Anatomical and Molecular Study of *Capsicum* L. Taxa (Solanaceae Family) Cultivated in Iraq

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ABSTRACT

Background: The anatomy of the stem and leaves is important to separate and identify species. The present work includes comparative anatomical and molecular studies of six taxa belonging to the genus *Capsicum* L. which are grown widely in Iraq. Anatomical and phylogenetic traits were observed for the identification of different taxa. **Methods**: For the anatomical study, stem, leaves and petioles of leaves were microscopically investigated, while for phylogenetics traits DNA was extracted from fresh and young leaves from each sample using RAPD and ISSR indices. **Results**: The anatomical results indicate that all taxa have anomocytic type of stomata. The glandular trichomes were found in the taxa *C. frutescens* and the taxa *C. annum var.kwari gochu* and *C. annum* was free from it, and these characteristics were important in the classification of the taxa of this genus. Phylogenetic results showed that 10 decamers of primers from 20 observed primers had diversity in cultivars of the genus *Capsicum* L. The genetic set of 10 RAPD primers and 8 Inter Simple Sequence Repeats (ISSR), and the pooled RAPD and ISSR data analyses support a genetic similarity range of 0.54, and 0.5 sequentially, and the taxa *C. frutescens* formed a single cluster in both molecular analyses in the genetic tree. The phylogenetic tree results also showed that other *Capsicum* cultivars are divided into two subgroups, one of which refers to the same variety *C. annuum var. annuum*, and it was among the most important taxa studied, as it showed that it has two stages of maturity, physiological and vegetative maturity, so it appears morphologically different, but genetically integrated with the same taxa (*Capsicum*). **Conclusion**: The anatomical and molecular study of *Capsicum* taxa cultivated in Iraq provides valuable insights into the diversity and relationships between different species of this taxa.

Keywords: Phylogeny, Anatomical study, *Capsicum* taxa, RAPD, ISSR.

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1-INTRODUCTION

Solanaceae (nightshade) is a large flowering plant family that includes about 2,300 species (1) and contains many important cultivated plants such as *Capsicum*. The genus *Capsicum* contains approximately 25 wild species with five cultivated species and taxa (2). *The C. annuum plant variety produces both spicy (chili or hot pepper) and mild (sweet pepper) fruits, which are widely utilized as spices, condiments, and vegetables.* These species are widely cultivated worldwide and only the Asian continent accounted for more than 70% of its total production during the year 2021 (3). India is the biggest producer accounting for more than 43 % of the world's total dry chili fruits production (3). Iraq produced 58781 tons of *Capsicum* and *Pimenta* in an area of 5391 ha during the year 2021 (3). Molecular and anatomical studies have helped to elucidate the taxonomic relationships among the various Capsicum taxa. The genetic diversity of *Capsicum* has been previously analyzed using several molecular techniques worldwide. Lefebvre *et al.* (4) worked on genetic diversity analysis among *Capsicum* genus using restriction

fragment length polymorphism (RFLP) technique. Adetula (5) studied random amplified polymorphic DNA (RAPD), while the amplified fragment length polymorphism (AFLP) technique was first used on *capsicum* by (6). Microsatellites or simple sequence repeats (SSRs) (7-8) and direct amplification of minisatellite DNA (DAMD-PCR) by (9-10) was also used for genetic diversity analysis among *Capsicum* genus. (11) Studied the slight Intra and interspecific biochemical diversity and detected six taxa of *C. frutescens* and *C. annuum*. Inter simple sequence repeat (ISSR) requires a very small amount of template and is convenient in result recording and highly reproducible (12). These studies have revealed several features that are useful in distinguishing between different taxa, such as the shape and size of the flowers and the number and arrangement of seeds within the fruit. Additionally, anatomical studies have helped to clarify the evolution of certain traits within the genus, such as the development of fleshy fruit walls in certain species.

Due to the selection processes in the *Capsicum* genus, varieties rise with new morphological characteristics (13), and have been seldom genetic diversity for proper understanding. This led to a very complex taxonomy of this genus. The great role of right taxon identification can be exemplified by the knowledge of the anatomical and morphological characteristics that are essential for studies of intergrading between plants and herbivores and other natural enemies (14).

In general, the integration of diagnostic anatomical characters associated with genetic fingerprints is normal to identify the characterization and evaluation of domesticated taxa, particularly interesting for gene bank curators (9, 15).

The main objective of this study is to show that the application of anatomical features along with the molecular study can be confirmed to be of great assistance in explaining problems related to classification. Thus, the requirement of including the results from the stem cross-section, leaf epidermis, and cross-section with data derived from the molecular study is very beneficial when evolving conclusions on the systematic of the *Capsicum* taxa.

2- MATERIAL AND METHODS

Plant material

Young samples of *Capsicum* cultivated taxa (stem, leaves and, petioles of leaves) were collected in 2019 from different geographical regions of Iraq. The collected taxa include *C. annum var. annum, C. annum var. kkwari-gochu, C. frutescens, C. annuum var. bola* and *C. annum*. The specimens were identified in the National Herbarium of Iraq (BAG) and Herbarium of the College of Education, University of Baghdad (BUE).

Microscopically Investigation

The epidermis of the leaf is prepared by following the method proposed by (16). At the first clearing of the epidermis of the leaf with distilled water, put in the 0.5% sodium hypochlorite for 10 minutes to remove the chlorophyll pigments. After cleaning, leaf epidermis was then put in ethanol alcohol for 10-15 minutes, finally, the samples were put on the slides and covered by cover slides then fixed by an Olympus KRÜSS light microscope then photographed using an Olympus Am scope camera.

For using the cross-sections of stem, leaf, and petiole, the fresh samples of it are kept in formalin acetic acid (FAA) which was prepared according to (17) for 24-48 hours and then preserved in 70% alcohol until the date of experiments.

The sectioning parts of the stem, leaf, and petiole by hand section, sectioned by a razor blade into thin and small pieces (4-6 cm) then putting in 0.5% of sodium hypochlorite for five min to clear the tissue and remove the chlorophyll pigment, then putting in the dish that contains the samples of plants a drop of 1% Safranin mixed with ethanol 70% for 30-45 min, finally putting the samples on the slide and mounted by cover slides and fixed by Olympus KRÜSS light microscope then photographed using AmScope camera.

DNA extraction:

Genomic DNA extraction was done according to the modified cetylrimethyl ammoniumbromide (CTAB) method of (18) and (19). The DNA samples were stained with 0.5 mg/ml ethidium bromide before being electrophoresed in 1% agarose gel. A total of 18 different primers (10 RAPD and 8 ISSR primers) were tested in this study (Table 1 and 2). The primers were supplied by Bioneer Company and screened. The master amplification reaction exists in (table 3). The Polymerase chain reaction (PCR) was initiated with a hot start method by using the single strand cDNA template on Labnet Thermocycler (USA). The PCR reaction was carried out according to the program of 40 amplification cycles (95°C for 1 min, 43.7°C for 1 min and 72°C for 1 min). The generated bands were compared.

Screening of PCR:

A total of 10 RAPD primers and 8 ISSR primers were experienced in this study (table 1, and 2) which were supplied and screened by Bioneer company. Ten primers that had previously been shown indicated results of band patterns, the master amplification Reaction existing in (table 3), the PCR was initiated with a hot start technique by using the single strand cDNA template on Labnet Thermocycler (USA). The PCR reaction was carried out according to the program of 40 amplification cycles (95°C for 1 min, 43.7°C for 1 min, and 72°C for 1 min). gel electrophoresis (Agarose 1%) is used for the analysis of PCR products for 60 minutes. The generated bands were compared, and the differential amplified bands were based on the presence or lack recorded as 1 or 0 of a band, within a size ranging between 150-1350 base pairs (bp).

Table 1. The sequences of RAPD primers used in this study

Primers	Primer ID	5' – 3' Sequences	Primers	Primer ID	5' – 3' Sequences
1	OPA-02	TGCCGAGCTG	11	OPD-08	GTGTGCCCCA
2	OPA-04	AATCGGGCTG	12	OPE-02	GGTGCGGGAA
3	OPA-06	GGTCCCTGAC	13	OPG-19	GTCAGGGCAA
4	OPA-08	GTGACGTAGG	14	OPJ -17	ACGCCAGTTC
5	OPA-09	GGGTAAGGCC	15	OPL-19	GAGTGGTGAC
6	OPC-08	TGGACCGGTG	16	OPN-15	CAGCGACTGT
7	OPC-09	CTCACCGTCC	17	OPP-09	GTGGTCCGCA
8	OPC-12	TGTCATCCCC	18	OPP-10	TCCCGCCTAC
9	OPD-02	GGACCCAACC	19	OPS-19	GAGTCAGCAG
10	OPD-06	ACCTGAACGG	20	UBC 1	CCTGGGCTTC

Table 2. Sequences of the ISSR primers used in this study.

Primers	Primer ID	5' – 3' Sequences
1	1M-1.01	ACACACACACACACACCG
2	1M-11.01	GGGGTGGGGTGGGGTG
3	1M-3.01	CTCTCTCTCTCTCTCTA
4	1M-5.01	TCTCTCTCTCTCTCTCA
5	1M-6.01	CACCACCACCACCAC
6	1M-7.01	CAGCAGCAGCAGCAG
7	AD2.01	AGCAGCAGCAGCAGCAGCG
8	AD6.01	GTCACCACCACCACCACCACCCAC

Table 3. The master amplification reaction

Materials	Final concentration	Volume for 1 tube
PCR pre mix	1x	5 µl
Deionized D. W	——	11 µl
Primer	(10 pmol/ µl) 10 pmol /µl	2 µl
DNA template	100 mg	2 µl

Data analysis:

The matrix's data were anticipated to calculate the genetic similarity within taxa based on Jaccard's similarity coefficients, and used to analyze the RAPD and ISSR matrix in the NTSYS-pc statistical package version 2.1. A dendrogram displaying relationships among the 6 genotypes was structured with the Unweighted Pair Group Method (UPGMA) along with Arithmetic Mean.

3-RESULTS

Anatomical study:

The study shows that the outline of the stem cross-section of the taxa is square in shape but has some differences among the taxa *C. annuum* var. *annuum*, *C. annuum* var. *bola* and *C. annum* have rounded edge from the end of it full by collenchyma tissue. The epidermis in the taxa consists of one layer covered by cuticle and the taxa *C. annuum* var. *annuum* and *C. annuum* var. *bola* have uniglandular trichomes multicellular and uniseriate, and the taxa *C. frutescens* have glandular trichomes and the taxa *C. annum* var. *kkwari-gochu* and *C. annum* free from the trichomes. The cortex followed by the epidermis consists of two types of tissue, the collenchyma tissue located under the epidermis consists of 2-3 layers and the parenchyma tissue consists of ordinary parenchyma cells that have schizogenous intercellular space among the cells, and the vascular bundles it takes the form of a quad continuous loop, located after the cortex consists from xylem and phloem, the pit position in the center consist from ordinary parenchyma tissue and have a cavity in the taxas such as *C. annuum* var. *annuum*, *Capsicum annuum* var. *bola* and *Capsicum frutescens* (Figure 1 and 2).

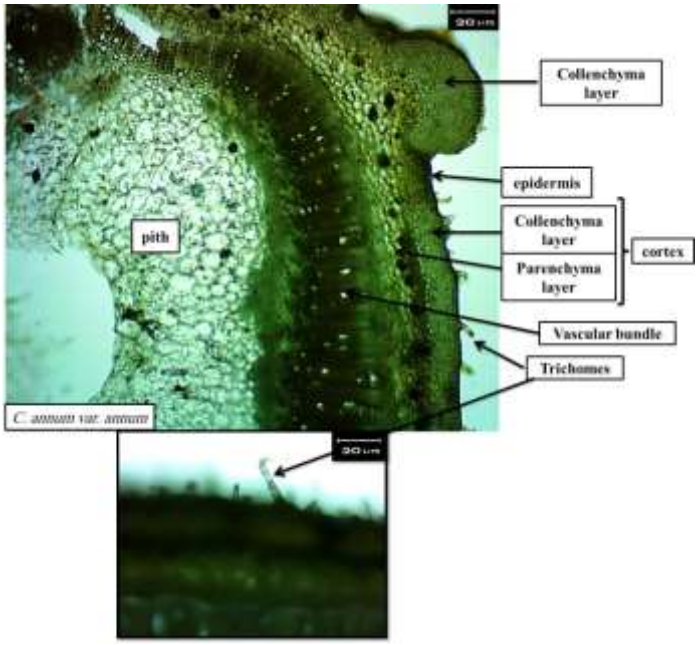

Figure (1): Cross section of the stem in the taxa *C. annum var. annum.*

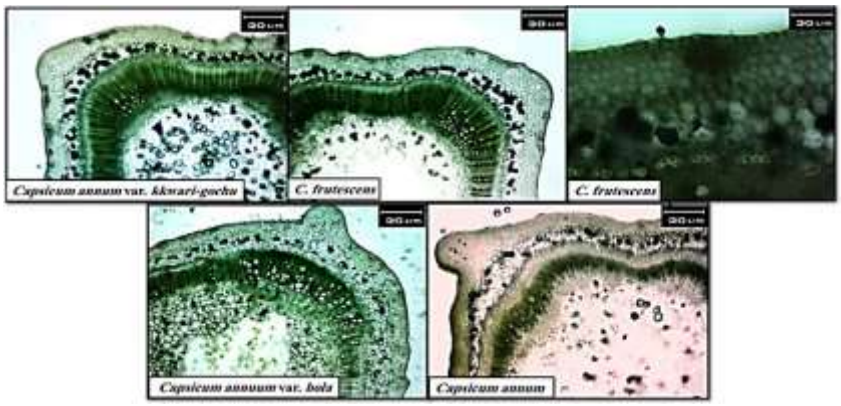

Figure (2): Cross section of stem in the taxa *Capsicum.*

The study also indicates that the leaves anatomy and the shape of the epidermal cells of the adaxial and abaxial surfaces was irregular and sinuous, and amphistomatic that's means having stomata at both the adaxial and abaxial surface of the leaf in the studied taxa of *Capsicum* (Figure 3 and 4).

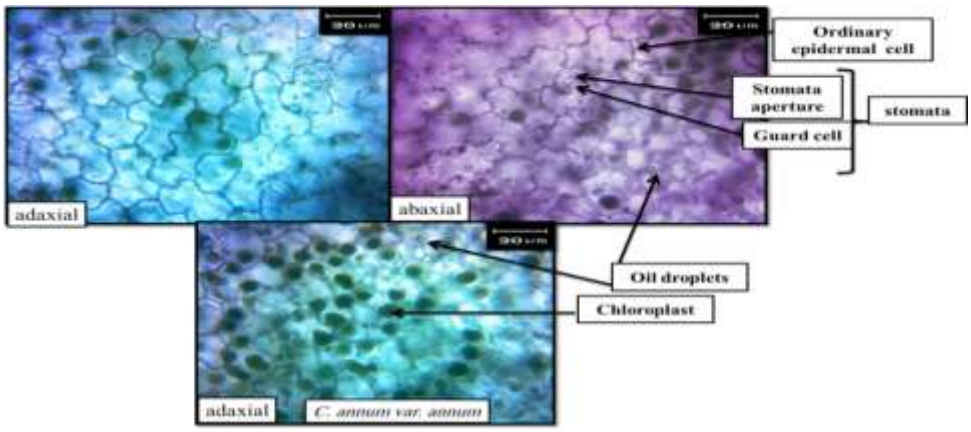

Figure (3): Epidermis and stomata of leaves in the taxa *C. annum var. annum.*

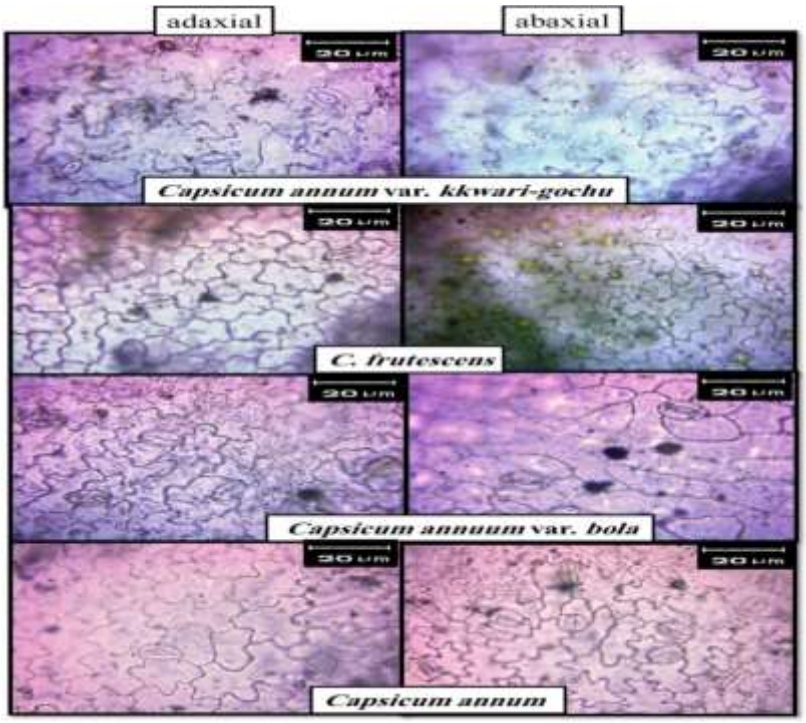

Figure (4): Epidermis and stomata of leaves in the *Capsicum* taxa.

The cross-section of the leaf blade in the taxa consist of the upper and lower epidermis, each of them consisting of one layer of ovoid cells and the mesophyll that consists two layer of palisade cell on the upper side followed by spongy layers (Figure 5). The midrib of leaf in the taxa takes different shapes among the studied taxa. The taxa *C. annum* var. *annum* has heart apex, the taxa *C. annum* var. *kkwari-gochu* and *C. annuum* var. *bola* have rounded apex while the taxa *C. frutescens* and *C. annum* have broad apex. The cross-section of midrib in the taxa consist from one layer ovoid cells of the epidermis and can investigate many unglandular trichomes diffuse in the epidermis of the taxa *C. annum* var. *annum*, *C. annum* var. *kkwari-gochu*, *C. annuum* var. *bola* and *Capsicum annum*, and the taxa *C. annum* var. *kkwari-gochu* and *C. frutescens* have glandular trichomes, cortex and vascular bundle located in the center of midrib takes crescent shape in all studied taxa, and consist of xylem and phloem tissues (Figure 6).

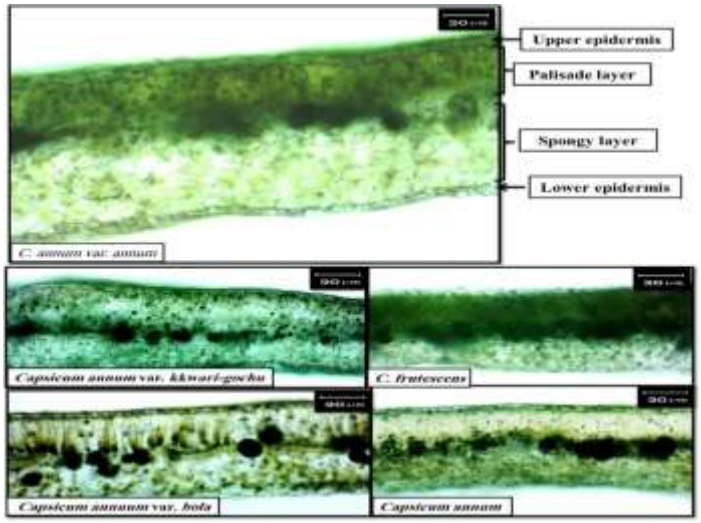

Figure (5): Cross section in the blade of leaf in the *Capsicum* taxa.

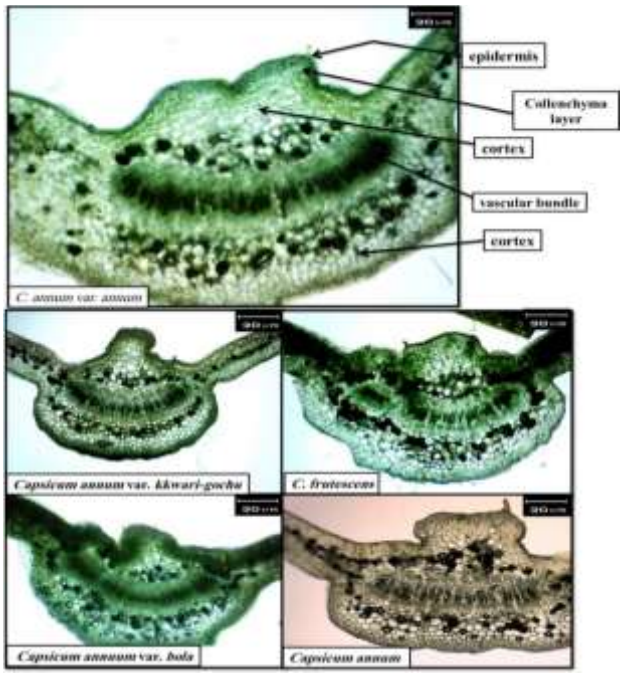

Figure (6): Cross section in the midrib of leaf in the *Capsicum* taxa.

The petiole cross-section also differs in shape among the studied taxa (Figure 7). The taxa *C. annum* var. *annum* takes a crescent shape with two long wings on the sides while the taxa *C. frutescens* take a crescent shape with two short wings on the sides and the remaining taxa have a crescent shape without wings. The cross-section of petioles consists of one layer of the epidermis, and taxa *C. annuum* var. *bola* only have unglandular trichomes in the epidermis of it and the remaining taxa are free of it. The cortex consisting of collenchyma tissue under the epidermis and ordinary parenchyma cells after it, and the vascular bundle located in the center of the petioles crescent shape in all studied taxa (Figure 7).

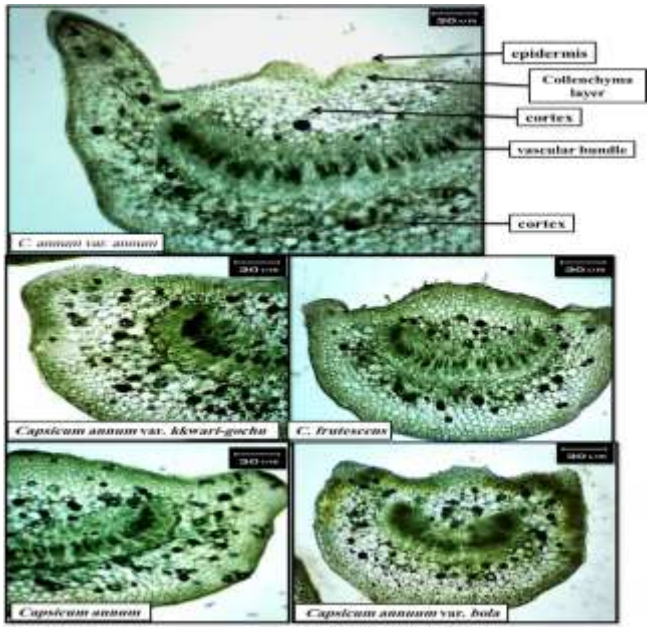

Figure (7): A cross section in the petiole of leaf in the *Capsicum* taxa.

Molecular study:
RAPD results

Amplification products of six genotypes with 10 primers yielded a total of 59 scoreable bands among which 49 were polymorphic (Table 4). The size of the amplification products ranged from 130 to 1350 bps. Primer OPA-06 and OPD -02 give the highest number of bands (8), while the lowest numbers (4) were obtained with primer OPD-09 and UBC-01 (Figures 8 and 9). Five primers cleared contraindications in their ability to produce unique product polymorphism with no monomorphism. Similarity matrices showed that taxa under study were divided into 2 groups, I. *C. frutescens* cultivate isolated from other cultivate with 0.54, while the II. Divided into 2 clusters with distinct separation about 0.42, A1: including 3 taxa, 2 of them showed the genetic analysis have same genotypes but the phenotype was different in the color of fruit (green and red) were *C. annuum* var. *annuum*, this verity separated from *C. annum* .var. *kkwari-gochu* with 0.27 similarity, A2 cluster contains 2 cultivate split up with 0.29 similarity.

Table (4). Amplified band numbers and size range of each primer of the RAPD method.

Primer name	Size range of bands(bp)	*AN	**PM	%	***MM	%
OPA-06	1200-150	8	8	100	0	0
OPA-08	800-200	6	6	100	0	0
OPA-09	1300-250	5	3	60	2	40
OPD-02	1250-130	8	8	100	0	0
OPD-06	1000-200	7	6	85.7	1	14.3
OPD-08	900-200	5	2	40	3	60
OPD-09	1350-400	4	4	100	0	0
OPP-09	1100-250	7	6	85.7	1	14.3
OPP -10	1200-150	5	2	40	3	60
UBC-01	1300-550	4	4	100	0	0
		59	49		10	

***AN = alleles number; **PM = Polymorphic bands; ***MM = Monomorphic bands.**

%PM= PM/Anx100% MM= MM/AN x100.

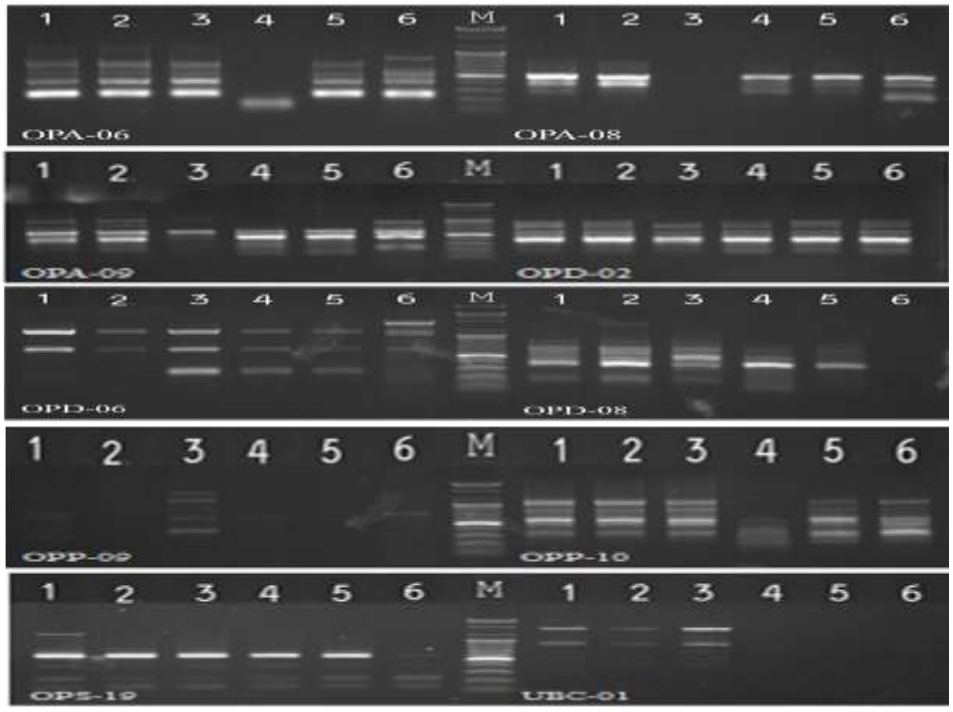

Figure (8): Profile of DNA of 6 taxa of genus *Capsicum* (molecular weight marker 1500-bp DNA Ladder).

***1, 2= C. annum* var. *annum, 3=C. annum* var. *kkwari-gochu,4= C. frutescens, 5= C. annuum* var. *bola* and *6=C. annum*.**

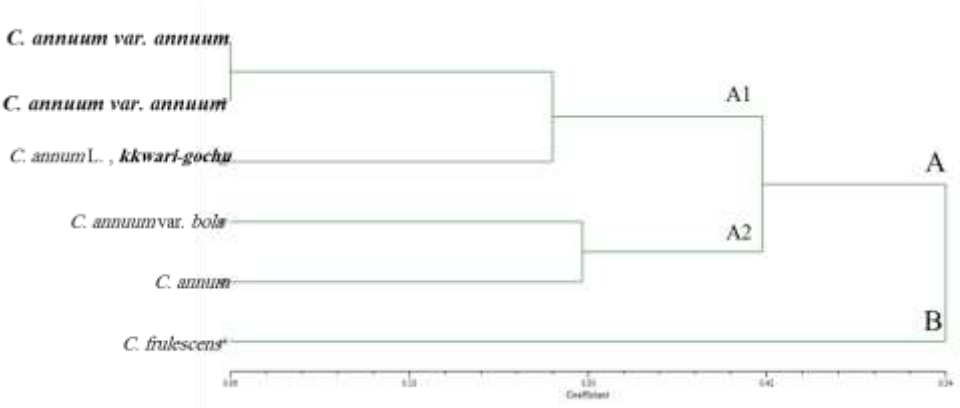

Figure (9): Dendrogram of genetic variety among *Capsicum* taxa by RAPD technique.

ISSR results

A total of 46 scorable bands were generated from 8 ISSR primers ranging from 250- 1200 bp that which 37 (80.4%) were polymorphic (Figures 10 and 11). The highest diversity was obtained in AD 6.01 was 10 alleles number, but 1M-3.01 primer had the lowest allele number (3). Three primers showed variation in their capacity to produce unique product polymorphism with no monomorphism (Table 5). The clustering showed in figure 10 and Dendrogram of genetic distances amongst all tested genotypes showed two distinct major clusters *C. frutescens* isolated from other cultivate with 0.5

Table (5): Amplified band numbers and size range of each primer of ISSR method.

Primer name	Size range of bands(bp)	*AN	**PM	%	***MM	%
1M-1.01	1100-250	5	3	60	2	40
1M-11.01	1000-300	6	4	66.7	2	33.3
1M-3.01	1100-300	3	3	100	-	0
1M-5.01	900-300	6	5	83.3	1	16.7
1M-6.01	1150-500	5	5	100	-	0
1M-7.01	1000-350	6	6	100	-	0
AD2.01	1100-400	5	2	40	3	60
AD6.01	1200-300	10	9	90	1	10
		46	37		9	

***AN = Alleles number; **PM = Polymorphic bands; ***MM = Monomorphic bands.**

%PM= PM/ANx100, % MM= MM/AN x100.

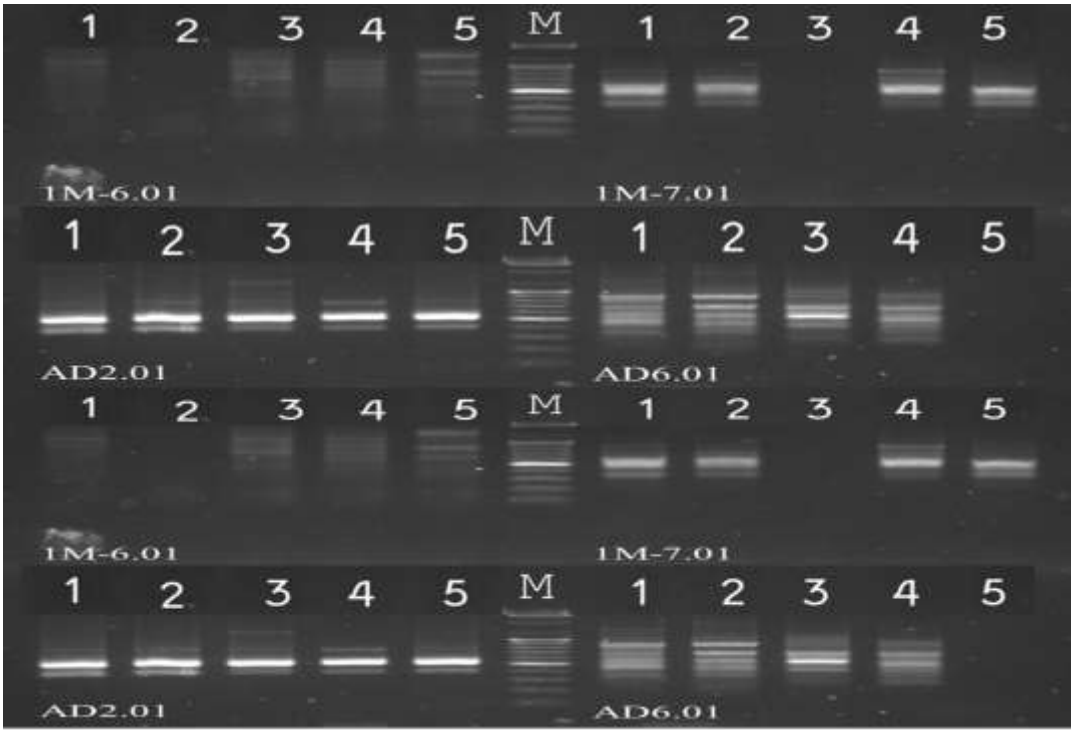

Figure (10): Profile of DNA of 6 taxa of genus *Capsicum* (molecular weight marker 1500-bp DNA Ladder.

1, 2= *C. annum var. annum,* 3=*C. annum* var. *kkwari-gochu,*4= *C. frutescens,* 5= *C. annuum* var. *bola* and 6=*C. annum*.

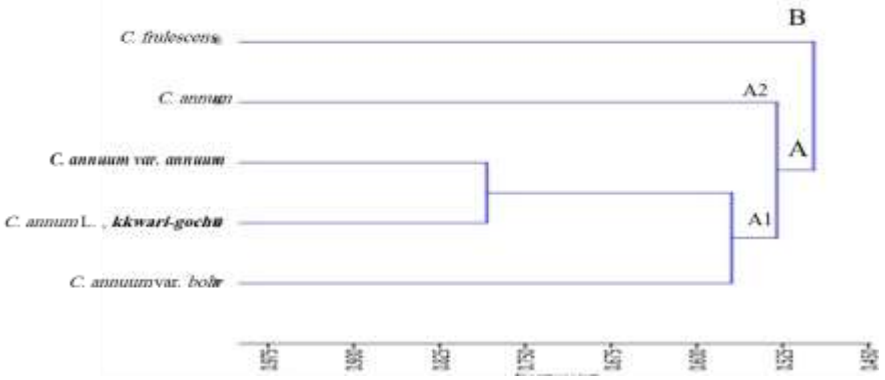

Figure (11): Genetic relationships Dendrogram among taxa of pepper by ISSR technique.

4-DISSCUSION

The anatomical features are one of the important characteristics, which help in differentiating the taxa (20). The anatomical feature can be isolated from the taxa from each other, and the result of the anatomical of stem agrees with (21). The type of stomatal complex in the *Capsicum* taxa was anomocytic, the indicates that the taxa are phylogenetically related (22), and previously investigated as many chloroplasts and oil droplets diffuse in the adaxial epidermis (Figure 3 and 4), the result agrees with (23).

The results of the cross-section in the blade of a leaf in the taxa *Capsicum* and cross-section in the midrib of a leaf in the taxa *Capsicum* agrees with (21) and (24). The petiole cross-section results were similar to the results of (25) and (20).

At first sight, RAPD results, and findings disagree with the researchers (26) and (27) who reported that *C. frutescens* as the closest taxa of *C. annuum*. (8) Also mentioned a partially closer connection between *C. annuum* and *C. frutescens* and were domesticated independently but closer area relatively. The results of (28) showed considering the tree two groups can be identified: 1 mainly grouping Mexican consisting of four chiltepíns and another containing mostly *C. annuum*. Hence indicating a possible link between those taxa and a common predecessor (29, 26).

ISSR results outcome confirms with the observation of (30) the two sub-clusters of *C. annum* were separated from other taxa in 0.52 but have the closer relationship between *C. annuum* var. *annuum*, and *C. annum* var. *kkwari-gochu* was 0.78. This finding was in accordance with those of (10) and (12) who detected that the genetic diversity among five peppers species was basically inter-specifically rather than intra-specifically. The converging could point to gene exchange between *C. annum* var. *annum*, *C. annum* var. *kkwari-gochu* and *C. annuum* var. *bola*. This theory is supported by (31) because the fertile hybrids were obtained between the taxa. Hence, RAPD and ISSR techniques act as stellar tools to know the genetic variety, map the genome, selection of parental lines for crossings (11) and the data from the molecular study to understand the existing genetic diversity or variation at the molecular level is the very first thing that should be studied. Overall, this study demonstrates the importance of both anatomical and molecular approaches in understanding the taxonomy and evolution of *Capsicum* taxa in Iraq. By combining these different approaches, researchers can gain a more comprehensive understanding of the relationships among different species and the factors that have major roles in their evolution.

5-CONCLUSION

The results obtained from this study can be used as a phylogenetic tool for a better understanding of the systematic study of *Capsicum* genus. Also, the integration between more than one aspect of studies provides more precise information that can serve as a genetic base and an understanding of genera to solve taxonomic problems.

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Ethics Approval and Consent to Participate

Only plant samples were used to carry out this research. No humans and/or animals participated in it.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding this work.

Availability of Data and Material

All data and materials mentioned in the manuscript contain all relevant raw data and it will be freely available to any researcher who uses it for non-commercial purposes.

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دراسة تشريحية وجزيئية لاصناف الفلفل (العائلة الباذنجانية) المزروعة في العراق

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الخلاصة

خلفية البحث: يتضمن العمل الحالي دراسات تشريحية وجزيئية مقارنة لستة أصناف تنتمي إلى جنس الفليفلة .Capsicum L والتي تزرع على نطاق واسع في العراق، **طرق العمل:** تناولت الدراسة التشريحية تشريح الساق والأوراق التي تم استخدامها لفصل وتحديد الأنواع.**النتائج:** تشير النتائج التشريحية إلى أن جميع الأصناف تتكون من ثغور من النوع الشاذ Anomocytic type، وبالنسبة للشعيرات الغدية glandular trichomes، وجدت في الصنف *C. frutescens* وكانت الأصناف *C. annum var.* kwari-gochu و *C. annum* خالية منها، وهذه الصفات مهمة في تصنيف الأصناف لهذا الجنس، النتائج التطورية للحمض النووي المستخرج من الأوراق الحديثة النمو و الطازجة من كل عينة باستخدام مؤشرات RAPD و ISSR، اذ تم اختبار 20 بادئة لدراسة مؤشرات التضاعف العشوائي متعدد الاشكال RAPD، وتبين ان 10 decamers من البادئات وجود تنوع في اصناف الجنس Capsicum L. وان تحليلات المجموعة الجينية لـ 10 من بادئات RAPD و 8 تكرار التسلسل البسيط (ISSR) ، وتحليلات بيانات RAPD و ISSR المجمعة تدعم نطاق التشابه الجيني 0.54 ، 0.5 بالتتابع ، وشكل الصنف *C. frutescens* مجموعة واحدة في كلا التحليلين الجزيئيين في الشجرة الوراثية. كما تظهر نتائج الشجرة الوراثية أن أصناف الفليفلة الأخرى تنقسم إلى مجموعتين فرعيتين، أحداهما تشير الى نفس الصنف *C. ann*uum var. annuum. وكان من بين اهم الأصناف المدروسة، اذ اظهر أن له مرحلتان من النضج، النضج الفسيولوجي والنضج الخضري، لذلك يبدو مختلفًا شكليًا ولكنه متكامل وراثيًا.**الاستنتاج:** ان الدراسات التشريحية والجزيئية للاصناف التي تنتمي إلى جنس الفليفلة .Capsicum L والتي تزرع على نطاق واسع في العراق توفر قيمة عالية حول التقارب الوراثي بين اصناف الفليفلة المختلفة.

الكلمات المفتاحية: دراسة تشريحية، اصناف الفلفل Capsicum ، RAPD، ISSR.