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Molecular Identification of *Morus* ssp. in Duhok Using Nuclear ITS Region and Chloroplast Matk Gene

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Abstract: Since Mulberries (*Morus*)is a tree species with a considerable plant variety. Molecular techniques are methods used to distinguish between species accurately, easily and quickly. This study examines a Molecular method for distinguishing different *Morus* species in the Duhok - Kurdistan region/ Iraq. The method is based on the use of four techniques: matK gene, the ITS region, PCR-RFLP, and SRAP markers. Twelve *Morus* species have been selected for this study from different region of Duhok. The ITS region's PCR result was 700 bp, but the matK gene's PCR produce was 900 bp. The same restriction site was found for all utilized cultivars when the 700bp of ITS fragment was used for PCR-RFLP with two restriction enzymes, RsaI GT/AC and HaeIII GG/CC. This study also used six combinations of SRAP markers to aid in grouping and identifying genetic similarities. The results of PCR-RFLP demonstrated an insufficient link between *Morus* physical appearance and genetic traits, but differences across studied cultivars could be identified using SRAP markers. Furthermore, this study demonstrated the possibility of DNA barcoding *Morus* cultivars, as well as additional sequence analysis and the identification of probable SNP between cultivars.

Keywords: DNA barcoding, ITS region, *Morus*, matK gene, PCR- RFLP, SRAP markers.

Introduction

Mulberries are members of the Moraceae family. Around 12 distinct *Morus* species have been grown worldwide for its aesthetic value, fruit, or timber (Zhang *et al.,* 2011). *Morus* is endemic to temperate Asia and North America. *Morus* is abundant in fibre, antioxidants, flavonoids, vitamins, and minerals, all of which have health benefits (Yan *et al.,* 2023). They improve heart health by controlling blood flow, reducing cholesterol, and decreasing the risk of some types of cancer. *Morus* can also aid with digestion, blood sugar control, blood circulation, immunity, and weight loss. Mulberries contains compounds such as anthocyanins and resveratrol, which help to prevent cancer cells and maintain liver function. *Morus* is also abundant in vitamin C, which can help reduce risk of developing a range of illnesses (Yuan & Zhao, 2017; Kadam *et al.,* 2019). Ecologically this plant perform a vital role in preventing and managing sand erosion, as well as treating stony desertification and saline alkali soil (Yan *et al.,* 2023).

Molecular markers could be used to distinguish between closely related plants as in Apricot genotype (Al-Janabi & Alhasnawi 2021). Molecular markers are powerful tools for describing genetic differences and finding

links between mulberry genotypes and cultivars, so many researchers have resorted to them compared to methods based on phenotypic or chemical characterization (İpek *et al.,* 2012; Mondal 2020). Recently DNA barcoding is one of the mostly used choice in plant biology to purpose of phylogenies for related taxa as well as species differentiation (Mosa *et al.,* 2019).

Attempts were undertaken in mulberry to examine the molecular characterisation of mulberry cultivars and genotypes using RAPD markers (İpek *et al.,* 2012), as well as employing ISSR markers to determine genetic links among local *Morus* land races (Salih *et al.,* 2022), furthermore SRAP markers was used for genetic diversity identification (Hu *et al.,* 2015).

Several studies have used genetic approaches to study diversity within *Morus*. Nepal & Ferguson (2012); used sequence data from ITS of the nrDNA and the chloroplast *trnL-trnF* intergenic spacer to study phylogenetic relationships of *Morus*. Meanwhile Zeng *et al.,* (2015) could define eight mulberry species in the genus *Morus* by internal transcribed spacer-based phylogeny.

The current study aimed to establish genetic relatedness among the most cultivated *Morus* in the region by utilizing conservative regions such as the ITS region and the matK gene in the plant's genetic composition as well as utilizing SRAP markers for genetic diversity identification.

Materials & Methods

Collecting Plant Samples

Leaf samples from 12 *Morus* cultivars for this study have been collected using car transportation from various regions of Duhok (Table 1) and brought the samples to the laboratory which was preserved in 4^0C till genomic DNA isolation.

Table (1): Cultivars used in this study.

no.	Sample name	Area of collection
m1	*Morus alba* L. pendula (weeping white mulberry)	Duhok/ tanahi/ avrocity
m2	*Morus alba* L. pendula (weeping white mulberry)	Duhok/ tanahi/ avrocity
m3	*Morus alba* L. pendula (weeping white mulberry)	Duhok/ tanahi/ avrocity
m4	*Morus alba* L. pendula (weeping white mulberry)	Duhok/ tanahi/ avrocity
m5	*Morus alba* L. pendula (weeping white mulberry)	Duhok/ tanahi/ avrocity
m6	*Morus alba* L. pendula (weeping white mulberry)	Duhok/ tanahi/ avrocity
m7	*Morus rubra* L. (red mulberry)	Duhok/ malta
m8	*Morus rubra* L. (red mulberry)	Duhok/ malta
m9	*Morus alba* L.	Duhok/ tanahi
m10	*Morus alba* L.	Duhok/ tanahi
m11	*Morus alba* L.	Duhok/ tanahi
m12	*Morus nigra* L. (black mulberry)/ Tooshambi	Duhok/ tava rashe

Genomic DNA isolation and PCR amplification:

The genomic DNA was extracted using the CTAB methods described by Hussein & Jubrael (2021) with minor changes.

For amplification of both region using two set of primers (Table 2), and SRAP markers (Table 3), A 20µl PCR reaction mixture was prepared containing 2 µl of DNA (25-50 ng), 2 µl (10 pmol) of each forward and reverse primers and 10 µl of (Go Taq G2 green)

(Promega, USA) along with 4 µl of DDs sterile water. The following settings were used to conduct the PCR amplification in an ABI Applied Biosystems PCR System 2720 thermal cycler: for ITS region. The optimized thermocycler conditions for the reaction were initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and final extension at 72 °C for 10 min. While for matk gene the same condition was applied except for annealing 58ºC for 30 sec. For SRAP marker was completely different program was followed: Initial denaturation step involved 5 min at 94˚C. followed with five cycles of denaturation for 1 min at 94˚C., annealing for 1 min at 35˚C., extension for 1 min at 72˚C. Followed by 38 cycles including 1 min at 94˚C, Annealing for 1 min at 50˚C, and elongation for 1min at 72˚C. Final extension for 5 min at 72˚C.

Table (2): The primer used in this study.

primer name	sequence of Forward	sequence of Reverse	banding size bp.
matk gene	5'CGATCTATTCATTCAATATTTC3'	5'TCTAGCACACGAAAGTCGAAGT3'	**900**
ITS region	5'TCCTCCGCTTATTGATATGC3'	5'TCCGTAGGTGAACCTGCGG3'	**700**

Table (3): SRAP primer combination used for this research.

Reverse primer	Sequence	Forward primer	Sequence
Em8	GACTGCGTACGAATTAGC	Me6	TGAGTCCAAACCGGATG
Em15	GACTGCGTACGAATTCTG	Me8	TGAGTCCAAACCGGTGT
		Me15	TGAGTCCAAACCGGTCA
		Me16	TGAGTCCAAACCGGGAC
		Me17	TGAGTCCAAACCGGGTA

PCR-Restriction Fragment Length Polymorphism (PCR- RFLP)

PCR-RFLP studies were performed on all ITS region (gene) PCR products. Enzymes with smaller and lower-molecular-weight fragments were chosen. Using these parameters, the enzymes RsaI GT/AC and HaeIII GG/CC (Jena bioscience, Germany) were chosen to digest the amplified 700bp gene. Individual digestion conditions were carried out in a final volume of 50µl, as recommended by the manufacturer: 5µl of 10x universal buffer, 1µg of PCR product, 10 units of enzyme, then the reaction was made up to 50µl with PCR grade water, and the mixture was incubated at 37 ºC for 2 to 4 hours. The product of the digested results was then analyzed using 3% agarose gel electrophoresis.

Data analysis

Based on the electrophoresis findings, DNA fragments in allele size were classified as present (1) or absent (0) (Kosman & Leonard 2005). These scores were used to assess genetic diversity. Various characteristics were

assessed to validate the potential of the selected markers and differentiate them among the cultivars examined, as well as to estimate genetic diversity.

For clustering and dendrogram construction, NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System) software version 2.1 was used for. The data were analyzed with the Similarity for Qualitative Data program, which established a genetic similarity index using the unweighted pair-group method with arithmetic mean (UPGMA) (Nei & Li, 1979).

Results & Discussion

12 *Morus* samples were selected to identified and categorized the genetic diversity using the internal transcribed spacer (ITS) region, matk gene, PCR-RFLP and SRAP markers.

It could be possible to identify several *Morus* species in Duhok Province using DNA barcoding, such in the ITS region was invariant in length for used genotypes (700bp) (Fig. 1), while for matk gene showing (900bp) (Fig. 2). In addition to successful amplification of the ITS region, further analysis was applied the 700bp PCR product of both universal primers ITS1/ ITS4 of the ITS region in PCR-RFLP analysis using both *Hae* III and *Rsa* I restriction enzymes. The result of PCR-RFLP digestion applying RsaI restriction enzyme showing two fragments of 580bp and 120bp through all samples (Fig. 3), while for applying HaeIII enzyme it could result of same two band of 400bp and 300bp among all morus samples (Fig. 4). Therefore; the result of PCR-RFLP could not characterize the genetic diversity among selected *Morus* samples.

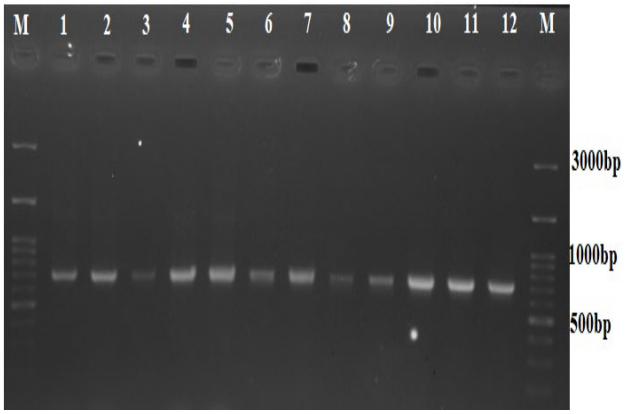

Fig (1): Represents a 1.5% agarose gel electrophoresis of the PCR product of ITS region for 12 samples of *Morus*. Lane M, DNA molecular weight 100bp marker, lane 1-12 *Morus* species.

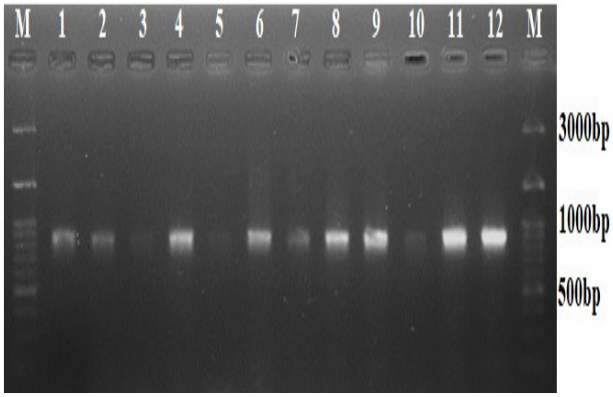

Fig (2): Represents a 1.5% agarose gel electrophoresis of the PCR product of matK region for 12 samples of *Morus*. Lane M, DNA molecular weight 100bp marker, lane 1-12 *Morus* species.

Furthermore, identifying particular genetic variations among studied species were identified using SRAP markers combinations. In this research 6 combination of SRAP primers could reveal reproducible result as shown in table (4).

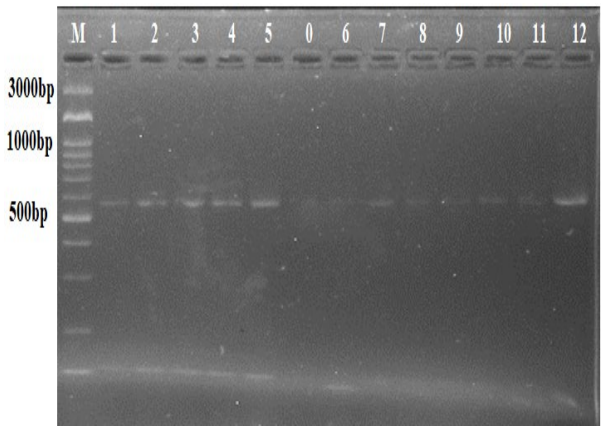

Fig (3) Represents a 3% agarose gel for digestion of 700bp ITS region using *Rsa*I restriction enzyme. Lane M, DNA molecular weight 100bp marker, lane 1-12 *Morus* species.

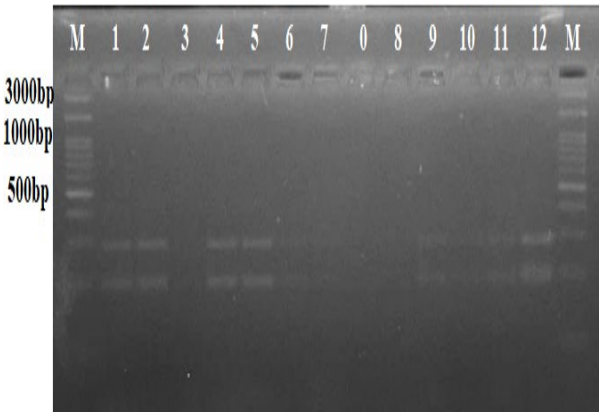

Fig (4) Represents a 3% agarose gel for digestion of 700bp ITS region using *Hae* III restriction enzyme. Lane M, DNA molecular weight 100bp marker, lane 1-12 *Morus* species.

Table (4): Data analysis for used SRAP primer combination.

Primer combination	Total namber of bands	Polymorphic bands	Monomorphic bands	percentage of polymorphic bands
Em8/ Me15	11	11	0	100
Em15/ Me6	9	5	4	55.5
Em15/ Me8	5	4	1	80
Em15/ Me15	12	11	1	91.6
Em15/ Me16	7	7	0	100
Em15/ Me17	8	5	3	62.5
Total	52	43	9	489.6
mean	8.6	7.1	1.5	81.6

In the fig. (5) the result of SRAP marker combination using Em8/Me15 were shown a clear banding pattern among all 12-sample used for this study. The use of SRAP markers revealed significant variations in genetics across Duhok Province *Mours* samples. The SRAP markers generated a significant number of reproducible and highly polymorphic fragments, which will be useful in population genetic research. The result was in agreement with Zhao *et al.,* (2009) which used 12 combination of SRAP primers and result of 83 total number of bands with average of 6.9 band per combination and an average of 71.1% polymorphic percentage.

Acording to resulted bands from all combination it could identify the genetic simelarity (Table 5) and clustering analysis (Fig. 6) of the samples used for this study. The table of genetic similarity could identifyed that the maximum similarity was 100% (1.0000) among cultivar (m4, m5) and (m10, m11), while the minimum similarity 0.1818 could result between both m6 and m9 samples.

The genetic simelarity analysis it was possible to identify the samples grouping in 3 clusters. The first cluster involved (m1, m2, m3, m4 and m5) this grouping was in agreement with the same geographical area of Duhok. The second cluster both (m8 and m9)

relatedness of about 81%. The third cluster was included (m6, m7, m10, m11 and m12).

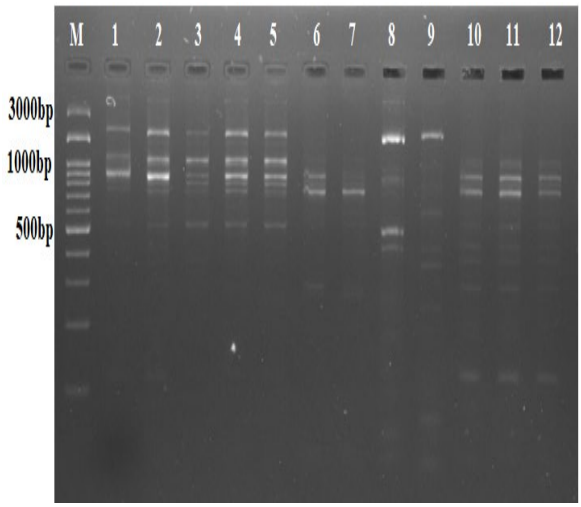

Fig. (5): SRAP marker combination of primer Em8/Me15 using 2% agarose gel.

The detected differences may be attributed to several factors such as the geographic obstacles, restricted gene flow, or local adaption to distinct ecological circumstances might all contribute to this difference. Identification of such patterns contributes to a better understanding of the evolutionary history of *Mours* species in the region (Sexton *et al.,* 2014). Furthermore, the high polymorphism of SRAP markers may allow for the discovery of significant characteristics, as well as SRAP markers can provide insights into the genetic basis of local adaptation and perhaps find genes involved in crucial ecological processes by correlating certain genetic markers to adaptive features (Ouborg *et al.,* 2010).

Table (5): Represent the Genetic similarity among all *Morus* cultivars.

	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10	m11	m12
m1	1.0000000											
m2	0.8181818	1.0000000										
m3	0.7272727	0.7272727	1.0000000									
m4	0.8181818	0.8181818	0.9090909	1.0000000								
m5	0.8181818	0.8181818	0.9090909	1.0000000	1.0000000							
m6	0.6363636	0.4545455	0.7272727	0.6363636	0.6363636	1.0000000						
m7	0.5454545	0.3636364	0.6363636	0.5454545	0.5454545	0.9090909	1.0000000					
m8	0.7272727	0.5454545	0.4545455	0.5454545	0.5454545	0.3636364	0.2727273	1.0000000				
m9	0.5454545	0.3636364	0.2727273	0.3636364	0.3636364	0.1818182	0.2727273	0.8181818	1.0000000			
m10	0.4545455	0.4545455	0.3636364	0.2727273	0.2727273	0.6363636	0.5454545	0.3636364	0.3636364	1.0000000		
m11	0.4545455	0.4545455	0.3636364	0.2727273	0.2727273	0.6363636	0.5454545	0.3636364	0.3636364	1.0000000	1.0000000	
m12	0.3636364	0.3636364	0.4545455	0.3636364	0.3636364	0.7272727	0.6363636	0.2727273	0.2727273	0.9090909	0.9090909	1.0000000

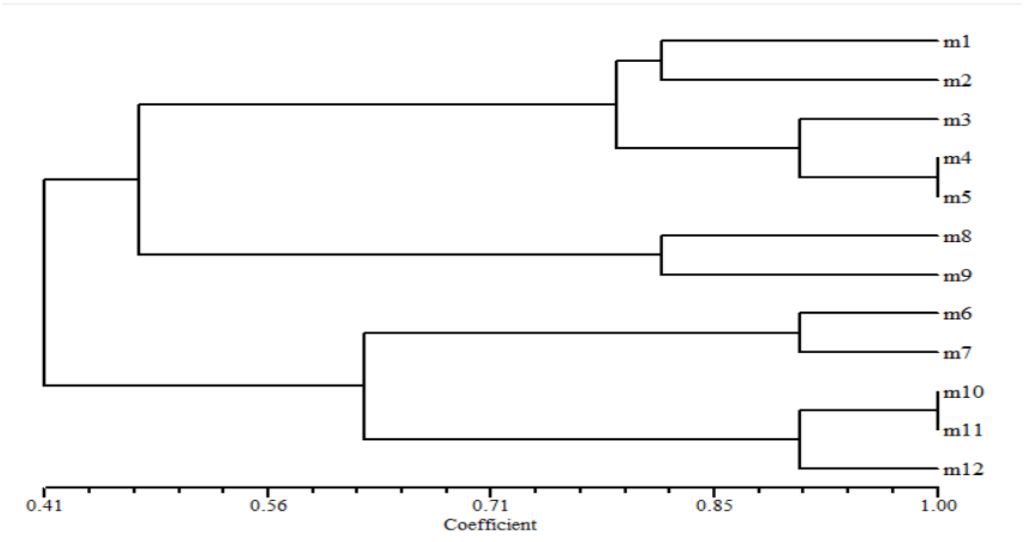

Fig. (6): Represent the clustering of used *Morus* samples according the data of SRAP marker.

Conclusion

The use of DNA barcoding, PCR-RFLP, and SRAP markers for Mours species in Duhok Province provides important tools for analyzing the genetic diversity, population structure, and evolutionary connections of these extraordinary plants. The use of the ITS region and matK markers to identify *Morus* cultivars in this study highlights the utility of these markers in plant genetic investigations. The information generated from these marker approaches may be used to develop effective conservation strategies and management plans for Mours species in Duhok Province.

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Conflicts of interest

The author declare that they have no conflict of interests.

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التعريف الجزيئي لـ (*Morus* ssp.) في دهوك باستخدام المواقع الجينية ITS region وجين الكلوروبلاستmatK .

دلال يوسف خضر سنجاري

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المستخلص: نظراً لأن التوت (*Morus*) هو نوع من الأشجار يحتوي على تنوع نباتي كبير. التقنيات الجزيئية هي طرق تستخدم للتمييز بين الأنواع بدقة وسهولة وسرعة. تتناول هذه الدراسة الطريقة الجزيئية لتمييز أنواع التوت المختلفة في دهوك– إقليم كردستان– العراق. تعتمد الطريقة على استخدام أربع تقنيات: جين matK، ومنطقة ITS، وPCR–RFLP، وعلامات SRAP. تم اختيار اثني عشر نوعا من التوت لهذه الدراسة من مناطق مختلفة من دهوك. وكانت نتيجة PCR لمنطقة ITS 700 زوج قاعدة أساس، ولكن إنتاج PCR لجين matK كان 900 زوج قاعدة أساس. تم العثور على نفس موقع التقييد لجميع الأصناف المستخدمة عندما تم استخدام 700 زوج قاعدة أساس من جزء ITS لـ PCR–RFLP مع اثنين من إنزيمات التقييد، RsaI GT/AC و HaeIII GG/CC. استخدمت هذه الدراسة أيضًا ست مجموعات من علامات SRAP للمساعدة في تجميع وتحديد أوجه التشابه الجيني. أظهرت نتائج PCR–RFLP وجود صلة غير كافية بين المظهر المورفلوجي والصفات الوراثية، ولكن يمكن تحديد الاختلافات بين الأصناف المدروسة باستخدام علامات SRAP. علاوة على ذلك، أظهرت هذه الدراسة إمكانية ترميز الحمض النووي لأصناف التوت *Morus*، بالإضافة إلى تحليل التسلسل الإضافي وتحديد SNP المحتمل بين الأصناف.

الكلمات المفتاحية: ترميز DNA الشريطي، منطقة ITS، التوت، جين matK، PCR– RFLP، علامات SRAP.