207 | 0,191 | 0,224 | 0,268 | 0,214 | 0,278 | | | |
| **DIF** | 0,222 | 0,164 | 0,230 | 0,182 | 0,246 | 0,265 | 0,207 | 0,284 | 0,222 | 0,241 | 0,409 | 0,366 | | |
| **ARV** | 0,139 | 0,161 | 0,170 | 0,193 | 0,127 | 0,171 | 0,189 | 0,188 | 0,143 | 0,175 | 0,284 | 0,230 | 0,247 | |

Table 3C- Jaccard's coefficient of similarity matrix, based on combined RAPD and SSR data

| TAXA | RES | PHY | FRA | REPM | REPR | HYBA | HYBE | NIG | RET | DUB | MIC | SPA | AUR | CAM | PAT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **RES** | | | | | | | | | | | | | | | |
| **PHY** | 0,283 | | | | | | | | | | | | | | |
| **FRA** | 0,280 | 0,263 | | | | | | | | | | | | | |
| **REPM** | 0,188 | 0,198 | 0,248 | | | | | | | | | | | | |
| **REPR** | 0,181 | 0,128 | 0,191 | 0,253 | | | | | | | | | | | |
| **HYBA** | 0,149 | 0,187 | 0,161 | 0,258 | 0,244 | | | | | | | | | | |
| **HYBE** | 0,143 | 0,134 | 0,200 | 0,208 | 0,222 | 0,249 | | | | | | | | | |
| **NIG** | 0,106 | 0,121 | 0,132 | 0,171 | 0,134 | 0,193 | 0,220 | | | | | | | | |
| **RET** | 0,162 | 0,138 | 0,158 | 0,225 | 0,206 | 0,217 | 0,200 | 0,227 | | | | | | | |
| **DUB** | 0,142 | 0,133 | 0,159 | 0,147 | 0,196 | 0,180 | 0,155 | 0,141 | 0,189 | | | | | | |
| **MIC** | 0,178 | 0,160 | 0,183 | 0,223 | 0,216 | 0,197 | 0,171 | 0,130 | 0,249 | 0,290 | | | | | |
| **SPA** | 0,164 | 0,117 | 0,160 | 0,179 | 0,156 | 0,158 | 0,139 | 0,142 | 0,198 | 0,282 | **0,400** | | | | |
| **AUR** | 0,085 | 0,105 | 0,124 | 0,144 | 0,154 | 0,149 | 0,128 | 0,088 | 0,158 | 0,244 | 0,191 | 0,226 | | | |
| **CAM** | 0,137 | 0,103 | 0,143 | 0,151 | 0,159 | 0,112 | 0,164 | 0,103 | 0,186 | 0,199 | 0,245 | 0,221 | 0,224 | | |
| **PAT** | 0,157 | 0,114 | 0,175 | 0,145 | 0,176 | 0,144 | 0,148 | 0,087 | 0,129 | 0,191 | 0,242 | 0,222 | 0,234 | 0,247 | |
| **PRA** | 0,167 | 0,129 | 0,172 | 0,141 | 0,148 | 0,120 | 0,147 | 0,122 | 0,155 | 0,156 | 0,169 | 0,138 | 0,100 | 0,173 | 0,126 |
| **PAN** | 0,100 | 0,123 | 0,111 | 0,101 | 0,109 | 0,103 | 0,128 | 0,085 | 0,106 | 0,102 | 0,117 | 0,091 | 0,078 | 0,127 | 0,130 |
| **SCA** | 0,113 | 0,143 | 0,133 | 0,169 | 0,135 | 0,149 | 0,152 | 0,140 | 0,157 | 0,147 | 0,167 | 0,131 | 0,127 | 0,131 | 0,118 |
| **STR** | 0,129 | 0,135 | 0,131 | 0,129 | 0,124 | 0,126 | 0,130 | 0,108 | 0,123 | 0,124 | 0,157 | 0,124 | 0,104 | 0,138 | 0,122 |
| **HIR** | 0,140 | 0,133 | 0,154 | 0,183 | 0,130 | 0,128 | 0,105 | 0,111 | 0,154 | 0,108 | 0,174 | 0,136 | 0,107 | 0,163 | 0,120 |
| **CON** | 0,137 | 0,095 | 0,120 | 0,163 | 0,118 | 0,099 | 0,095 | 0,120 | 0,156 | 0,093 | 0,132 | 0,132 | 0,127 | 0,122 | 0,130 |
| **ECH** | 0,161 | 0,100 | 0,140 | 0,165 | 0,101 | 0,146 | 0,140 | 0,138 | 0,158 | 0,139 | 0,168 | 0,176 | 0,115 | 0,125 | 0,118 |
| **PAL** | 0,147 | 0,105 | 0,130 | 0,151 | 0,132 | 0,124 | 0,119 | 0,111 | 0,147 | 0,103 | 0,143 | 0,128 | 0,081 | 0,147 | 0,115 |
| **MEDE** | 0,133 | 0,105 | 0,108 | 0,133 | 0,097 | 0,104 | 0,095 | 0,111 | 0,164 | 0,118 | 0,133 | 0,144 | 0,112 | 0,118 | 0,137 |
| **MEDM** | 0,135 | 0,117 | 0,132 | 0,122 | 0,088 | 0,103 | 0,081 | 0,076 | 0,154 | 0,083 | 0,116 | 0,105 | 0,079 | 0,093 | 0,133 |
| **OCH** | 0,133 | 0,114 | 0,112 | 0,128 | 0,109 | 0,119 | 0,128 | 0,136 | 0,153 | 0,093 | 0,153 | 0,101 | 0,078 | 0,103 | 0,114 |
| **BOC** | 0,135 | 0,136 | 0,104 | 0,126 | 0,098 | 0,137 | 0,121 | 0,112 | 0,134 | 0,114 | 0,120 | 0,083 | 0,075 | 0,115 | **0,070** |
| **DIF** | 0,120 | 0,095 | 0,113 | 0,102 | 0,084 | 0,120 | 0,130 | 0,095 | 0,139 | 0,098 | 0,104 | 0,118 | 0,095 | 0,099 | 0,110 |
| **ARV** | 0,127 | 0,102 | 0,114 | 0,122 | 0,136 | 0,157 | 0,112 | 0,092 | 0,126 | 0,154 | 0,137 | 0,121 | 0,115 | 0,126 | 0,130 |

Table 3C- Jaccard's coefficient of similarity matrix, based on combined RAPD and SSR data (Continued)

| TAXA | PRA | PAN | SCA | STR | HIR | CON | ECH | PAL | MEDE | MEDM | OCH | BOC | DIF | ARV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PRA** | | | | | | | | | | | | | | |
| **PAN** | 0,230 | | | | | | | | | | | | | |
| **SCA** | 0,195 | 0,225 | | | | | | | | | | | | |
| **STR** | 0,224 | 0,251 | 0,234 | | | | | | | | | | | |
| **HIR** | 0,226 | 0,181 | 0,200 | 0,264 | | | | | | | | | | |
| **CON** | 0,227 | 0,151 | 0,195 | 0,192 | 0,330 | | | | | | | | | |
| **ECH** | 0,221 | 0,176 | 0,195 | 0,250 | 0,251 | 0,270 | | | | | | | | |
| **PAL** | 0,249 | 0,187 | 0,234 | 0,233 | 0,240 | 0,216 | 0,246 | | | | | | | |
| **MEDE** | 0,169 | 0,153 | 0,183 | 0,196 | 0,202 | 0,187 | 0,173 | 0,272 | | | | | | |
| **MEDM** | 0,165 | 0,139 | 0,182 | 0,199 | 0,187 | 0,171 | 0,201 | 0,245 | 0,296 | | | | | |
| **OCH** | 0,173 | 0,178 | 0,188 | 0,185 | 0,173 | 0,207 | 0,153 | 0,162 | 0,148 | 0,174 | | | | |
| **BOC** | 0,181 | 0,145 | 0,180 | 0,159 | 0,195 | 0,164 | 0,160 | 0,191 | 0,188 | 0,182 | 0,198 | | | |
| **DIF** | 0,182 | 0,161 | 0,171 | 0,160 | 0,167 | 0,219 | 0,166 | 0,221 | 0,178 | 0,178 | 0,266 | 0,259 | | |
| **ARV** | 0,142 | 0,142 | 0,142 | 0,148 | 0,145 | 0,167 | 0,169 | 0,174 | 0,128 | 0,181 | 0,240 | 0,220 | 0,236 | |