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TAXONOMIC STUDY OF SOME *EUPHORBIA* L. SPECIES BY LEAF ANATOMICAL AND MOLECULAR CHARACTERISTICS USING *RBCL* AND *MATK* GENES

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SUMMARY

A current study assessed the leaf anatomical and molecular characteristics of eight species of the genus *Euphorbia* (Euphorbiaceae), i.e., *E. craspedia* Boiss, *E. denticulate* Lam., *E. falcate* L., *E. hirta* L., *E. helioscopia* L., *E. peplus* L., *E. kansuensis* L., and *E. macroclada* Boiss ,collected from several districts of the Middle and Northern Iraq. The anatomical result refers to a uniseriate epidermis (single layer), except for the species *E. kansuensis and E. peplus,* wherein the upper epidermis recorded a variable thickness among the species. The highest epidermis thickness (28 µm) was visible in the species *E. falcata,* while the mean decreased to 17 µm in the species *E. helioscopi*a. Based on the anatomical attributes, further species groupings resulted in two. In the first group, the leaf's cross-section was unifacial, with the palisade tissues on both sides of the blade. This group included *E. hirta*, *E. peplus, E. macroclada?,* and *E. denticulata*. Contrastingly, the second group has a bifacial leaf, with the mesophyll distinguished as palisade and spongy. The said group comprised the *E. craspedia* and *E. helioscopia species.* In the genus *Euphorbia* species, the study of the genetic relationship continued according to the sequencing method of *rbcl* and *matk* genes. The results revealed that most species samples showed light bands, characterized by their intensity ranging from 800 to 1000 bp. The highest recorded genetic affinity through the *rbcl* gene emerged in *E. denticulata* and *E. kansuensis*, whereas the lowest was in *E. macroclada and E. hirta*. Based on *matk* gene results, the highest genetic affinity observed resulted in the species *E. kansuensis* and *E. denticulata* with an average value of 0.0160, with the lowest recorded in the species *E. helioscopia and E. peplus* with a value of 0.1307.

Keywords: *Euphorbia*, taxonomy, leaf anatomical traits, *rbcl* and *matk* genes sequencing

Key findings: Taxonomic Study, including molecular and anatomy of leaves and anatomical characteristics' molecular diagnosis, used the *rbcl* and *matk* genes to distinguish the eight species of the genus *Euphorbia* L. (Euphorbiaceae) that grows in the central and northern areas of Iraq. Based on anatomical and genetic similarities and differences, the study results classified the species into various groups.

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INTRODUCTION

Euphorbiaceae is the largest family of higher flowering plants, with 300 genera and 8000 species, belonging to the order Euphorbiales, one of the immense orders of angiosperms (Neeraj and Lal, 2019). The family, comprising herbs, shrubs, trees, and sometimes succulent, is characteristic of secreting milky juice (Acharya and Vaidya, 2017). It is also one of the most complex families due to species richness, and several taxonomists have attempted to solve taxonomic problems within the family Euphorbiaceae (Nichodemus and Ekeke, 2021). The family Euphorbiaceae also has five diverse tribes, i.e., Euphorbieae, Hippomaneae, Hureae, Pachystomaleae, and Stomatocalyeae. The genus *Euphorbia* belonged to the first tribe, and most of its species are poisonous, irritating to the skin, and contain rubber and milk (Willis, 1973; Thakur and Patil, 2012).

Euphorbia plants can grow well in temperate and tropical regions, adapt to various environments, and show wider differences in growth forms. These plants vary, from annual grasses to perennial trees that have a unique structure of their flowers, and it is a universal genus that has many species in different non-tropical regions, such as, Southern USA, South Africa, the Mediterranean basin, and the Central East (Zahra *et al*., 2014).

Anatomical traits are critically important in modern taxonomic studies, which often support phenotypic features. Taxonomists gave greater attention to such studies, expanding them to find more qualities that could help them better separate the species, genera, and even families (Aziz *et al*., 2016; Mohsin *et al*., 2023). Anatomical data has long been benefitting traditional classification because differences within species, genera, and families are usually a reflection of anatomical features.

Anatomical features of stems, leaf epidermis, stomata, and other traits are helpful in various studies. There has also been remarkable development in the past five decades on vascular plant anatomy and their uses in taxonomy (Ahmad *et al*., 2010). Findings of Metcalfe and Chalk (1957) revealed the anatomical features of *Euphorbia* found in a wide range of variations in terms of diversity of environments, and no single feature is notable in the various tribes into which the family split. The taxonomy science development also employed several morphological characteristics (Aloush, 2014).

In past studies investigating leaf epidermal features of 50 species of *Euphorbia,* their findings revealed that papillae of different shapes and sizes were evident in these cells, as well as, the different types of stomata and forms of epidermal cells also appeared (Kakkar and Paliwal, 1974). The study of the anatomical features represented by leaf epidermal cells, stomata complexes, and Anisocytic, Paracytic, and Anomocytic stomata complexes had taxonomic significance supporting the phenotypic characteristics in the diagnosis and isolation of the orders of genus *Euphorbia* (Sleibi, 2015). Talebi *et al*. (2017) conducted a study on the attributes of the leaf epidermis of 18 species of *Euphorbia* in Iran, and their results showed that the type of stomata was similar among all the species.

Determining the relationship between the species is crucial to obtaining better results in biological studies (Ji *et al*., 2021). The differences and similarities among the species are their genetic relationship evidence, which contributes to understanding the evolutionary relationship. Therefore, several molecular marker techniques have provided detailed information about the genomes, which was also impossible to obtain based on phenotypic classification methods. With the advancement of plant molecular biology in recent years, advances in genetic research have introduced various new marker methods, including targeted and functional genetic markers, which developed several innovative DNA-based marker systems (Sun *et al*., 2022).

Past genetic research revealed that the taxonomy of species of the family Euphorbiaceae, particularly of the genus *Euphorbia,* is very complex, showing several taxonomic variations compared with the traditional classification, which relied only on morphological parameters (Webster, 1994). Universal coding genes, such as, the plant DNA regions *matK* and *rbcL,* as well as, transcribed internodes, ITS and ITS2, are short strands of DNA that can help identify, discriminate, and assign taxonomy at the family, genera, and species levels (Staats *et al*., 2016). Therefore, based on the above discussion, the presented study sought to determine the taxonomic relationship among the *Euphorbia* species using the leaf anatomical characteristics and the genetic sequence of *rbcL* and *matK* genes.

MATERIALS AND METHODS

Genetic material and study location

The plant samples collection of the genus *Euphorbia* species occurred during the 2021–2022 season in several Districts of Middle and Northern Iraq. The species included were *E. craspedia, E. denticulata*, *E. falcata, E. hirta*, *E. helioscopia, E. peplus, E. kansuensis,* and *E. macroclada.*

Anatomical study

Mature leaves, collected from the plants' selected species of the genus *Euphorbia*, proceeded with the samples fixed in FAA (formalin-glacial acetic acid-alcohol) (5 ml [40%]:5 ml:90 ml [70%]). Preparing the cross-section of the leaves used the microtome, with the samples stained by safranin (1%) (Aloush, 2014). Anatomical observations performed employed a compound microscope model Novel.

Molecular analysis

DNA extraction

DNA isolated from the plant leaves of the studied genus *Euphorbia* species followed the process according to the CTAB method described by Weigant *et al.* (1993), obtaining an amount of 50–70 micrograms per 1 g of leaves for each species. The purity ranged between 1.7–2 MS, measured using a nanodrop device, and adjusting the DNA sample dilution to reach a concentration of 50 ng/microliter as the appropriate concentration for conducting PCR reactions. The large size of DNA molecules in plant cells made it difficult to obtain and purify the DNA without being destroyed. There are several ways to separate DNA; however, the method of Weigant and his group is the most appropriate, being characterized by ease and efficiency in the isolation process, as one of the difficulties researchers face in the DNA isolation from plant cells is the thick cell wall surrounded by the cell membrane.

Purifying the DNA from the rest of the cell components had the study employ chloroform: isoamyl alcohol (1:24) as an organic solvent that removes CTAB and keeps the DNA in the aqueous phase after centrifugation (Sambrook *et al*., 1989). Isomal alcohol works to form the foaming resulting from CTAB (Maniatis *et al.,* 2001) and to convert the DNA from the soluble state to the insoluble one, adding cooled isopropanol alcohol followed, which works to precipitate it (Clapp, 1996). Thus, the DNA appears in white dense blocks, then dissolved in a solution (TE) or deionized distilled water and kept at 20 °C temperature until use (Maniatis *et al*., 2001).

Gel electrophoresis

Genomic DNA samples electrophoreses ensued on a 1% agarose gel to ensure the presence and integrity of the DNA, then compared with the Marker's index by molecular weight (Sambrook *et al*., 1989).

Polymerase chain reaction

The primers used were from the Korean company BioNEER, as apparent in Table 1. We used the components of the Pre-Mix kit in the PCR. In this study, the volume of the PCR reaction was 20 microliters, with the elements

Table 1. Primers for amplification of *rbcl* and *matk* genes.

No.	Primer Name	Sequence 5′→→→ 3′	Size (bp)	Annealing
1	Rbcl	F) TGGGTTGCTAACTCAATGG R) ATTGAATGAATTGATCGTA	1200–1100 bp	52
2	Matk	F) GAAGTAGTAGGATTGATTCTC R) TACAGTTGTCCATGTACCAG	1048 bp	52

required to conduct this reaction supplied by BioNEER in 0.2 ml Eppendorf tubes.

DNA sequencing analysis

Determination of the nucleotide sequence of the amplified gene of the *matK* and *rbcL* proceeded in the direction of Forward immediately after obtaining the product of the gene duplication by sending 25 µl of the PCR product and 100 µl of each initiator at a concentration of 17.5 pmol from each initiator to the Korean company, Macrogen. Then, running the results through a computer program on the Internet (BLAST in situ basic search tool for nucleotide sequences) compared them against the database of the National Center for Biotechnology Information (NCBI), which inserts all the nucleotide sequences into the BLAST field to perform alignment, sequence comparison, and sequence matching. The genetic data of the plant samples for diagnosis and knowing their type and genus with the sequences in the previously known and diagnosed indexes helped further convert these sequences into a statistical application that expresses the existence of a significant positive correlation whenever the expected E-Value decreases. Using the MEGAX program processed the sequences received from the company to remove and trim the abnormal successions (Badgujar and Mahajan, 2013).

Genetic tree analysis

Sequence data analysis used the dendrogram based on the genetic dimension. The genetic relationship diagram, prepared based on the genetic dimension table, utilized the UPGMA method (The unweighted pair group method for the arithmetic average) and the MEGAX program (Fayed *et al*., 2020).

RESULTS AND DISCUSSION

Anatomical study

The results showed that in cross-sections of leaves, the upper and lower epidermis were uniseriate, except for the two species *E. kansuensis and E. peplus* that have the epidermis as biseriate on both surfaces. The upper epidermal cells were heterogeneous in shape and semispherical-spherical in the species *E. macroclada*, *E. falcata,* and *E. peplus.* However, the species with oval and epidermis covered with a layer of cuticle was at the highest level in *E. kansuensis* (8 µm), and species *E. macroclada, E. falcata, and E. craspedia* recorded the lowest mean value (4 µm). The cuticle thickness decreased in the lower epidermis compared with the upper, and the highest mean (5 µm) for that emerged in the species *E. kansuensis* and *E. denticulata.* The value decreased to 2 µm surfaced in the species *E. macroclada and E. craspedia*. The upper epidermis recording a variable thickness among the species had the highest mean (28 µm) observed in *E. falcata,* while it decreased to 17 µm in *E. kansuensis* and *E. helioscopia*. The maximum thickness of the lower epidermis was 20 µm, which lowered to 11 µm in the species *E. craspedia and E. kansuensis.* The variations in the tissue pattern among the studied *Euphorbia* species divided the species into several groups (Table 2, Figures 1 and 2).

In Group 1, the *Euphorbia* species were characteristic of being unifacial, with the palisade tissue located on both sides of the blade under the upper epidermis and above the lower epidermis. In between these two layers exists a spongy tissue whose cells were spherical-irregular and large, depicting the palisade layer as having more than one row in the five species, i.e., *E. hirta, E. peplus, E. macroclada, E. denticulata*, and *E. kansuensis.*

Table 2. Quantitative characteristics of the cross-section of leaf blades in *Euphorbia* species (μm).

Characteristics / Species		Cuticle thickness		Epidermis thickness		Number of rows/ Palisade	Palisade thickness	Spongy thickness	The Leaf thickness
		Upper	Lower	Upper	Lower				
1	*E. macroclada*	4	2	23	17	3-2	55	35	138
2	*E. hirta* L.	5	3	25	15	4-3	62	38	155
3	*E. falcate* L.	4	3	28	20	-	-	-	95
4	*E. craspedia*	4	2	18	11	4-3	60	40	139
5	*E. helioscopia*	5	3	25	20	5-4	65	35	158
6	*E. peplus* L.	7	4	27	13	4-3	65	43	162
7	*E. kansuensis*	5	3	19	13	4-3	67	48	179
8	*E. denticulate*	8	5	17	11	5-4	89	53	185

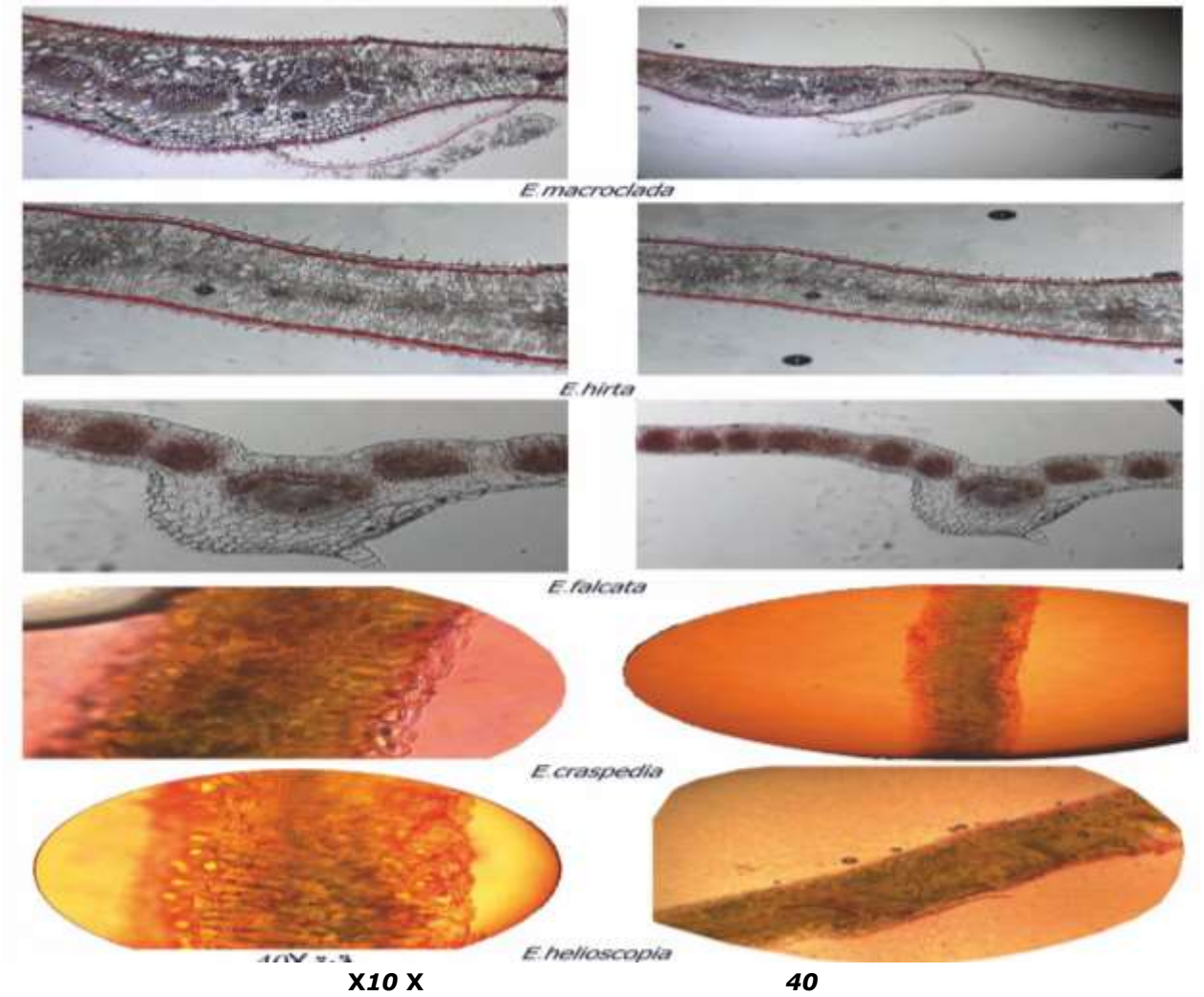

Figure 1. Cross-section of leaf blades in *Euphorbia* species.

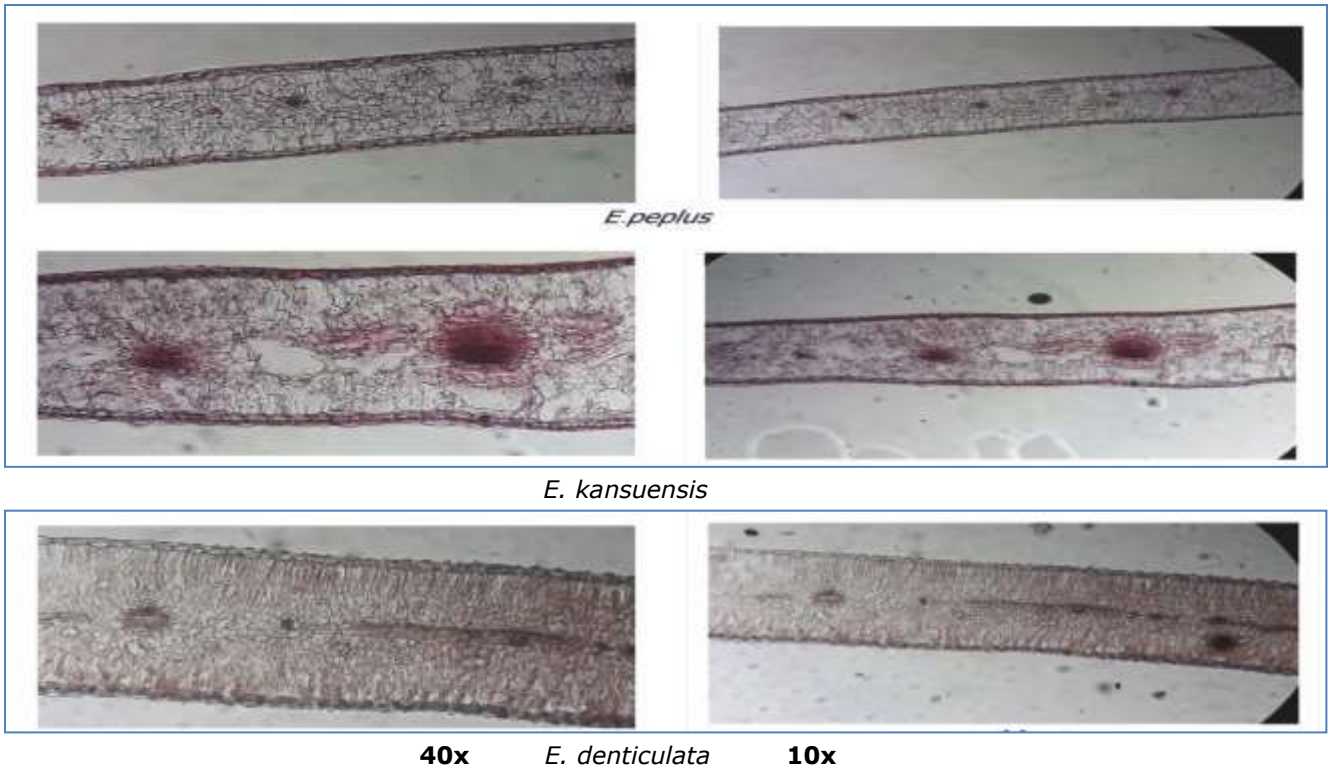

Figure 2. Cross section of leaf blades in *Euphorbia* species.

The results further revealed the presence of air holes in the leaf blade of the species *E. kansuensis,* with the rest of the *Euphorbia* species having more compact tissues (Figures 1 and 2). The Group-2 species illustrates bifacial (Dorsiventral) leaves, distinguishing the mesophyll into two tissues. The palisade layer formed below the upper epidermis had the spongy layer following it. This group comprised two species, viz., *E. craspedia* and *E. helioscopia.*

In Group 3, containing the species *E. falcata*, the tissues were not distinct and heterogeneous, and palisades had spongy tissue, with some milky glands distributed within the tissue in a rhombic to circular shape. The results further showed that the number of rows of palisade cells ranged between 4–5 in the two species, i.e., *E. kansuensis* and *E. denticulata,* while the number of rows decreased to 2–3 in *E. macroclada* and *E. denticulata* that excelled by registering the highest rate of palisade and spongy tissue thickness, which reached 98 µm and 61 µm, respectively. However, the rate decreased at the lowest level for both tissues at 55 µm for the palisade tissue and 35 µm for the spongy tissue in the species *E. macroclada*. The rest of the *Euphorbia* species ranged between these two values; however, no recording of the palisade cells in the species *E. falcata* occurred (Table 2, Figure 1).

Based on the total leaf thickness, *Euphorbia* species can also differ into several groups. The first group has an average leaf thickness of 95–140 µm and includes the three species *E. macroclada*, *E. craspedia,* and *E. falcata*. In the second group, average leaf thickness ranged between 140–185 µm and included the four species *E. hirta*, *E. helioscopia, E. peplus,* and *E. kansuensis.* The third group comprised *E. denticulata*, recording a leaf thickness of 215 µm. The concentration of large vascular bundles closed in the midrib region. In general, displaying diagonal rows and the bark forming a strip of several rows, it was in a crescent shape directed toward the lower epidermis. The number of vascular

bundles differed according to the species. In species *E. macroclada* and *E. falcata*, the number of bundles ranged from 4 to 8. It is worth mentioning that the two species, *E. macroclada* and *E. hirta,* were distinct by the presence of glandular hairs on both leaf surfaces.

The current results were consistent with past findings on the recorded variations in shapes of the epidermal cells of the leaf blades, their thickness and the number of rows, and the average thickness of the upper and lower epidermis cells varying between 18–126 µm in the two *Euphorbia* species *E. macrocarpa* and *E. peplus,* respectively (Sleibi, 2015). The average thickness of the epidermis ranged from 10.8 to 34.6 µm for the upper epidermis and 10.4 to 31.3 µm for the lower epidermis in species *E. prostrata* and *E. denticulata*, respectively. The average thickness of the palisade layer was 183.5 and 117.3 µm in the species *E. kansuensis* and *E. milii*, respectively, and was indistinct from the rest of the studied *Euphorbia* species. The thickness of the spongy layer ranged from 16.3–222.8 in the species *E*. *prostrata and E. denticulata*, respectively. The leaf blade thickness varied from 84.7 to 319.8 µm in *E*. *macrocarpa and E. kansuensis,* respectively.

Molecular study

The plant contains different amounts of compounds, such as, proteins, phenolic compounds, and polysaccharides, which become contaminants if they get mixed with the isolated nucleic acid, and this leads to the formation of a very viscous liquid, inhibiting PCR reactions (Do and Adams, 1991). The presence of polysaccharides also affects outcomes, especially those that depend on RAPD indicators (Pandey *et al*., 1996). Hence, making some modifications to obtain an appropriate amount of DNA and good purity to carry out the reactions, and also adding PVP to get rid of phenolic compounds transpired to easily remove them as they stick to the DNA, reducing its purity, affecting subsequent reactions (Porebski *et al*., 1997).

Manual crushing uses a ceramic mortar to break the plant cell walls while adding liquid nitrogen. The low temperatures (-196) stopped the action of released nuclear enzymes when the walls shattered, with the mixture then directly exposed to a temperature of 65 °C. In the presence of CTAB, which leads to the binding of CTAB with the DNA and the formation of a CTAB-Nucleic acid complex, it replaces the cleaved proteins, keeping the DNA in the aqueous phase, working to prevent it from sedimenting with other components during the subsequent rejection stages. The EDTA substance included in the extraction solution acts as a chelating agent that pulls magnesium ions necessary for the activity of nuclear enzymes that analyze nucleic acids, inhibiting the action of those enzymes (Liabata *et al*., 2019).

For purifying the DNA from the rest of the cell components, the use of chloroform: isoamyl alcohol (1:24) as an organic solvent aided in removing CTAB and keeping the DNA in the aqueous phase after centrifugation (Sambrook *et al*., 1989). Isomal alcohol works to form the foaming resulting from CTAB (Maniatis *et al.,* 2001) and to convert the DNA from the soluble state to the insoluble one attained addition of cooled isopropanol alcohol, which works to precipitate it (Clapp, 1996). Thus, the DNA appears in white dense blocks, then dissolved in a solution (TE) or deionized distilled water and kept at 20 °C temperature until use (Maniatis *et al*., 2001).

The molecular weight determination of the extracted DNA samples according to the studied primers *rbcL* and *matK* also continued. The results of the relevant study showed that most of the samples provided bands after being electrically transmitted, distinguishable by their intensity, and ranging between 800–1000 bp after comparing them with the DNA Lader (molecular marker) (Figure 3). The presence of bands with almost the same molecular size indicates that they belong to a single genetic origin, as it gives a clear picture of the strength of interdependence between the different tribes of identical origin (Aloush, 2014). The use of these specialized genes contributed to authenticating the interdependent relationship among the studied *Euphorbia* species.

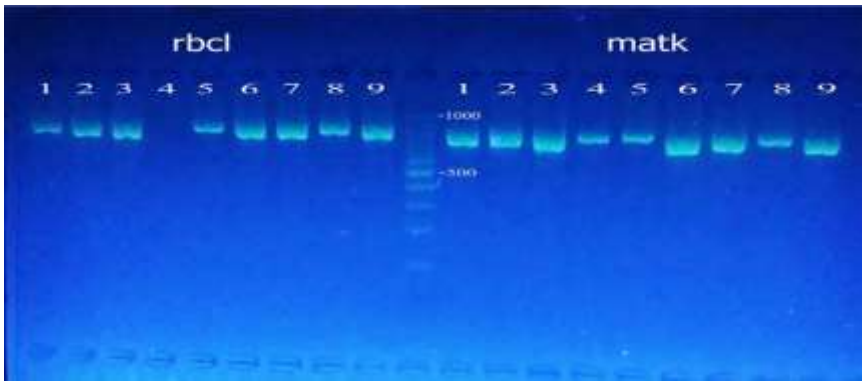

Figure 3. Electrophoresis of DNA extracted from samples of species belonging to the genus *Euphorbia* using *rbcL* and *matK* (*1-E. macroclada 2- E. hirta, 3- E. falcata, 4- E. craspedia, 5- E. helioscopia, 6- E. peplus, 7-E. kansuensis,* and *9-E. denticulata*). Note: The species' 8 subspecies was not included in this study

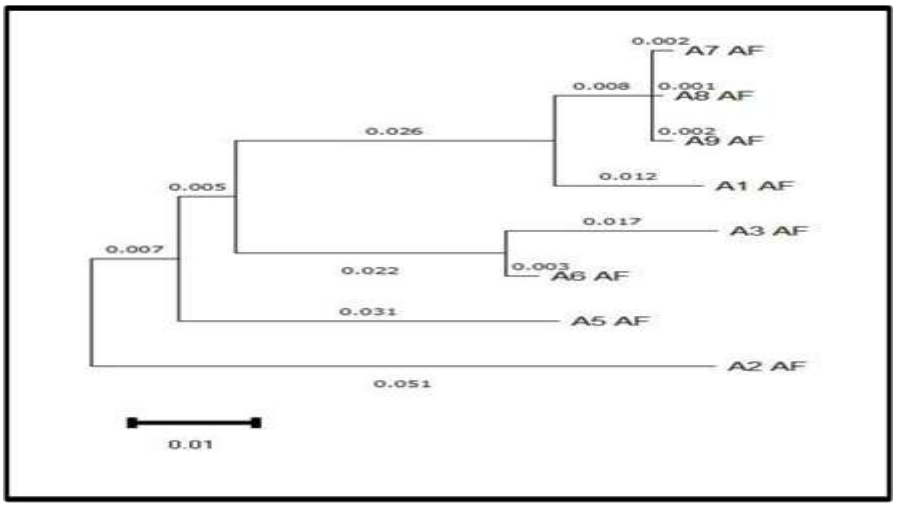

Figure 4. UPGM dendrogram showing the relationship among species of the genus *Euphorbia* L. according to the *rbcL* gene (1- *E. macroclada 2- E. hirta, 3- E. falcata, 4- E. craspedia 5- E. helioscopia, 6- E. peplus, 7- E. kansuensis, and 9- E.denticulata)*. Note: The species' 8 subspecies was not included in this study.

Table 3. The genetic distance between species of the genus *Euphorbia* L. according to the *rbcL* gene. (1- *E. macroclada 2- E. hirta, 3- E. falcata, 4- E. craspedia, 5- E. helioscopia, 6- E. peplus, 7-E. kansuensis, and 9- E. denticulata*). Note: The species' 8 subspecies was not included in this study.

Species	A1_AF	A2_AF	A3_AF	A5_AF	A6_AF	A7_AF	A8_AF
A2_AF	0.09129						
A3_AF	0.07290	0.09738					
A5_AF	0.07098	0.08553	0.06673				
A6_AF	0.05776	0.07949	0.01956	0.05580			
A7_AF	0.02181	0.08522	0.07047	0.06957	0.05644		
A8_AF	0.02083	0.08631	0.06942	0.06745	0.05542	0.00278	
A9_AF	0.02084	0.08845	0.06838	0.06950	0.05240	0.00371	0.00278

Genetic relationship

The studied species of the genus *Euphorbia* underwent comparison with the samples recorded in the Gen Bank, confirming their diagnosis (Table 3). According to the indicators of the primer *rbcL*, it revealed that the highest genetic affinity was between the two species *E. denticulata* and *E. kansuensis*, and the anatomical study recorded a similarity of the nature of the mesophilic tissue, where the cross-section of the leaf was unifacial, indicating the similarity in the path of photosynthesis, considering the characteristic as one of the genetic ones. The similarity in the anatomical attributes enhances the results of genetic affinity, while the minimum genetic affinity (highest genetic distance) was evident between *E. macroclada* and *E. hirta*. The *rbcL* has a primary role in determining the paths of photosynthesis for its role in the ribulose-1-phosphate synthesis and counting it as one of the plastid nuclear indicators that provide the common origins between species. Its demonstration in the dendritic diagram specifies the species classified into several groups (Figure 4, Table 3).

The first group included the species *E. hirta*, depicting isolation genetically from the rest of the species. This species was unique in its herbaceous growth, as well as, in its uniqueness in anatomical characteristics, such as, heterogeneity in the shape of the cells on the upper surface of the leaf (irregular) and the internal and external walls. In addition to giving more evidence for stomata on the two leaf surfaces and its distinction with a high rate of the thickness of the leaf blade, it also indicates an increase in the thickness of the palisades and spongy layers and an increase in photosynthetic units associated to the genetic sequence of the starter *rbcL*.

The second group comprised the two species *E. peplus* and *E. falcata*. Notably, the collection areas of the two species were similar, as the two species grew adjacent to each other in the central and northern regions of Iraq. The nature of their growth was herbaceous, and it may indicate genetic similarity and the extent of convergence.

The second group, in turn, has two secondary groups representing it. The first secondary group included the two species, *E. kansuensis,* and *E. denticulata*, and these species were similar in terms of the nature of the mesophilic tissue, as it was unifacial. The results of the current study also support what previous studies have demonstrated on the common monophyletic origin of all the species in the genus *Euphorbia* (Steinmann and Porter, 2002; Bruyns *et al*., 2006; Zimmermann *et al*., 2010; Horn *et al*., 2012).

The significant results were also in analogy with several other past findings that chose *matK* and *rbcL* for studying the genetic affinity and the stages of development of plant families. The Consortium for the Barcode of Life Plant Working Group (CBOL, 2009) considered the *rbcL* and *matK* genes the best genetic region for identifying the plants. The presented results also showed that the primer *matK* is one of the genetic primers that can be reliable for species identification and diagnosis. Table 4 shows that the highest genetic affinity appeared between the species *E. kansuensis* and *E*. *denticulate,* with a value of 0.0160, whereas the lowest occurring between the two *Euphorbia* species *E. helioscopia* and *E. peplus,* with a value of 0.1307.

The *Euphorbia* species could separate further into several groups and branches (Figure 5). The first secondary group included *E. peplus* and *E. falcata*, being genetically close to each other by 0.055, and characteristic of their herbaceous growth nature and adjacent distribution in the collection areas within the study. The second secondary group splits into two parts. The first secondary group included *E. denticulata* and *E*. *kansuensis,* recording the highest genetic affinity compared with the rest of the species (Table 4). These two mentioned *Euphorbia* species were similar in rows of palisade cells and the average thickness of the mesophyll tissue, indicating similarity in the developmental characteristics of both species, especially concerning the path of photosynthesis.

The second secondary group also splits into two groups. The first group contained *E. macroclada*, which was genetically isolated.

Table 4. The genetic distance among the species of the genus *Euphorbia* L. according the *matK* gene (1- *E. macroclada, 2- E. hirta, 3- E. falcata, 4- E. craspedia, 5- E. helioscopia, 6- E. peplus, 7- E. kansuensis,* and *9- E. denticulata*).

Species	B1_BF	B3_BF	B4_BF	B5_BF	B6_BF	B7_BF
B3_BF	0.0893					
B4_BF	0.0355	0.1114				
B5_BF	0.0657	0.1078	0.0485			
B6_BF	0.1012	0.0371	0.1290	0.1307		
B7_BF	0.0289	0.0790	0.0517	0.0843	0.0935	
B9_BF	0.0345	0.0884	0.0548	0.0835	0.0989	0.0160

Note: The species' 8 subspecies was not included in this study.

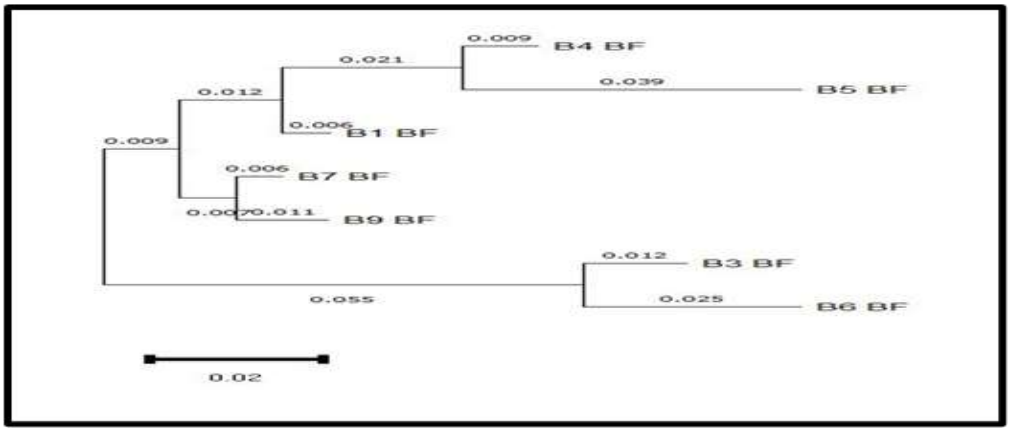

Figure 5. UPGM dendrogram showing the relationship among species of the genus *Euphorbia* L. according to the *matK* gene (1- *E. macroclada, 2- E. hirta, 3- E. falcata, 4- E. craspedia, 5- E. helioscopia, 6- E. peplus, 7- E. kansuensis, and 9- E. denticulata*). Note: The species' 8 subspecies was not included in this study.

This species was distinguishable by the nature of its shrubby growth and a decrease in the average thickness of the mesophyll tissue. The second group included *E. craspedia* and *E. helioscopia*, as genetically the same, indicating the common genetic origin. It is worth noting that these two species have a shrubby and delusional nature of growth. This study also agrees with the findings demonstrated by Dorsey (2013) that the *matK* region is optimal for studying the genetic relationship among the genus *Euphorbia* species.

The results concluded that most of the *Euphorbia* species samples showed the bands after being electrocuted, and these bands were prominent by their intensity ranging from 800 to 1000 bp after comparing them with the DNA Lader. It revealed that the lowest genetic affinity appeared between the species *E. macroclada* and *E. hirta* through the *rbcL* gene, which was a plastid nuclear indicator of a common ancestry between species. The *matK* gene results showed that the highest genetic affinity emerged between *E. kansuensis* and *E. denticulata,* with a value of 0.0160, and the lowest was evident in the two *Euphorbia* species, i.e., *E. helioscopia* and *E. peplus,* with a value of 0.1307.

CONCLUSIONS

The results suggested that anatomical characteristics of the leaf can help identify and classify various species of the genus *Euphorbia*. Some other attributes can serve to

describe the studied species, such as, the tissues' distribution in blades. The leaf was unifacial in the *Euphorbia* species *E. hirta*, *E. peplus, E. macroclada, and E. denticulata.* However, in species *E. craspedia* and *E. helioscopia,* the leaves were bifacial. Besides anatomical traits, molecular studies provided more accurate results in separating the species based on the genetic similarities and differences between them. The primers *matK* and *rbcL* regions were optimal for studying the genetic closeness among the genus *Euphorbia* species. The investigations through leaf anatomy and molecular markers can better help understand and distinguish the various *Euphorbia* species.

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REFERENCES

Acharya D, Vaidya M (2017). Anatomical study of *Euphorbia hirta* L. World *J. Pharm. Res*. 6(7): 1407-1416.

Ahmad KT, Ahmad M, Shaheen NM, Abdul N (2010). Taxonomic diversity in epidermal cells of some sub-tropical plant species. *Int. J. Agric. Biol*. 12: 115-118.

Aloush RH (2014). Biosystematics study of species of genera *Lathyrus* L. (Papilinaceae) in Northern and Middle Iraq. Thesis, College of Pure Science, Tikrit University, Iraq (in Arabic).

Aziz SA, Azmi TKK, Sukma D, Qonitah FZ (2016). Morphological characters of triploids and tetraploids produced by colchicine on buds and flowers of *Phalaenopsis amabilis*. *SABRAO J. Breed. Genet*. 48(3): 352-358.

Badgujar SB, Mahajan RT (2013). Peptide mass fingerprinting and N-terminal amino acid sequencing of glycosylated cysteine protease of *Euphorbia nivulia* Buch.-Ham. *J. Amino Acids* doi: 10.1155/2013/569527.

Bruyns P, Ruvimbo V, Mapaya J, Hedderson T (2006). A new subgeneric classification for Euphorbia (Euphorbiaceae) in Southern Africa based on ITS and psbA-trnH sequence data. *Taxon* 55(2): 397-420.

CBOL (2009). Consortium for the Barcode of Life Plant Working Group. A DNA barcode for land plants. *Proc. Nat. Acad. Sci.* USA 106: 12794–12797.

Clapp JP (1996). Species diagnosis protocols PCR and other nucleic acid methods (Methods in Molecular Biology 50), Pub Humana, pp. 427.

Do N, Adams RP (1991). A simple technique for removing plant polysaccharide contaminants from DNA. *BioTechniq* 10(2):- 162-164.

Dorsey B (2013). Phylogenetics and morphological evolution of *Euphorbia* subgenus *Euphorbia*. Thesis, University of Michigan, USA.

Fayed AAA, Ahamed MS, Faried AM, Mohamed MH (2020). Leaf morphology and venation patterns of *Euphorbia* L. (Euphorbiaceae) in Egypt with special notes on their taxonomic implications. *Jordan J. Biol.* Sci. 13(2): 165-176.

Horn JW, Morawetz J, Riina R, Steinmann VW, Berry PE, Wurdack KJ (2012). Phylogenetics and the evolution of major structural characters in the giant genus *Euphorbia* (Euphorbiaceae). *Mol. Phylogen. Evol*. 63: 305-326.

Ji Y, Yang J, Landis JB, Wang S, Yang Z, Zhang Y (2021). Deciphering the taxonomic delimitation of *Ottelia acuminata* (Hydrocharitaceae) using complete plastomes as super-barcodes. *Front. Plant Sci*. 12: 681270. (that is the correct)

Kakkar L, Paliwal GS (1974). Studies on leaf anatomy of *Euphorbia* L. V. Epidermis. *Proceed. Indian Nat. Sci. Acad*. 40: 55-67.

Liabata P, Richter J, Faus I, Słomiňska-Durdasiak K, Zeh LH, Gadea J, Hauser MT (2019). Involvement of the eIF2 α kinase GCN2 in UV-B responses. *Front. Plant Sci.* 10: 1492.

Maniatis T, Fritsch E, Sambrook J (2001). In Vitro Application of DNA by the Polymerase Chain Reaction in Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory Press, New York, USA, 691.

Metcalfe CR, Chalk L (1957). Anatomy of the Dicotyledons, Leaves, Stem, and Wood in Relation to Taxonomy with Notes on Economic Uses. Vol. 1. Oxford, Great Britain: Oxford Univ. Press. pp: 502.

Mohsin RM, Abd Asal KN, Kamaluddin AA, Zaky AA (2023). Genotypes and storage duration effects on the quality of cut flower - gerbera (*Gerbera jamesonii* Hook). *SABRAO J. Breed. Genet*. 55(1): 260-267. http://doi.org/10.54910/sabrao2023.55.1.24.

Neeraj B, Lal S (2019). A survey of some medicinally important plants of the Euphorbiaceae family used by the Santhal tribes of Santhal

Pargana*. Ind. J. Trad. Knowl.* 18(3): 610-614.

Nichodemus CO, Ekeke C (2021). Morpho-anatomical and histological characters in the systematics of the croton species (Euphorbiaceae: Crotonoideae) in Southern Nigeria. *Phytol. Balcanica J*. 27(2): 187-202.

Pandey RN, Adams RP, Flournoy LE (1996). Plant polysaccharides. *Plant Mol. Biol. Rep*. 14: 17-22.

Porebski S, Bailey LG, Baum BR (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol. Biol. Report.* 15: 8-15.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual (No. Ed. 2). Cold Spring Harbor Laboratory Press. USA.

Sleibi AT (2015). A comparative biosystematics study of some species of the genus *Euphorbia* L. (Euphorbiaceae) in Iraq. Department of Biology, College of Education and Pure Science, University of Baghdad, Iraq.

Staats M, Arulandhu AJ, Gravendeel B, Holst-Jensen A, Scholnines I, Peelen T, Prins TW (2016). Advances in DNA metabarcoding for food and wildlife forensic species identification. *E. Anal. Bioanal. Chem*. 408: 4615-4630.

Steinmann VW, Porter JM (2002). Phylogenetic relationships in Euphorbieae (Euphorbiaceae) based on ITS and ndhF sequence. *Ann. Mo. Bot. Gard.* 89: 453-490.

Sun YH, Jiang F, Zeng X, Pan X, Wu YQI, Wu X (2022). Species identification and genetic diversity of Alcea (Malvaceae) using SCoT molecular markers: Medicinal plants. *Genetika 54*(1): 369-378.

Talebi S, Noori M, Naniz HA (2017). A study of epidermal leaf anatomy of 18 Euphorbia taxa from Kerman Province, Iran. *Biologija* 63(2): 126-133.

Thakur HA, Patil DA (2012). The family Euphorbiaceae: Anatomical conspectus. *J. Sci. Technol.* 2(6): 51-57.

Webster GL (1994). Synopsis of the genera and suprageneric taxa of Euphorbiaceae. *Ann. Miso. Bot. Gard*. 81(1): 133-144.

Weigant F, Baum M, Udupa S (1993). DNA molecular marker techniques*. Techn. Manu. No. 20*. ICARDA.

Willis JC (1973). A Dictionary of Flowering Plants and Ferns. 8th Ed. Camb. Univ. Press. pp. 441-443.

Zahra NB, Ahmad M, Shinwari ZK, Zafar M, Sultana SH (2014). Systematic significance of anatomical characterization in some Euphorbiaceous species. *Pak. J. Bot*. 46(5): 1653-16.

Zimmermann NFA, Ritz CM, Hellwig FH (2010). Further support for the phylogenetic relationships within *Euphorbia* L. (Euphorbiaceae) from nrITS and trnL-trnF IGS sequence data. *Plant Syst. Evol.* 286: 39-58.