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MASTER'S PROGRAM WITH THESIS

DETERMINATION OF GENETIC VARIATIONS IN BLACK MULBERRY (*Morus nigra*) GENOTYPES FROM TÜRKİYE AND IRAQ

Master Thesis

Prepared By

Ala Asi Mohammed Al-Salihi

First Supervisor

Assoc. Prof. Dr. Onur SARAÇOĞLU

Second Supervisor

Assist. Prof. Dr. Ibrahim SAYGILI

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ETHICS CONTRACT

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TOKAT GAZİOSMANPAŞA UNIVERSITY

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With this document, I declare that the Master's thesis named “Determination of genetic variations in black mulberry (*Morus nigra*) genotypes from Türkiye and Iraq” is an original work that I prepared in accordance with the guidelines of Tokat Gaziosmanpaşa University - Institute of Graduate Studies and under the consultancy of Assoc. Prof. Dr. Onur SARAÇOĞLU and Assist. Prof. Dr. Ibrahim SAYGILI. And I hereby declare that all the information in the current thesis has been gathered and presented in line with academic rules and ethical principles, and as a prerequisite for these rules and principles, I have cited all data, thoughts, and results that do not belong to me.

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The Student Who Prepared the Thesis

Ala Asi Mohammed Al-Salihi

JURY ACCEPTANCE AND APPROVAL

The defines examination of the thesis study titled “Determination of Genetic Variations in Black Mulberry (*Morus Nigra*) Genotypes from Türkiye and Iraq’’, which was prepared by Ala Asi Mohammed AL-SALIHI was held on 02/08/2023. It was accepted by the jury members of that session as a Master Thesis at Tokat Gaziosmanpaşa University, Graduate Education Institute, Department of Horticulture.

Jury Members (Title, Name Surname) Signature

Chairman: Prof. Dr. Çetin ÇEKİÇ

Member: Assoc. Prof. Dr. Onur SARAÇOĞLU

Member: Assist. Prof. Dr. Ibrahim SAYGILI

Member: Assoc. Prof. Dr. Hakan KELES

Member: Assist. Prof. Dr. Sinem ÖZTÜRK ERDEM

CONFIRMATION

/ /2023

Doç. Dr. Yusuf TEMÜR

DIRECTOR OF GRADUATE EDUCATION INSTITUTE

ABSTRACT

DETERMINATION OF GENETIC VARIATIONS IN BLACK MULBERRY (*Morus nigra*) GENOTYPES FROM TÜRKİYE AND IRAQ

ALA ASI MOHAMMED AL-SALIHI

MASTER'S THESIS, HORTICULTURE DEPARTMENT

Thesis first supervisor: **Assoc. Prof. Dr. Onur SARAÇOĞLU**

Thesis second supervisor: **Assist. Prof. Dr. İbrahim SAYGILI**

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This study was conducted to investigate the genetic diversity of 47 *Morus nigra* genotypes from Iraq and Türkiye using Simple Sequence Repeat (SSR) markers and IPBS markers. In the research was focused on comparing populations of *Morus nigra* from Iraq and Türkiye to gain insights into the genetic variations and relationships within and between these regions. The main objective was to assess the genetic diversity within and between populations of *Morus nigra* using SSR and IPBS marker systems. A total of 47 *Morus nigra* samples from Iraq and Türkiye were analyzed using ten SSR markers, revealing a range of polymorphism with a PIC (Polymorphic Information Content) value ranging from 0.51 to 0.68. The number of alleles was 27 and the mean polymorphism rate of alleles was 75%. The IPBS analysis resulted in a PIC value ranging from 0.26 to 0.50, a total number of bands were 138, of which 108 bands were polymorphic. The percentage of polymorphic bands was 74%. The resulting data were subjected to various statistical analyses, including genetic diversity indices and Principal Coordinate Analysis (PCoA). Results show that *Morus nigra* genotypes have lower variation in SSR loci. But IPBS marker systems have quite high variations, indicating powerful usage of retrotransposons.

Keywords: Genetic diversity, IPBS marker, Microsatellite, Molecular characterization

ÖZET

TÜRKİYE VE IRAK'TAN TOPLANAN KARADUT (*Morus nigra*) GENOTİPLERİNDE GENETİK VARYASYONLARIN BELİRLENMESİ

ALA ASI MOHAMMED AL-SALIHI

YÜKSEK LİSANS TEZİ, BAHÇE BİTKİLERİ BÖLÜMÜ

Tez birinci danışmanı: **Doç. Dr. Onur SARAÇOĞLU**

Tez ikinci danışmanı: **Dr. Öğr. Üyesi İbrahim SAYGILI**

Ağustos 2023, + 40 sayfa

Bu çalışma, Irak ve Türkiye'den 47 *Morus nigra* genotipinin bu bölgeler içindeki ve arasındaki genetik çeşitliliğini araştırmak amacıyla Simple Sequence Repeat (SSR) markörleri ve Inter Primer Binding Site (IPBS) markörleri kullanılarak yapılmıştır. Araştırma kapsamında *Morus nigra* genotiplerinin genetik çeşitliliğinin tespiti için IPBS markör sistemleri ilk kez kullanılmıştır. Irak ve Türkiye'den toplam 47 *Morus nigra* örneği, 10 SSR belirteci kullanılarak analiz edilmiş ve PIC (Polimorfik Bilgi İçeriği) değeri 0.51 ile 0.68 arasında değişen bir polimorfizm ortaya çıkarılmıştır. Çalışmada elde edilen alel sayısı 27 olup, alellerin ortalama polimorfizm oranı %75'tir. IPBS analizi, 0.26 ile 0.50 arasında değişen bir PIC değeri ile sonuçlanmıştır. Elde edilen toplam bant sayısı 138'dir ve bunların 108'i polimorfiktir. Polimorfik bantların yüzdesi %74 olarak bulunmuştur. Elde edilen veriler, genetik çeşitlilik endeksleri ve Temel Koordinat Analizi (PCoA) dahil olmak üzere çeşitli istatistiksel analizlere tabi tutulmuştur. Sonuçlar, *Morus nigra* genotiplerinin SSR lokuslarında düşük varyasyonlara sahip olduğunu göstermektedir. Ancak IPBS markör sistemi, retrotranspozonların güçlü bir şekilde kullanıldığını gösteren oldukça yüksek varyasyonların belirlenmesini sağlamıştır.

Anahtar Kelimlere: Genetik çeşitlilik, IPBS marker, Mikrosatelite, Moleküler karakterizasyon

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CONTENTS

LIST of TABLES

LIST of FIGURES

SYMBOLS AND ABBREVIATIONS

BAU:	Expected amplicon length
CTAB:	Cetyltrimethyl Ammonium Bromide
DNA:	Deoxyribonucleic Acid
IBA:	Indole-3-Butyric Acid
PAT:	Primary Adhesion Temperature
PCoA:	Principal Coordinates Analysis
PCR:	Polymerase Chain Reaction
PIC:	Polymorphism Information Content
IPBS:	Inter-Primer Binding Site
SSR:	Simple Sequence Repeats
RAPD:	Relative Afferent Pupillary Defect
RFLP:	Restriction Fragment Length Polymorphism
ISSR:	Inter Simple Sequence Repeats
TBE buffer:	Tris-borate-EDTA Buffer
NaCl:	Sodium Chloride
LTR:	Long Terminal Repeats
UPGMA:	Unweighted Pair Group Method with Arithmetic Mean
µM:	Micrometre
^{0}C:	Degrees Celsius
%:	Percent

1. INTRODUCTION

Local and unnamed genotypes play a crucial role in preserving the gene pools and promoting biodiversity, ensuring the stability and functionality of the ecosystem. In Türkiye and Iraq (Aziz at el. 2018), black mulberry genotypes (*Morus nigra* 2n=22x=308, decosaploid) (Dalkiliç and Dalkiliç, 2018) exhibit significant variations. Although morphological triats can be used to differentiate mulberry genotypes (Orhan, 2009), these traits can vary in different environments and are limited in number (Pinto et al., 2018). For the study of the genetical variations in mulberry genotypes, morphological characterization alone is insufficient, necessitating the employment of additional methods.

The various mulberry genotypes are not well known in Iraq. So, in order to determine their relationships and qualities, cutting-edge molecular genetic approaches can be applied. Yet, research on recognized mulberry species and comparable cultivars have previously been conducted in Türkiye.

DNA markers have always been useful for figuring out the cool stuff about genetic diversity (Baloch et al., 2015). One of these markers is simple-to-sequence repeats (SSR) or microsatellites. SSR markers have considerable advantages, such as high frequency, reliability, and reproducibility. SSRs defined the genetic diversity in mulberry genotypes and species (Pinto et al., 2018; Garcia-Gómez et al., 2019). Turkish mulberry genotypes were also characterized with SSR (Orhan et al., 2020) and other marker systems ISSR (Keskin et al., 2022), and AFLP (Kafkas et al., 2008). Although they are pretty reliable marker systems, SSRs have some disadvantages. they are in a limited number (Pinto et al., 2018) and need specific agarose or acrylamide gels and other expensive amplicons detection systems. Due to these problems, SSR markers may not be sufficient and practical to specify the level of population polymorphism in a population mostly belonging to a black mulberry species. However, there is always a need for the power of SSR for reliability and repeatability.

Most of the plant genome comprises retrotransposons, which are present in most eukaryotic species. Due to their genetic diversity, retrotransposons are a valuable source of molecular markers. Retrotransposon markers have been utilized in many evolutions, and genetics has been a major field of study due to their wide applicability, simple implementation, and

genotype resolution systems. Recently, a new Polymerase Chain Reaction (PCR)-based method has been defined that can isolate long terminal repeat LTR transposons in almost any organism and is used as a global marker system. Inter primer binding site (IPBS) is a method that relies on the amplification of reverse transcriptase primer binding sites in LTR retrotransposons. The isolation methods of retrotransposons are based on conserved protein-coding regions, but IPBS primers directly show polymorphism for retrotransposon regions in the genome (Kalendar, 2010). Various retrotransposon marker systems are commonly used in numerous genetic and evolutionary diversity studies due to their general applicability, simplicity of implementation, and genotype resolution (Feschotte et al., 2002; Kalendar et al., 2004). To count the positive aspects of the IPBS system, retrotransposons constitute an important part of the plant's genetic material, the primers used once used in any organism can be counted, and the primers are universal.

DNA markers have been pretty awesome in crop breeding, especially when studying genetic diversity and keeping those genes safe. Scientists use cool techniques like RAPD, ISSR, AFLP, and SSRs (or microsatellites) to get the job done (Gupta et al., 2000). Since there's no solid scientific proof about the Mulberry types in Iraq, it's crucial to understand their genetic diversity better. This way, how to properly use and protect them can be determined. This research tries to estimate the genetic diversity with relatedness among the most important Mulberry genotypes. Their population structure in Iraq and Türkiye was enhanced for Mulberry genotypes using IPBS and SSR markers for the first time. In this thesis, genetic diversity was studied in *Morus nigra* using SSR and IPBS markers.

2. LITERATURE REVIEW

2.1 General Characteristics of *Morus nigra*

Mulberry, belonging to the *Morus spp*. of the *Moraceae* family, is a resilient, perennial tree that exhibits rapid growth. Mulberry plants are typically monoecious, occasionally dioecious and rely on cross-pollination for reproduction. The tree's chromosome count ranges from 28 to 308 (2n = 28–308) (Kruthika et al. 2023). Mulberry is distributed globally across regions with warm, humid climates from 50°N to 10°S latitudes. These regions include Southeast Asia, Europe, and North and South America. Asia, in particular, boasts the most mulberry species, with China housing 24 species and Japan housing 19 species.

Conversely, India has four reported species: (*M. laevigata, M. alba, M. indica, and M. serrata*) (Sarkar et al., 2017). Mulberry exhibits considerable ploidy-level variation in its natural occurrence and is extensively cultivated in India, primarily under irrigation systems. India's Central Sericultural Germplasm Resources Centre conserves around 1200 mulberry accessions, encompassing indigenous and exotic varieties. This conservation effort aims to preserve the diverse genetic resources of mulberry. Mulberry thrives in temperate or subtropical regions and adapts to various climatic conditions, topographical settings, and soil types (Jan et al., 2021).

Mulberry holds significant agricultural and historical importance in Türkiye , where it has been cultivated for centuries. The cultivation of mulberry species in Türkiye dates back more than 400 years (Ercisli and Orhan, 2007). In Türkiye , three primary species of mulberry are cultivated for fruit production: black mulberry (*Morus nigra*), red mulberry (*Morus rubra*), and white mulberry (*Morus alba*) (Ercisli and Orhan, 2008). The black mulberry, originally from Iran, is predominantly grown in Southern Europe and Southwest Asia, particularly valued for its fruits and recognized as the most important species in Mediterranean countries (Tutin, 1996). Among Asian countries, black mulberry is widely found in Iran, Iraq, Afghanistan, Pakistan, and India (Ahlawat et al., 2016). In Türkiye , black mulberry (known as "Kara Dut") is extensively cultivated. It thrives in Mediterranean conditions and in the northeast, where it is grown in orchards for its delicious edible fruits (Ercisli and Orhan, 2008).

Mulberry fruits, including mulberries, hold significant economic importance worldwide. Their appeal has grown recently due to their abundance of health-promoting vitamins, antioxidants,

and other valuable nutrients. Mulberries, particularly, are rich sources of vitamins and minerals, notably anthocyanin (Sakar et al., 2023). They are also abundant in phenolic compounds with broad-spectrum antimutagenic and anticarcinogenic properties. The fruit of *Morus nigra* contains substantial levels of total phenolic compounds and various phytonutrients, including ascorbic acid (He et al., 2022). *Morus nigra* is also recognized in folk medicine and is considered a medicinal plant.

Mulberry demonstrates adaptability to diverse climatic conditions, ranging from tropical and subtropical regions to temperate areas. It is widely distributed across various geographical and ecological zones, being a deciduous tree native to warm, temperate, and subtropical regions of Asia, Africa, North America, and Southern Europe (Pérez-Gregorio et al., 2011). While *Morus alba* is originally from China, *Morus nigra* and *Morus rubra* are associated with Iran and North America, respectively (Uzun and Bayır, 2010). Black mulberry, scientifically known as *Morus nigra L.,* is an indigenous tree of Western Iran extensively cultivated in Southern Europe, Southwest Asia, and Mediterranean countries. It is valued for its succulent fruits and is a source of valuable industrial, medicinal, and ornamental products. Mulberries can be categorized into ornamental, fruit, foliage, or timber varieties (Ipek et al., 2012).

The mulberry plant, characterized by its rapid growth and woody nature, is frequently pruned to encourage its development as a bush or small tree (Prithivirajan et al., 2019). It can withstand repeated lopping and pruning and features a taproot system with few shallow roots. Additionally, it exhibits strong pruning capabilities. The leaves of the mulberry plant are either entire or often deeply lobed, displaying a simple structure and an alternate arrangement. They are stipulate, petiolate, and may take on an ovate or broadly ovate shape with serrate or crenate-serrate margins. Mulberry plants are typically monoecious, occasionally dioecious, and their inflorescences form catkins that bear unisexual flowers hanging from a pendant or drooping peduncles. Female inflorescences tend to have a limited number of flowers closely arranged together. These flowers possess a one-celled ovary, bifid stigma, and four persistent perianth lobes. Mulberries primarily rely on wind as their main pollinator. Plant Propagation can be achieved through either seeds or cuttings (Koul et al., 1979).

The fruits of the black mulberry (*Morus nigra*) consist of two parts: the fruit stalk, the portion to which the flower stalks are attached, and the fleshy parts of the fruit, which correspond to

the flower stalks. Black mulberry fruits are medium to large, slightly elongated, and nearly round (Kostić et al., 2013). The fruits are matte black or dark red (Figure 1).

Figure 1. Sample of *Morus nigra* fruits

When fully ripe, the fruits of the mulberry tree are characterized by their juiciness, pleasant sweetness, and aromatic qualities (Singhal et al., 2010). The fruit harvesting period extends over a remarkably long duration, typically lasting for 8 to 10 weeks. This extended period of fruit availability offers a significant advantage to producers and consumers of black mulberry (*Morus nigra*) (Kilincer, 2023). Furthermore, the fruit of the black mulberry can be frozen and stored for extended periods, enhancing its shelf life.

From a nutritional standpoint, a vital role is played by fruits in promoting human health and well-being. They can be mentioned as a valuable source of dietary fibre, crucial vitamins, and they do not contain salt. Additionally, their appealing appearance increases appetite and provides high caloric content (Karaca, 2009). Mulberry fruits are highly esteemed for their nutritional value (Ercisli, 2004). Certain species, like (*M. laevigata, M. rubra, M. nigra, and M. alba*), are grown specifically for their delicious fruits since their leaves aren't ideal for feeding silkworms. Similarly, the black mulberry (*Morus nigra*) is extensively grown in Türkiye for its fruits (Gökmen 1973). Moreover, mulberry trees are widely employed for landscaping purposes across Asia, Europe, and America (Tipton, 1994).

2.2 Molecular markers

The field of genetics has made significant contributions to agricultural research, particularly in the latter part of the 20th century. Agricultural genetics utilizes molecular marker techniques to identify genetic variations among cultivars, leading to discoveries in gene source identification, genetic mapping, and characterization (Dar et al., 2019). Molecular marker technology offers substantial advantages over traditional methods, notably the ability to yield prompt results unaffected by environmental factors. Genetic characterization, a prominent application of molecular marker technology, represents a highly researched area globally (Kaçar, 2001).

To check out the genetic variation, morphological, biochemical and molecular markers are widely employed. Molecular markers pertain to DNA-level traits and are extensively utilized for detecting DNA polymorphisms between individuals through PCR (Kalendar et al., 2010). Molecular marker techniques have been widely adopted in gene source characterization, offering advantages such as the ability to analyse an entire genome using minute tissue samples and minimal interference from growth conditions. As a result, plant genetic resources can now be described with higher clarity and precision. However, it is essential to perceive these marker systems not as alternatives but as complements of morphological markers, taking a holistic approach. Understanding the genetic variants of the parental plants can help to predict the performance of the resulting hybrid individuals; hence discovering genetic differences can assist in choosing acceptable parent plants for breeding experiments. As a result, the selection process can be significantly expedited, safeguarding valuable genetic resources (Gülşen and Mutlu, 2005).

2.3 Application of DNA Markers in Mulberry

Breeding in mulberries presents considerable challenges for breeders due to the distinctive attributes of this cross-pollinating tree, characterized by high heterozygosity and an extended period of immaturity. These challenges encompass multiple aspects. Initially, acquiring homozygous plants through inbreeding or anther/pollen culture proves arduous, demanding a profound comprehension of heterosis mechanisms and the capacity to forecast hybrid performance (Vijayan, 2007).

Furthermore, the identification of valuable genetic factors within diverse mulberry populations or lines fluctuates temporally and spatially as these factors become apparent through observable traits. Breeders also face the daunting tasks of selecting hybrids that possess desired characteristics, integrating favourable genetic components into superior breeding lines, and alleviating the adverse effects of linked genes. Lastly, breeders must acknowledge and consider genotype interactions and the environment.

Plant breeding studies rely on the morphological and genetic identification of plant resources to harness gene reservoirs. It is often misleading to assume that plant materials studied are distinct genotypes based solely on morphological similarities (Vijayan, 2007). Therefore, characterizing plant materials using different methods becomes necessary. In this context, DNA fingerprinting methods have proven to be highly advantageous. Scientists often use various DNA markers techniques like ISSR , SSR , RAPD ,AFLP and IPBS to study genetic diversity (Kafkas et al., 2008; Nemli et al., 2015; Orhan et al., 2022; Salih et al., 2022).

Breeding studies, on the other hand, mainly rely on morphological markers, which makes genotypes more vulnerable to environmental influences and less likely to convey their true characteristics accurately. Therefore, by establishing the genetic variations of mulberry genotypes, it becomes possible to record their genetic structures and effectively preserve, evaluate, and practically utilize plant genetic resources. The genetic differences identified through DNA fingerprinting methods yields significant benefits in selecting parent plants for crossbreeding in breeding studies. Understanding the genetic traits of the chosen parent aids in predicting the vegetative characteristics of the resulting F_1 individuals. Consequently, the lengthy selection period, a major challenge in the selective breeding of higher plants, can be significantly reduced with DNA markers (Gülşen and Mutlu, 2005).

2.3.1. SSR Markers

SSR markers are short segments of DNA containing repetitive sequences of 1 to 6 base pairs. During the replication process, the DNA polymerase enzyme exhibits a high error rate within these repetitive regions, leading to a significantly higher occurrence of mutations, approximately 1000 times more than in other regions. Consequently, SSR loci exhibit noticeable variation even among closely related individuals. The codominant SSR marker system has applications outside of the initial laboratory settings. These markers work well with semi-automated processes like capillary electrophoresis, fluorescence labeling, and computer-assisted allele length assessment. Leveraging the characteristics of SSR markers, they find frequent application in various fields, including genetic resource conservation, variety identification, population genetic studies, marker-assisted selection, phylogenetic analysis, and molecular mapping (Gülşen and Mutlu, 2005).

2.3.2. IPBS Markers

The molecular tool known as inter-primer binding site (IPBS) has been proposed (Kalendar et al., 2010). It proves advantageous for DNA fingerprinting in various organisms, including plant and animal kingdoms. One notable advantage of IPBS is its ability to detect genetic variation without prior sequencing knowledge, making it a valuable technique (Demirel et al., 2018; Karık et al., 2019). IPBS markers exhibit a high polymorphism rate and are particularly suitable for detecting genetic variation in clonally propagated polyploid species like black mulberry, as they generate informative multiple bands. Several studies have utilized IPBS markers to genetically characterize crops, including *Cicer* spp. (Andeden et al., 2013), *Vitis vinifera* (Guo et al., 2014), *Lens* spp. (Baloch et al., 2015), *Phaseolus vulgaris* (Nemli et al., 2015), *Prunus armeniaca* (Čechová et al., 2012), *Solanum tuberosum* (Shah et al., 2016), and *Nicotiana tabacum* (Yaldiz et al., 2018), yielding significant insights into genetic variation. However, no known research currently employs IPBS markers for mulberry species.

IPBS markers and SSR markers offer several advantages compared to other marker systems. IPBSs allow discrimination among genotypes without requiring prior sequence knowledge and demonstrate high reliability because of the stringent annealing temperature employed (Guo et al., 2014). SSRs are scarce, but they are still in use because of their great reliability and reproducibility. Given this knowledge, IPBS is an emerging marker system that has the

promise to study cultivated plants. Consequently, this thesis project aims to employ the IPBS marker system for the molecular characterization of black mulberry (*Morus nigra*) genotypes and the determination of genetic distances between these genotypes. This research project, IPBS markers are firstly applicated in *Morus nigra*, and will make original contributions to the field.

2.3.3. Studies with SSR Molecular Markers

Kilincer (2023), conducted a study to genetically characterize 344 sour black mulberry and 18 mulberry samples cultivated in Türkiye and found the all the black mulberry accessions shows identical results across the 15 loci. The The analysis revealed 132 alleles at these loci when considering the entire population. Various calculations were carried out, including expected heterozygosity (He = 0.92), observed heterozygosity (Ho = 0.51), and Polymorphism information content (PIC) (= 0.41) values for the entire population. The application of the UPGMA cluster analysis successfully distinguished different *Morus* species. Unlike other fruit species, black mulberry displayed limited genetic diversity in the region, suggesting that Türkiye and Anatolia may not be the native homeland of sour black mulberry. These findings highlight the unique genetic structure of sour black mulberry among cultivated woody fruit species.

Öztürk et al. (2022), was assessed the genetic diversity in 29 genotypes of *Cucurbita pepo* L. species specifically in Erzincan, using SSR markers. The analysis of morphological traits within the genotypes revealed significant variation in plant, flower, fruit, and leaf characteristics. All primers used in the evaluation showed a 100% polymorphism rate. The seven SSR markers yielded 15 polymorphic bands, with the number of alleles per marker ranging from 2 to 3 and a mean of 2.14 alleles. The PIC ranged from 0.06 (GMT-M61) to 0.247 (GMT-P41), with a mean PIC value of 0.152 for each marker. Based on Nei genetic distance, cluster analysis categorized the 29 genotypes into four main groups. The germplasm of the spider plant consisted of 18 domestic and foreign linkages, and the study utilized seven polymorphic simple sequence repeat (SSR) markers. The SSR markers revealed 46 alleles at seven loci, with an average of 6.57 alleles per locus. The average polymorphism knowledge index was recorded as 0.69. Analysis based on the Nei genetic distance indicated a slight population difference between domestic and exotic spider plants, as evidenced by a low fitting index value of 0.024 and a high Nm (10.20). The resolve of molecular variance did not show

remarkable dissimilarity between local and foreign linkages or within these groups. Furthermore, when accessions were grouped based on stem color, there was minimal population difference with an Fst value of 0.007. In the two hierarchical clusterings using the unconstrained pair group method with arithmetic mean (UPGMA) and principal coordinate analysis (PCoA), the local joint 'ML-3-KK' was distinctly grouped, indicating its uniqueness compared to other accessions and its potential for selection programs aimed at genetic improvement of spider plants. UPGMA and PCoA yielded similar results in grouping the 18 spider plant accessions into four clusters.

Ozturk et al. (2021) took a closer look at the diversity of 14 different squash types from the *Cucurbita maxima* species grown in Erzincan. They used both physical characteristics and genetic traits to explore the variation. They could dig deeper into genetic diversity using fancy DNA markers called SSR. When they checked out the looks of these squash, they found that the plants, flowers, fruits, and leaves had quite a range of features. The genetic analysis used seven SSR markers and found 23 different gene variations. Each marker had between 2 to 5 alleles, averaging 3.286. They also calculated PIC which measures the diversity of the markers. PIC values ranged from 0.00 (for a marker called GMT-M61) to 0.202 (for GMT-P25), with an average PIC value of 0.130 per marker.

Orhan et al. (2020) conducted a study that employed 16 simple sequence repeat (SSR) markers to analyse 73 mulberry collected from various regions of eastern Anatolia in Türkiye . The results yielded are the calculation of 83 alleles across the 16 SSR markers. The PIC ranged from 0.43 to 0.84, averaging 0.61 per site. Among the SSR markers used, MulSTR3, MulSTR5, MulSTR6, SS04, and SS20 were identified as the most effective markers for detecting genetic variations among strawberry cultivars, genotypes, and strains. The genetic similarity coefficients ranged from 0.24 to 0.94, with an average similarity value of 0.41, indicating a substantial level of polymorphism among the strawberry samples. Based on the Jaccard similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA), the samples were classified into three major clusters corresponding to different species.

Arunga et al. (2015) investigated the genetic diversity of 36 French bean varieties cultivated in Kenya, utilizing a combination of morpho-agronomic and SSR markers. in the study they

measured 20 morpho-agronomic traits over two growing seasons, followed by diversification of genetic sources at 26 SSR loci. The analyses reveals the significant variation in traits such as seed coat diameter, seed coat number and weight, and seed weight. However, traits such as flowering time, leaf width and length, and plant height did not contribute significantly to the observed diversity. Of the 26 SSR primer pairs amplified in the 36 DNA samples, 18 provided informative data, with an average allele count of 2.17 and a PIC value ranging from 0.17 to 0.41. The diversity level ranged from 0.19 to 0.50. By combining morpho-agronomic and DNA-based markers provided the advantageous features of both marker types.

Wangari et al. (2013) focused on analyzing the genetic diversity between two mulberry species, *M. alba and M. indica*, which are extensively cultivated in Kenya. They examined five genotypes from each species using 13 SSR markers. The SSR markers exhibited a high level of polymorphism, detecting 35 polymorphic bands and 74.47% polymorphic loci. The average observed heterozygosity per locus was 0.3670, indicating significant variation. Analysis through Molecular Variance (AMOVA) revealed that 95% of the observed variation occurred among the species, while only 5% was within the species. Basic coordinate analysis (PCoA) differentiated the samples into three groups.

Weiguo et al. (2007) employed ISSR and SSR markers to examine the genetic diversity of 27 cultivated mulberry varieties, including 8 wild varieties. The study compared ISSR and SSR markers in their informativeness term and activity for assessing genetic diversity and nexus in mulberry genotypes. The results indicated that SSR markers exhibited higher polymorphism and provided more informative data than ISSR markers. Analysis of the genetic diversity revealed that both markers demonstrated higher genetic diversity in cultivated varieties than in the wild species. The average genetic similarity coefficients, as determined by ISSR and SSR matrices, were 0.7677 and 0.6131, respectively, indicating distinct genetic distances among mulberry genotypes. The UPGMA clustering analysis based on ISSR and SSR markers further confirmed that wild species are genetically distant from the cultivated types examined in this study.

2.3.4. Studies with IPBS Molecular Markers

Gencer and Serçe et al. (2022) conducted a comprehensive study on local apple varieties from forty-eight samples collected from twenty-nine different places at various altitudes (1125-1726 m) in Niğde, Türkiye. The research encompassed morphological, pomological, and molecular analyses to better understand the use of IPBS primers in apples. The molecular results revealed a sample similarity range of 0.61-1.00. Based on these findings, the molecular data effectively differentiated all individuals studied except sample pairs, suggesting that the observed differences were primarily attributed to genotypic variations and environmental conditions.

Yildiz et al. (2022) conducted a study to define the genetic diversity and population structure of 38 Daucus accessions using 21 retrotransposon markers (IPBS). They made an UPGMA dendrogram. They found four different groups. One group comprised Daucus species with 2n=18 chromosomes, called a subclade (A'). In their separate groups, it differed from two other species, *D. pusillus, and D. muricatus*. The rest of the Daucus crew was put in Group B. They noticed some things were mixed up between the different groups. When they looked at the differences between the clusters, they found that 41.85% came from their variations, and the other 58.15% were just differences within each cluster. The biggest population difference was between clusters 2 and 3 (0.579), while clusters 1 and 4 had the smallest difference with an F_{st} value of 0.16.

Kizilgeci et al. (2022) went on a cool adventure to study the genetic diversity and population structure of 70 wild wheat species found in parts of Türkiye. They used 35 IPBS markers and found that 11 marker were pretty awesome at showing the differences between the wheat species. These 11 markers revealed 61 different allele. On average, they found about 5.6 different allele for each marker. The PIC values ranged from 0.22 to 0.47, with an average of 0.35. According to Nei's measurement, the genetic diversity (H) was 0.4861, and Shannon's information index (I) was 0.6791. They used a fancy UPGMA method to group the 70 wild wheat species into three main clusters. Principal coordinate analysis (PCoA) indicated that three principal coordinates accounted for 62.33% of the total variation. Furthermore, population structure analysis revealed the formation of three sub-populations comprising all genotypes. The expected heterozygosity values were all over the place, ranging from 0.2666 for the first group to 0.2330 for the third group. On average, it was around 0.2500. They also measured population differentiation, which was 0.3716 for the first group, 0.3930 for the

second group, and 0.4804 for the third group. So they found that the different wheat types had varying levels of genetic diversity. This study shows that IPBS markers were good at figuring out the genetic differences between different types of genotypes.

Shimira et al., (2021) investigated scarlet eggplant germplasm consisting of fifty-two accessions using IPBS-retrotransposon markers. They used twelve highly variable primers, resulting in a total of 329 bands, of which 85.03% showed polymorphism. The average of polymorphism information content was 0.363. The average effective number of alleles, Shannon's information index, and gene diversity were recorded as 1.298, 0.300, and 0.187, respectively. When they looked at the different collections from the Musanze district, they found a high level of diversity among them. They used different analysis methods like model-based structure, neighbour-joining, and principal coordinate analysis (PCoA), and all of them showed clear patterns of genetic differences among the scarlet eggplant samples based on where they were collected. They found that most of the variation, about 81%, was within the populations themselves rather than between them.

Phong et al. (2016) conducted a study to assess the genetic diversity and relationships among 15 tea (*Camellia sinensis*) accessions cultivated in Vietnam. They used 6 IPBS primers and looked at 21 different physical traits. They compared the IPBS analysis the morphological analysis, which used the Euclidean distance coefficient. UPGMA dendrograms that displayed the genetic links among the different tea types were produced with the assistance of these analyses. Comparing the IPBS analysis to the morphological analysis showed some minor variations. It showed that the Shan and Assam types formed their group separating from the China types. On the other hand, the IPBS analysis grouped the 15 tea genotypes into two main categories. One group included small-leaved China, Assam-small-leaved China hybrid, and Shan-large-leaved China hybrid types, all with small leaves. The other group mainly consisted of Shan, Assam, and Shan-small-leaved China types, which had large leaves. This results shows that IPBS markers are good tools to differentiate local genotypes or landrace grown in various regions or a long time .

Nemli et al. (2015) employed a IPBS marker system to examine the genetic diversity of common bean species. Utilizing 47 IPBS primers, they identified 180 polymorphic bands across 67 common bean species. On average, each primer exhibited four polymorphic band.

The genetic similarity among the species, determined using NTSYS pc software, ranged from 0.09 to 0.99. The IPBS marker demonstrated an average PIC value of 0.73. Population structure analysis used structure software that resulted in the formation of four distinct populations. The study successfully demonstrated that IPBS markers are robust and highly informative, making them advantageous for assessing the genetic diversity of common bean species.

Guo et al. (2014) evaluated the molecular diversity of 35 grape varieties using IPBS markers. Fifteen IPBS-selected primers generated 99 polymorphic DNA bands, resulting in an overall polymorphism rate of 86.3%. The efficacy of IPBS markers was comparable to, or even surpassed, other retrotransposon-based markers commonly used in grapes. The study highlighted IPBS markers as a simple, informative, reproducible, and convenient method for assessing genetic diversity in grapes.

Andeden et al., 2013 detected 130 scorable bands across 10 IPBS primers on five annual wild species and one cultivated species (*Cicer arietinum*). IPBS markers identified 130 bands with complete polymorphism, averaging 13.0 bands per primer. The average PIC value for IPBS was 0.91. The clustering patterns of accessions and species within groups showed substantial similarity.

3. Material and Method

Since it was stated in previous studies on black mulberries that the plants in Türkiye were closely related, therefore Türkiye's samples were only collected from different regions of the province of Tokat (Kilincer, 2023; Orhan et al., 2020; Kafkas et al., 2008). The genetic characterization process of 47 mulberry genotypes has been collected from Türkiye and four different cities in Iraq (32 genotypes from 4 cites in Iraq and 15 genotypes in TOKAT from Türkiye). The names of the genotypes and their coordinates are given in Table. 1 and Figure. 2, respectively. Other species used in dendrogram of SSR markers, a total 19 genotypes of the *Morus rubra*, *Morus laevigata*, and *Morus alba*, were collected in Iraq and Türkiye (Table. 2).

Figure 2. Cities where genotypes are collected

*21:Sartak, 22:Erbil, 23:Halabja, 24:Duhok, 60:Tokat.

Table 1. *Morus* species and genotypes used in the research

G.Number	Country	City	Code	Latitude	Longitude
1	Iraq	Sartak	I-21-N1	34.94994	45.73558
2	Iraq	Sartak	I-21-N2	34.95033	45.73618
3	Iraq	Sartak	I-21-N3	34.95028	45.73632
4	Iraq	Sartak	I-21-N4	34.94908	45.72325
5	Iraq	Sartak	I-21-N5	34.94899	45.72344
6	Iraq	Sartak	I-21-N6	34.94906	45.72341
7	Iraq	Sartak	I-21-N7	34.94894	45.7229
8	Iraq	Sartak	I-21-N8	34.94898	45.72255
9	Iraq	Sartak	I-21-N9	34.94907	45.72261
10	Iraq	Sartak	I-21-N10	34.94909	45.72263
11	Iraq	Duhok	I-24-N1	37.03709	43.37102
12	Iraq	Duhok	I-24-N2	37.03626	43.72824
13	Iraq	Erbil	I-22-N1	36.50216	44.47353
14	Iraq	Erbil	I-22-N2	36.54835	44.43335
15	Iraq	Erbil	I-22-N3	36.50586	44.47481
16	Iraq	Erbil	I-22-N4	36.50235	44.47268
17	Iraq	Erbil	I-22-N5	36.50235	44.47268
18	Iraq	Erbil	I-22-N6	36.50235	44.47268
19	Iraq	Erbil	I-22-N7	36.50235	44.47268
20	Iraq	Erbil	I-22-N8	36.50235	44.47268
21	Iraq	Erbil	I-22-N9	36.51991	44.45494
22	Iraq	Erbil	I-22-N10	36.51991	44.45494
23	Iraq	Halabja	I-23-N1	35.18948	45.97326
24	Iraq	Halabja	I-23-N2	35.18816	45.97311
25	Iraq	Halabja	I-23-N3	35.18850	45.97330
26	Iraq	Halabja	I-23-N4	35.16689	45.94597
27	Iraq	Halabja	I-23-N5	35.16706	45.94595
28	Iraq	Halabja	I-23-N6	35.16706	45.94595
29	Iraq	Halabja	I-23-N7	35.16706	45.94595
30	Iraq	Halabja	I-23-N8	35.15937	45.94334
31	Iraq	Halabja	I-23-N9	35.1892	45.97191
32	Iraq	Halabja	I-23-N10	35.18711	45.98536
33	Türkiye	Tokat	T-60-N1	40.32894	36.28384
34	Türkiye	Tokat	T-60-N2	40.32726	36.28587
35	Türkiye	Tokat	T-60-N3	40.31519	36.54238
36	Türkiye	Tokat	T-60-N4	40.37891	36.64309
37	Türkiye	Tokat	T-60-N5	40.21275	36.3846
38	Türkiye	Tokat	T-60-N6	40.34362	36.54710
39	Türkiye	Tokat	T-60-N7	40.31532	36.54106
40	Türkiye	Tokat	T-60-N8	40.37547	36.62970
41	Türkiye	Tokat	T-60-N9	40.33300	36.47696
42	Türkiye	Tokat	T-60-N10	40.33300	36.47696
43	Türkiye	Tokat	T-60-N11	40.33312	36.47857
44	Türkiye	Tokat	T-60-N12	40.33312	36.47857
45	Türkiye	Tokat	T-60-N13	40.33219	36.47825
46	Türkiye	Tokat	T-60-N14	40.33219	36.47825
47	Türkiye	Tokat	T-60-N15	40.34256	36.53052

Table 2. Other *Morus* species used in the research

G.number	Country	City	Code	Species
1	Iraq	Duhok	I-24-L1	*Morus laevigata*
2	Iraq	Duhok	I-24-L2	*Morus laevigata*
3	Iraq	Duhok	I-24-L3	*Morus laevigata*
4	Iraq	Duhok	I-24-L4	*Morus laevigata*
5	Iraq	Duhok	I-24-L5	*Morus laevigata*
6	Iraq	Duhok	I-24-L6	*Morus laevigata*
7	Iraq	Duhok	I-24-L7	*Morus laevigata*
8	Iraq	Sartak	I-23-L1	*Morus laevigata*
9	Türkiye	Tokat	T-L1	*Morus laevigata*
10	Türkiye	Tokat	T-L2	*Morus laevigata*
11	Türkiye	Tokat	T-L3	*Morus laevigata*
12	Iraq	Duhok	I-24-R1	*Morus rubra*
13	Türkiye	Tokat	T-R1	*Morus rubra*
14	Türkiye	Tokat	T-R2	*Morus rubra*
15	Türkiye	Tokat	T-AW1	*Morus alba*
16	Türkiye	Tokat	T-AW2	*Morus alba*
17	Türkiye	Tokat	T-AW3	*Morus alba*
18	Türkiye	Tokat	T-AW4	*Morus alba*
19	Türkiye	Tokat	T-AP1	*Morus alba*

3.1. Hardwood cutting of *Morus nigra*

Hardwood cuttings were taken in December 2021 for propagation from the determined *Morus nigra* genotypes. The cuttings were planted in perlite medium at 20°C (in the Reproduction Unit of Tokat Gaziosmanpaşa University, Research and Application Center). Before planting, the bottom part of the cuttings was immersed in a 6000 ppm IBA solution. Three months later, the cuttings have been ripped off and planted in pots.

Figure 3. Fresh leaf sample

3.2. DNA extraction

The genomic DNA from the leaves was extracted with CTAB method (Doyle and Doyle 1990) with a modification. A fresh leaf sample (Figure 3), approximately 4-5 cm long (approximately 200 mg), taken from young seedlings in two-three leaves state, was ground in a 1.5 ml microcentrifuge tube in liquid nitrogen and using a 1 ml pipette tip and extracted with cetyltrimethylammonium bromide (CTAB) hot extraction buffer [50 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB and 1% (v/v) b-mercaptoethanol]. The mixture was incubated at 60 °C for 2 hours, followed by two extractions with chloroform/isoamyl alcohol (24:1). Isopropanol was used to precipitate nucleic acids, and the DNA pellet was dissolved in Tris–EDTA (TE) buffer (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA, pH 8.0). RNA was removed by digestion with deoxyribonuclease-free ribonuclease. The remaining impurities were extracted with chloroform. Total DNA was precipitated using cold ethanol (Doyle and Doyle 1990) (Figure 4).

Figure 4. DNA extraction

The quality and amount of the obtained genomic DNA were adjusted to 50 ng/µl by controlling with a 1% agarose gel and spectrophotometer (Figure 5). Genomic DNAs of insufficient quality and quantity were re-isolated. Agarose gel electrophoresis was performed and investigated under UV light to control DNA quality. Then, the amount of DNA was determined, and the tube containing the DNA of the samples was stored at +4 °C for subsequent PCR (Figure 6).

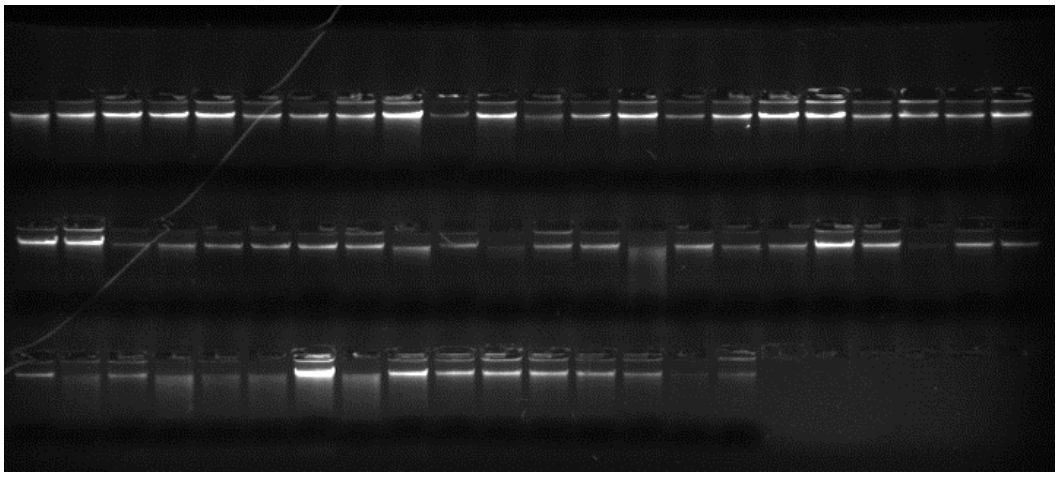


Figure 5. Genomic DNA in 1% agarose gel image

3.3. IPBS analysis

There are 83 IPBS markers (Kalendar et al., 2010) Since it is unknown which markers produce a PCR product of scoreable quality in *Morus nigra* species in this marker system, all markers should be investigated first. Therefore, to use the resources effectively, all markers were studied on 8 *Morus nigra* genotypes randomly chosen among the genotypes used in the experiment. As a result of using 13 IPBS markers that are predictable, scorable, strong, and polymorphic were studied. Some properties related to IPBS markers are given in Table 3.

Table 3. Primer names, their sequence, and annealing temperature

Markers	Sequence	Annealing tempreture℃
IPBS 2074	GCTCTGATACCA	50 ℃
IPBS 2081	GCAACGGCGCCA	60 ℃
IPBS 2232	AGAGAGGCTCGGATACCA	50 ℃
IPBS 2242	GCCCCATGGTGGGCGCCA	55℃
IPBS 2245	GAGGTGGCTCTTATACCA	50 ℃
IPBS 2253	TCGAGGCTCTAGATACCA	50 ℃
IPBS 2257	CTCTCAATGAAAGCACCA	50 ℃
IPBS 2295	AGAACGGCTCTGATACCA	50 ℃
IPBS 2381	GTCCATCTTCCA	50 ℃
IPBS 2382	TGTTGGCTTCCA	50 ℃
IPBS 2388	TTGGAAGACCCA	50 ℃
IPBS 2390	GCAACAACCCCA	50 ℃
IPBS 2395	TCCCCAGCGGAGTCGCCA	60 ℃

The PCR reaction volume for IPBS was 25 µl. Approximately 25-50 ng (1 µl) of genomic DNA was used as tamplate. The PCR reaction contained 1 µM for 12-13 base pair primers, 0.6 µM for 18 base pair primers, 5X Taq PCR buffer, 1.5 mM MgCl2, and 1 unit of Taq DNA polymeraseThe PCR program consisted of 1 cycle at 95°C for 3 min; 28–30 cycles at 95°C for 15 s, 50–60°C (see above) for 60 s, and 68°C for 60 s; a Final extension step of 72°C for 5 min, depending on primer temperature (Table 2) (Kalendar et al., 2010). The resulting PCR products were electrophoresed in 1 X TBE buffer on a 1.7% agarose gel, visualized with a gel imaging system (Vilber Lourmat CN-08), to which ethidium bromide was added to the gel. The bands have been scored as "1" in the presence of the band and "0" in the absence of the band.

3.4. SSR analysis

Ten SSR markers were used in this research. SSR markers named MulSTR (Aggarwal et al. 2004), SS (Zhao et al. 2005), and MulSTRt G79725 (Pinto et al., 2018) were selected for high polymorphism information content of SSR markers. Some characteristics of SSR markers were given in Table 4.

Table 4. SSR markers' names, sequence, annealing temperature, and Expected amplicon length

Marker Name	Froward primer	Reverse primer	EAS (bp)	PAT (°C)
MulSTR1	GCCGTGTACCAGTGGAGTTTGCA	TGACCGTTTCTTCCACTTTACCTAATG	181-209	55
MulSTR2	CGTGGGGCTTAGGCTGAGTAGAGG	CACCACCACTACTTCTCTTCTTCCAG	163-206	55
MulSTR3	GGGTTGGGTAGATGGGCTTATGTTA	CCCTATTAACTTTTTGGTCACCTCTA	107-221	55
MulSTR4	GGTCAAGCGCTCCAGAGAAAAG	GGTGCAGAGGATGAAAGATGAGGT	112-146	68
MulSTR5	CCCCCTGCAATGCCCTCTTTC	TGGGCGAGGCAGGGAAGATTC	134-166	68
MulSTR6	TCCTTAGGTTTTTGGGGTCTGTTTACAT	CCTCATTCTCCTTTCACTTATTGTTG	119-181	58
SS04	CGAGGGAGGGATGAGGAGC	CACATTCATGCACCCTCCTATA	118-240	63
SS17	TACAGGGCTCGGGCAAATG	TGATCCGAAGCTTGGGGTCT	249-243	63
SS18	TCTTCGCCCGTTGTTCGC	AGCAATTTTCTTCAACTCACCTTCT	181-210	63
MULSTR G79725	GAGAACAGTGGGAGTGAACCA	CCCCTCTTGGCCTATATCTCTC	158-184	50

*EAS: Expected amplicon size, PAT: Primer annealing temperature

The PCR reaction volume of the SSR markers was 25 µl. Approximately 50 ng (1 µl) of genomic DNA has been used as a template. The PCR reaction contained 250 nM of the two primers, 0.2 mM of each nucleotide, 1.5 mM $MgCl_2$, and 1 unit of Taq DNA polymerase. PCR conditions were as follows. After five minutes, hot start at 94 ºC, 35 cycles of annealing at 94 ºC for 30 seconds, primer annealing at 50-68 ºC (depend on primers) for 30 seconds, and DNA elongation at 72 ºC for 30 seconds. After that, it consisted of a primer extension at 72 ºC for five minutes and at 4 ºC forever (Kandemir and Saygili 2021). The PCR products gained were run in 1 X TBE buffer in a 3% metaphor agarose gel. Bands were visualized via ethidium bromide added to the gel using a gel imaging system (Vilber Lourmat CN-08). The bands were scored with the BioCapt v.11.02 program. Binary data were created by scoring the bands according to their presence (1) and absence (0) (Ahmed et al. 2022).

3.5. Statistical Analysis

For SSR and IPBS marker data, genetic diversity was calculated using the computer program Popgene v.1.32 (Yeh et al., 2000). Genetic similarity and genetic distance between mulberry species were estimated according to Nei (1972) for pairwise comparison based on the proportion of shared bands produced by each primer. For SSR markers, the polymorphism information content (PIC) was calculated following {PIC = $1 - \sum_{i-1}^{n} pi^2$ }described by Serrote et al. (2020). For IPBS, The PIC was calculated following the formula described by (Thovhogi et al., 2021) {PIC = 1− [fi2 + (1− fi)2]}. A dendrogram was prepared for both marker systems.The mean Shannon information index (i) and gene diversity (h) were determined with the Popgene v.1.32 statistical software. Master coordinate analysis (PCoA) was performed using PAST 4.03 software (Gencer and Serçe, 2022).

4. Result and Discussions

4.1. Simple Sequence Repeats (SSR)

Four of ten SSR markers produced polymorphic bands on 47 *Morus nigra* genotypes (Table 4). The polymorphism rate of SSR markers (four polymorphic SSR markers in ten investigated markers 4/10) was low (40%). Kilincer (2023), who examined *Morus nigra* genotypes from Türkiye, detected 0% polymorphism rate in 15 SSR markers in 334 genotypes. On the other hand, Orhan et al. (2020), who examined the SSR markers (same markers with the present study) in 26 genotypes, determined 100% polymorphism. Low polymorphism rates in the SSR markers were due to the genotypes rather than the polymorphism levels of the markers. Because SSR markers used in the present study produced high polymorphism rate in other *Morus* species (Mathi Thumilan et al., 2016; Orhan et al. 2020). Orhan et al. (2020) reported also that genetic diversity was lower in *Morus nigra* than other *Morus species*. Therefore, it would not be wrong to say that variations in SSR loci were lower in *Morus nigra* than other *Morus species.*

A total of 28 alleles were detected at the 10 SSR loci analyzed on 47 *Morus nigra* genotypes. The number of alleles was 1 in SS04 and SS017, 2 in MUL STR4 and MUL STR5, 3 in MUL STR2, MUL STR6, MUL SAT G79725, SS18, and 5 in MUL SAT 1 and MUL SAT 3. The mean number was 2.8 per locus (Table 4). Orhan et al. (2020) evaluated 16 SSR loci among the mulberry species and defined 83 alleles with an average of 4.54 alleles per SRR markers. Kilincer (2023) found 130 alleles in 15 SSR loci of 362 genotypes (344 *Morus nigra*, 1, *M. macroura*, 15 *M. alba* and 2 *Morus spp*) with an average of 8.66 alleles per SRR markers. The partly high allele numbers in previous studies mentioned above were because researchers use different species. Kilincer (2023) also reported that there is no different allele among 344 *Morus nigra* genotypes. Results from previous studies and similarly from the present study show that the allele numbers of SSR markers in *Morus nigra* species are quite low. Therefore, it can be concluded that *Morus nigra* genotypes may have been distributed in these regions (Iraq and Türkiye) from a few trees due to low genetic diversity in *Morus nigra*. *Morus nigra* genotypes are clonally reproduced by hardwood cutting (Isbilir et al., 2022), and not by seeds which provide high genetic variations via recombination, supporting the interference that *Morus nigra* genotypes were spread from several trees.

Polymorphism information content (PIC) values were between 0.0416 - 0.0425 in polymorphic markers (Table 5). The average PIC value was 0.0419 for polymorphic markers. The highest PIC was determined in MulSTR3 (0.0425), while the lowest PIC in MULSAT G79725 (0.0416). Orhan et al. (2020) evaluated 16 SSR loci primers on mulberry species and reported an average PIC value of 0.61, identifying MulSTR3 as the most informative marker with a value of 0.84. Also, Wani et al. (2013) found that PIC varies between 0.260 (MulSTR3) - 0.623 (MulSTR4), with an average of 0.438 per locus, for 6 SSR primers in 17 mulberry genotypes. PIC values were very low in the present study than those of previous studies. Kilincer (2023) similarly examined only *Morus nigra* genotypes and found no polymorphism, so the PIC values are zero. These findings might imply that SSR markers can provide valuable information for the assessment of the genetic diversity of mulberry species except for *Morus nigra.*

Table 5. A number of detected alleles, the polymorphism information content of SSR markers in 47 *Morus nigra* genotypes

Marker Names	Polymorphism rate of alleles (%)	Number of alleles	Polymorphic alleles	PIC
MUL STR1	non-polymorphic	5	0	0
MUL STR2	66.7	3	2	0.0416
MUL STR3	100	5	5	0.0425
MUL STR4	non-polymorphic	2	0	0
MUL STR5	non-polymorphic	2	0	0
MUL STR6	66.7	3	2	0.0416
MUL STR G79725	66.7	3	2	0.0416
SS04	non-polymorphic	1	0	0
SS17	non-polymorphic	1	0	0
SS18	non-polymorphic	3	0	0
Mean	%75	2.7	2.5	0.63

PIC: Polymorphism information content.

In the SSR markers dendrogram, some genotypes of *Morus* species along with *Morus nigra* were used for confirmation of SSR markers for discrimination power. The dendrogram a total of 66 genotypes (47 *Morus nigra, 11 Morus laevigata, 5 Morus alba, 3 Morus rubra*) into two main clusters, whereas *Morus nigra* clustered in the dendrogram, indicating the similarity of *Morus* genotypes in Türkiye and Iraq (Figure 7). The first cluster represents 47 *Morus nigra* genotypes and is clearly distinguishable from other species. *Morus nigra* genotypes showed close genetic relationships. There was only one different genotype (I-24-N1) based on SSR

markers, indicating quite low genetic diversity within *Morus nigra* genotypes. The second cluster of 19 mulberry genotypes (*M. rubra*, *M. laevigata*, and *M. alba* 'white-purple') represented a distinct group of mulberry samples. The second cluster was further divided into three subgroups, which clearly distinguish between Morus species. Subgroups consist of eleven *Morus laevigata* genotypes, five *Morus alba* genotypes (white-purple), and three *Morus rubra* genotypes. Orhan et al. (2020), Cekic and Ozmen (2018), and Kilincer (2023) found that *Morus nigra* is distinctly different from other Morus species based on SSR marker data. Cekic and Ozmen (2018) found the polymorphism level was lowest in black mulberry genotypes, which were obtained from 15 ISSR primers with 38 mulberry genotypes. Kilincer (2023) also states that there is no different genotype among 344 genotypes. These results show that *Morus nigra* has no or very low polymorphism at SSR loci within the species. On the other hand, same results show that *Morus* species can be easily distinguished by SSR markers. Further, purple mulberry is closer to *Morus alba*, as also found by Kilincer (2023).

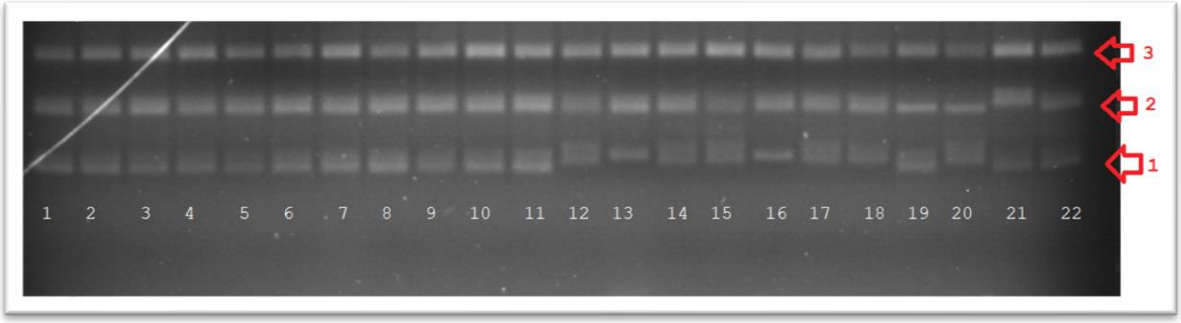
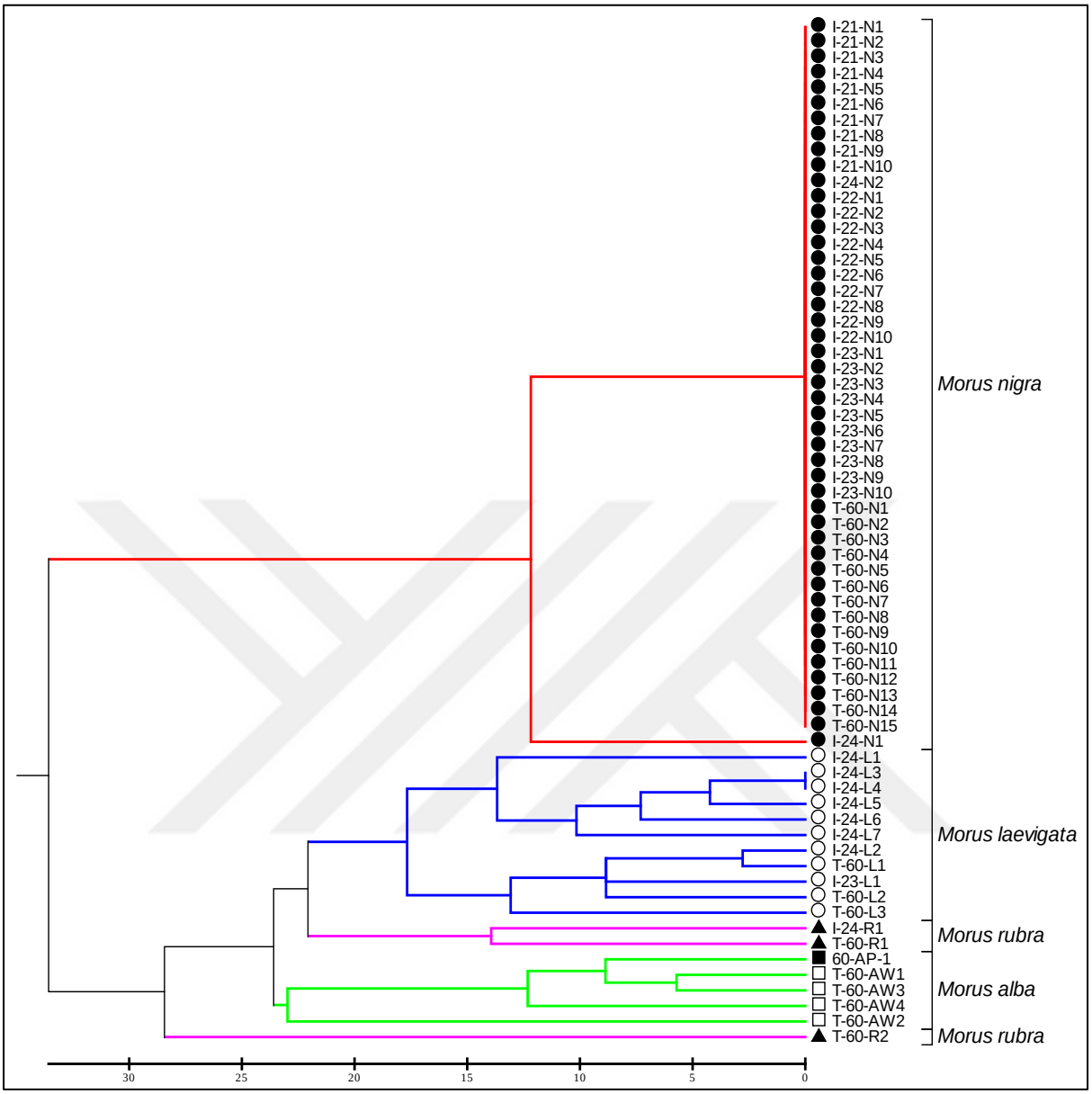


Figure 6. Gel image of SSR MUL SAT G97925 marker

*Band profiles of MUL SAT G97925 SSR marker on 66 mulberry species in 3% metaphor agarose. The row indicated with a 1 arrow indicates the first loading, the row indicated with a 2 arrow indicates the second loading, and the row indicated with a 3 arrow means the third loading.

Figure 7. UPGMA dendrogram based on SSR data



*The encodings for labeling genotypes are as follows. For regions; T: Türkiye, I: Iraq, 21: Sartak, 22: Erbil, 23: Halabja, 24: Duhok, 60: Tokat, and for species; N: *Morus nigra*, R: *Morus rubra*, L: *Morus laevigata*, AW: *Morus alba* - white, WP: *Morus alba* – purple.

As a result, the variations based on SSR data in *Morus nigra* was low. The ploidy level is too high in *Morus nigra* (2n=22X=308). They did not germinate without any pretreatment by seed (Gunes and Cekic, 2004). Therefore, the production of mulberry with seeds is limited and not used in general (Kruthika et al. 2023). *Morus* species are produced clonally by hardwood cutting to preserve the true characteristics of the genotypes. In addition, apomictic seed formation may also have contributed to the low genetic variation among other *Morus* species, and maybe in *Morus nigra*, which reduces genetic variation (Orhan et al., 2020; Kilincer,

2023). These are the reasons for the very low genetic variation in the SSR data investigated in the present study.

4.2. Inter-primer binding site (IPBS) retrotransposon markers

Thirteen IPBS retrotransposon marker produced a total of 138 bands in *Morus nigra* genotypes (Table 6). The average allele number per marker was 10.6. The highest allele number was 25 and obtained from 2074 marker. The lowest allele was 2 from 2381 IPBS retrotransposon marker. The total number of polymorphic alleles was 108, the mean number of polymorphic alleles per primer was 8.3. The highest number of polymorphic bands was 25 bands obtained from 2074 IPBS. The lowest number of polymorphic bands was one from IPBS 2381 retrotransposon primers (Table 6).

Morus nigra genotypes were first evaluated with IPBS markers in the present study. Therefore, the obtained results are discussed with implications from genetic variation detection studies conducted on different crops using IPBS. Shimira et al. (2021) researched 12 IPBS primers in 52 eggplants genotypes and found a total of 329 bands of which 85.03% were polymorphic. Andeden et al. (2013) researched 71 accessions of *Cicer* species using 13 IPBS primers. They reported that the number of bands per marker ranged from 8 (IPBS 2392) to 17 (IPBS 2074) in the *Cicer* species with an average of 13 bands per primer, and the highest number of bands detected in IPBS 2074 marker. Yildiz et al (2022) used 21 IPBS primers in 39 genotype of *Daucus, Apiaceae,* and higest band was detected 2074 IPBS marker (15). In the present study, it was found the higher polymorphic bands than in previous studies in IBPS. This actually an expected situation as *Morus nigra* has one of the highest ploidy level (*Morus nigra* 2n=22x=308, decosaploid) in cultivated plant. IPBS markers exhibit a high polymorphism rate and are particularly suitable for detecting genetic variation in clonally propagated polyploid species like *Morus nigra*. Several studies have utilized IPBS markers to assessment the genetic diversity in crops such as apple (Genger et al., 2022), *Cicer* spp. (Andeden et al., 2013), Lens spp. (Baloch et al., 2015), *Phaseolus vulgaris* L. (Nemli., 2015), Daucus carota L. subsp. sativus Hoffm. (Yildiz., 2022)and find out high levels of genetic diversity.

The average polymorphism percentage (P%) was 0.74%, and the highest polymorphism percentage was 100% in IPBS 2074, IPBS 2081, IPBS 2232, IPBS 2395, and the lowest range was 23.08% in IPBS 2253 (Table 6). Gencer and Serçe et al., (2022) has found 143

polymorphic bands on 48 local apples using 15 IPBS, and detected polymorphism rates of 5 - 94%. Also, the average polymorphism percentage (P%) was 99.03% of explored 21 IPBS with 38 *Daucus* accessions (Yildiz et al., 2022). Barut et al. (2020) studied 96 genotypes of Quinoa (*Chenopodium quinoa Willd*.) IPBS marker and polymorphism rate was 66.8%. Very high levels of polymorphism in iPBS markers confirmed that they are an good marker for detecting genetic variation in plant germplasm.

The highest polymorphism information content (PIC) was found in the IPBS 2245 primer (0.50), followed by the other high values with 0.49 in the IPBS 2074, 2257, 2395 and 0.48 in IPBS 2242. The lowest PIC value (in the IPBS 2253) was 0.26. The mean PIC value was determined as 0.43 (Table 6). Gencer and Serçe et al. (2022) reported that the PIC values of IPBS vary between 0.10-0.34 on apple genoytypes. Barut et al. (2020), studied 96 genotypes of Quinoa, determined PIC of 0.410. Kizilgeci et al. (2022) used 11 iBPS primers for 70 wild wheat species, and PIC values between 0.22 and 0.47. In the literature, it has been noticed that PIC values vary on crops and wild species in IPBS. This may be due to the number of genotypes, ploidy levels and sometimes number of species used. Results presented herein explain the presence of a good level of polymorphism in evaluated *Morus nigra* genotypes and also confirm the universal nature of the IPBS marker system, which can be used for the detection of polymorphism in any species.

Considering the gene diversity (H) obtained from the IPBS retrotransposons primers, the lowest gene diversity was 0.04 from the IPBS 2253 primer and the highest gene diversity value with 0.49 from the IPBS 2390 primer, the average of gene diversity was 0.35 (Table 5).The Shannon information index (Shannon information content; I) was obtained with the highest value (0.68) from the IPBS 2390 primer and with the lowest value (0.10) from the IPBS 2253 primer. The average Shannon information content was obtained as 0.35 (Table 5). The effective number (NE) in alleles of Primer 2390 and Primer 2253 displayed maximum and minimum of 1.95 and 1.04, respectively. The mean of Ne was 1.37 (Table 6).

As a result, mean values for Ne, H, and I were observed as 1.37, 0.25, and 0.35, respectively. The values of Ne, H, and I in this study are higher than in other studies. Shimira et al. (2021) obtained values of 1.32, 0.186 and 0.273 for Ne, H and I in 12 IPBS primers of 52 Scarlet eggplants. Also, Yaldiz et al. (2018) researched of 11 IPBS primer markers in 96 Turkish tobacco.

They found 1.27, 0.19 and, 0.31 for Ne, H, and I value. The common reasons of existing of higher values for different Gene diversity indices might be happened because of the high efficiency of the IPBS retrotransposon marker system in assessing the genetic diversity or the higher diversity might come from the nature of germplasm itself.

Table 6. Primer name, number of total bands, polymorphic bands, and some diversity parameters of the IPBS -retrotransposon primers used during this study

Markers	Total Band	Na	NE	H	I	P%	pic
IPBS 2074	25	25	1.11	0.3	0.18	100%	0.49
IPBS 2081	6	6	1.6	0.35	0.52	100%	0.43
IPBS 2232	13	13	1.52	0.3	0.45	100%	0.42
IPBS 2242	9	5	1.71	0.39	0.57	55.55%	0.48
IPBS 2245	12	11	1.21	0.2	0.25	91.67%	0.50
IPBS 2253	13	3	1.04	0.04	0.1	23.08%	0.26
IPBS 2257	10	9	1.28	0.18	0.3	90%	0.49
IPBS 2295	13	7	1.19	0.12	0.21	53.85%	0.39
IPBS 2381	2	1	1.07	0.08	0.14	50%	0.41
IPBS 2382	6	3	1.38	0.24	0.39	50%	0.45
IPBS 2388	7	6	1.4	0.27	0.43	85.72%	0.38
IPBS 2390	8	5	1.95	0.49	0.68	62.50%	0.47
IPBS 2395	14	14	1.33	0.25	0.36	100%	0.49
Total	**138**	**108**	**-**	**-**	**-**	**-**	**-**
Average	**10.6**	**8.3**	**1.37**	**0.25**	**0.35**	**0.74%**	**0.43**

*Na: number of polymorphic alleles, NE: number of effective alleles, H: gene diversity, I: Shannon information index, P%: Polymorphism percentage, PIC: polymorphism information contents.

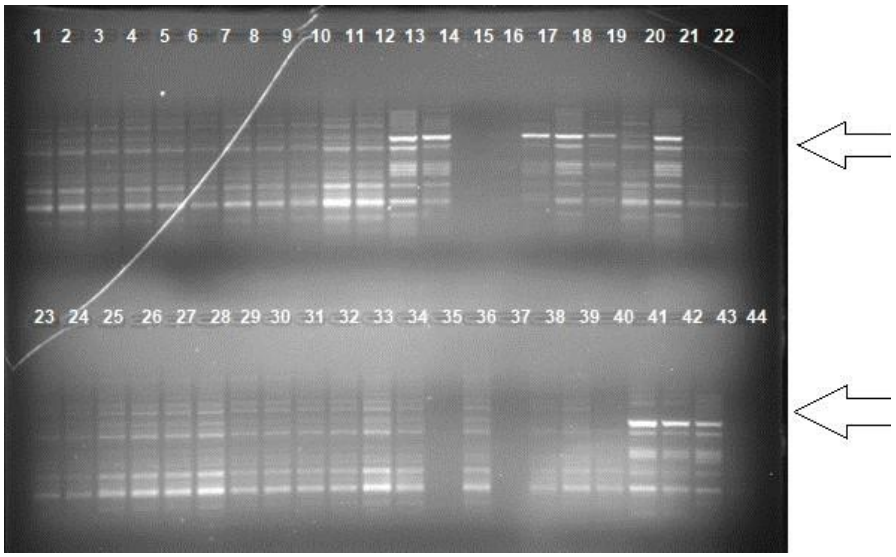


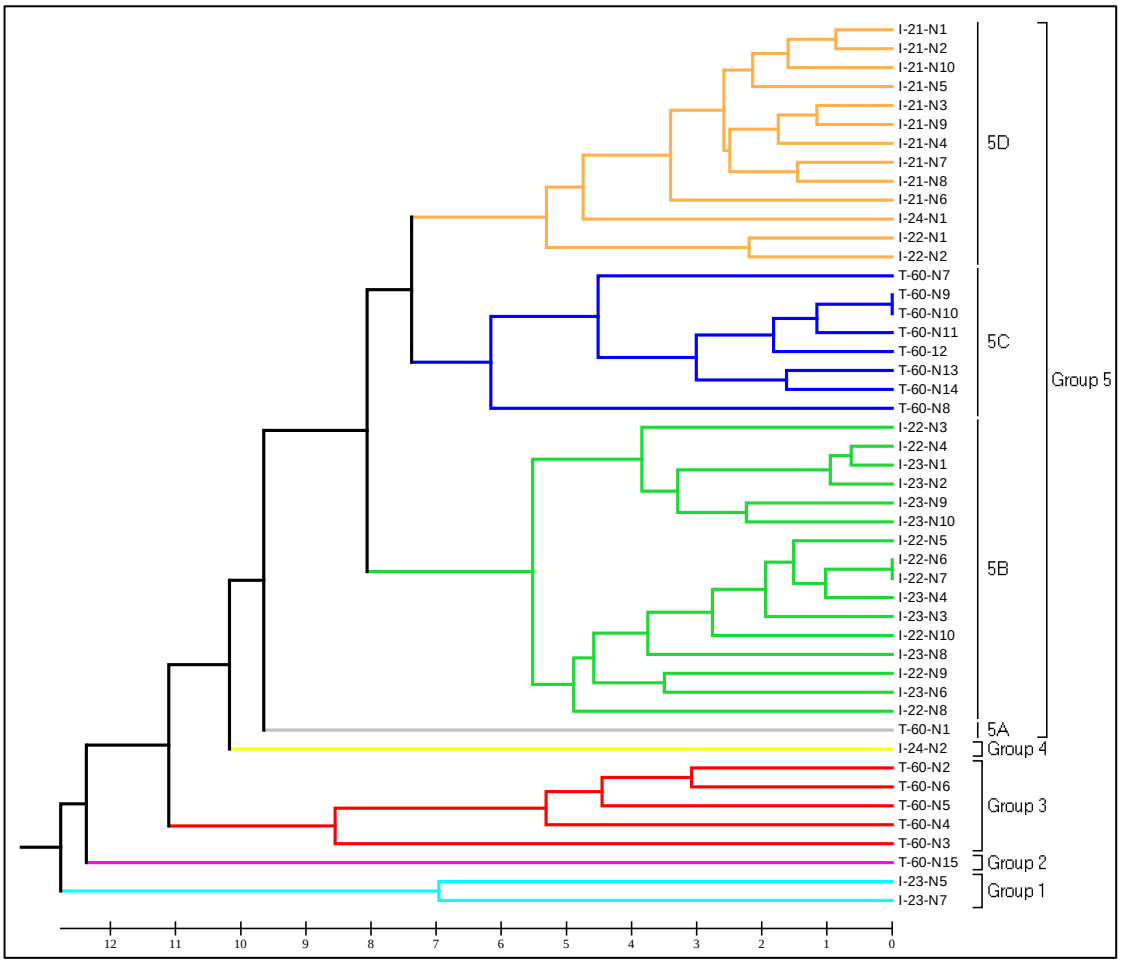
Figure 8. Gel image of IPBS2074 primer

*Scanning with IPBS 2074 primer. 1% agarose gel was used. The row indicated with a 1 arrow indicates the first loading, and the row indicated with a 2 arrow indicates the second loading.

In the dendrogram based on thirteen IPBS markers, there were only two identical genotypes, other 45 genotypes were different at least one allele any IPBS markers (Figure 9). Dendrogram consists of five groups. The first group is two genotypes from Iraq, the second group consists of one genotype from Türkiye. The third group consists of five genotypes from both of them. The fourth group consists of one genotype from Iraq. The fifth group (the largest group) was divided into four sub-groups: The 5A group consists of one genotype from Türkiye, the B group consists of 16 genotypes all of them from Iraq, the C group consists of eight genotypes from Türkiye, and the D group consist of 13 genotypes from Iraq. As a result, found diversity between the *Morus nigra* genotypes were high. Because of that *Morus nigra* genotypes were first evaluated with IPBS markers in the present study. In the last studies, the IPBS marker showed a high difference between genotypes in different crops (Gencer and Serçe et al. 2022), (Yildiz et al., 2022), and (Shimira et al., 2021). The iPBS markers are considered a reliable and polymorphic molecular tool since they allowed discrimination among *Morus nigra*, and determined the genetic diversity and population structure among *Morus nigra*.

As a result, the variations based on IPBS data in *Morus nigra* were high. The iPBS markers are considered a polymorphic molecular tool since they allowed discrimination among *Morus nigra*, and determined the genetic diversity and population structure among *Morus nigra*. In species with low genomic variation and high ploidy levels, such as *Morus nigra*, which are vegetatively propagated and difficult to propagate by seed supporting variation through genetic segregatins, IPBS markers offer a powerful advantage for genetic characterization.

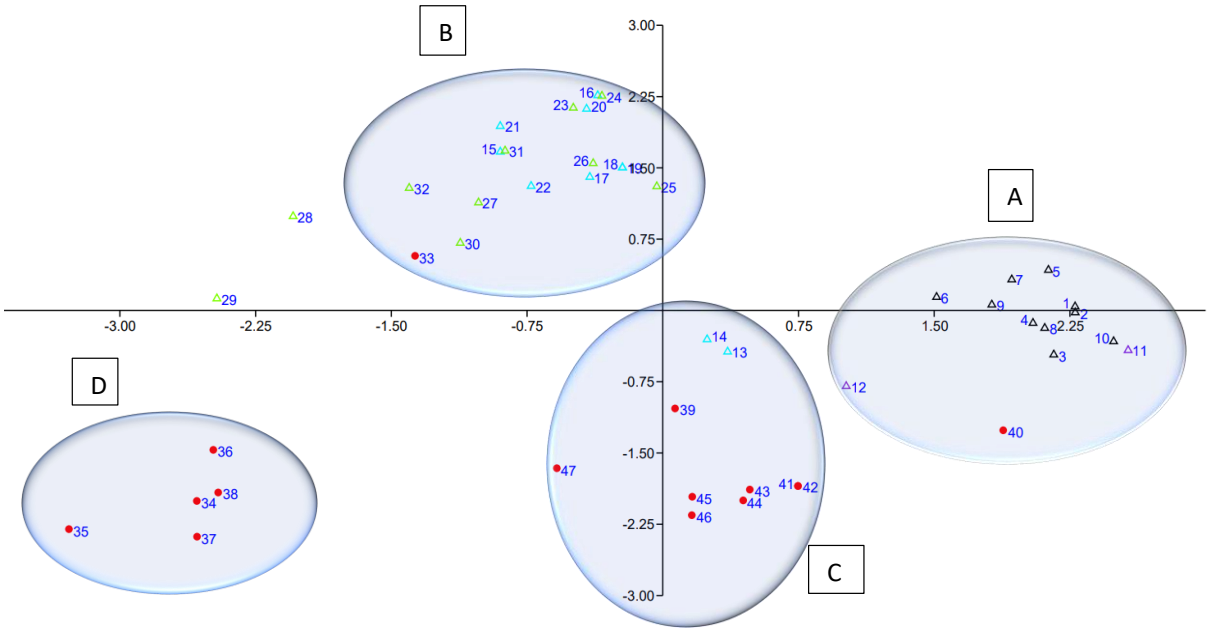
Figure 9. The UPGMA dendrogram based on IPBS markers



*The encodings for labeling genotypes are as follows. For regions; T: Türkiye, I: Iraq, 21: Sartak, 22: Erbil, 23: Halabja, 24: Duhok, 60: Tokat, and for species; N: *Morus nigra*

PCoA (Principal coordinate analysis) was obtained by IPBS markers data and showing the genetic similarity/difference between 47 *Morus nigra* (Figure 10). In PCoA, the samples of *Morus nigra* are divided into four main groups. Group A consists of 13 samples, all of which were taken from Iraq except genotype 40 (T-60-N8). Group B consists of 17 Iraq samples, except for genotype 33 (T-60-N1) from Türkiye. Due to Türkiye border neighbor with Iraq, it has been believed that these genotypes originally came from the same region. Group C consisted of a total 10 genotypes two of which them was from Iraq. Group D group consisted of 5 from Türkiye. The genetic distances between 47 *Morus nigra* cultivars supported the data obtained from the dendrogram (Figure 9). The genotypes examined were clustered together, mostly referring to the regions where they were collected. This indicates that the IPBS markers produced data confirming the regional distribution.

Figure 10. Principal coordinates analysis (PCoA) of 47 samples of *Morus nigra* and one related species based on 13 IPBS markers. The numbers at each dot correspond to those displayed in Table 1



*__Black__: Refers to genotypes obtained in Iraq-21, Purple: Refers to genotypes obtained in Iraq-24, Blue: Refers to genotypes obtained in Iraq-22, Green: Refers to genotypes obtained in Iraq-23, Red: Refers to genotypes obtained in Türkiye.

CONCLUSION

The main objective of this research is to determine the genetic diversity of *Morus nigra* trees growing in Turkey and Iraq. It was determined that there is genetic diversity in *Morus nigra* mulberries grown in different regions by using IPBS but very low in SSR markers. Although SSR markers couldn't show a high genetic diversity, but the IPBS marker was successful in showing genetic diversity this is due to being associated with high ploidy. Therefore, in crops with high polyploidy and vegetatively propagated we recommend using an IPBS marker to determine the genetic diversity.

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