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COMPLETE CHLOROPLAST GENOME OF *SALVIA MULTICAULIS* VAHL. (LAMIACEAE)

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ABSTRACT

This study was aimed to investigate the complete chloroplast (plastome) genome of *Salvia multicaulis* for the first time in Iraq and surrounding countries. In this study, the complete chloroplast genome was sequenced, assembled, and annotated. Then, phylogenetic analysis was performed. Accordingly, the closely related species were aligned and compared. The circular quadripartite plastome length was 151,373 base pair (bp) includes Large Single-Copy (LSC) region with 82682 bp, and Small Single-Copy (SSC) region with 17,682 bp, and two inverted repeat (IR) regions of 25,592 bp. The complete GC content of the plastome was 38 %. The phylogenetic tree analysis shows that there were two distinct clades. The first clade comprises of the taxa from center-west Asia/ mediterranean region. The second clade includes the East Asia taxa. The *S. multicaulis* was located on the first clade as a sister group to *S. officinalis* and *S. yangii*. Consequently, the analysis of the five clustered species demonstrated that the intergeneric spacers regions were highly variable compared to the coding region.

Key words: **Next Generation Sequencing, Phylogenetic Tree, Plastome *Salvia*.**

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INTRODUCTION

Salvia is one of the largest genera in the Lamiaceae family which includes 1015 accepted species. This genus has a cosmopolitan distribution from Australia to Americas including Mediterranean basin (POWO, 2023). The genus is significantly diverse due to the reasonable radiation of species to various locations. There are about 500 species in Mesoamerica/ South America, 350 species in West and west Asia and the Mediterranean regions (Alziar, 1988–1993; Walker and Sytsma, 2007). *Salvia* has considerable medicinal, ornamental, and ecological values (Sharifi-Rad et al., 2018). Likewise, many species have been applied in folk medicine such as anti-microbial, anti-inflammatory, and antitumor (Ulubelen, 2003; Şenol et al., 2010; Salih and Al Dabagh, 2021). This genus has been identified and classified morphologically, based on flower stamen shapes and numbers (Himmelbaur and Stibal, 1932). Approximately 11 various types of stamens have been recognized (Claßen-Bockhoff et al., 2003; Walker and Sytsma, 2007; Will and Claßen-Bockhoff, 2014). These great morphological variations proposed to be resulted from parallel evolution. Consequently, the work has been approved phylogenetically (Walker et al., 2015; Drew et al., 2017). Taxonomic position and evolutionary relations of *salvia* were examined. Notwithstanding, the genus was suggested to be monophyletic when morphological data were applied. In contrast, molecular chloroplasts approved that the genus is polyphyletic (Walker et al., 2004). This genus is represented in the Turkish and Iran floras by 161 species. 100 species were present in Turkey of which 53 species are endemic (Kahraman et al., 2018), whereas 61 species in Iran of which 17 are endemic (Jamzad, 2012). In Iraq there are inconsiderable studies on *Salvia* as a genus. Hedge (1982a,1982b)

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reported 23 species of *Salvia* from Kurdistan Region of Iraq including the endemic *S. kurdica* Boiss. & Hohen. ex Benth. Moreover, Abbas (2013) recognized 33 species of *Salvia* in various parts of Iraq. Four years later, the narrowly endemic *S. ali-askaryi* S.A. Ahmad were identified (Ahmad, 2016). Thus, the genus is currently represented in Kurdistan Iraq by 25 species, of which two are endemics. The native *Salvia multicaulis* Vahl. is widely distributed from W. Asia to Sinai (POWO, 2023.). This cryptic taxon is widespread in Iraq, Iran, and Turkey (Esra et al., 2011; Abbas, 2013; Talebi et al., 2017). This species is regarded as an aromatic (Jamzad, 2012), medicinal plants, treating many seasonal diseases; colds and flu (Cakilcioglu et al., 2010), inflammation of the tonsils (Tetik et al., 2013). *S. multicaulis* is morphological distinct due to the bract shapes and basal leaf petiole structures, flower frequency on the inflorescence cycle, and the calyx sizes (Talebi et al., 2017). In last few years, the Next Generation Sequencing (NGS) techniques are significantly applied to the most angiosperms genera including *Salvia* species. These techniques play important role in generating the complete chloroplast genome (plastome) as well as conducting the phylogenetic analysis to various taxa among this genus. Some *Salvia* chloroplast genomes were studied properly mainly in East and west Asia and Southern America*; S. przewalskii* and *S. bulleyana* (Liang et al., 2019), *S. miltiorrhiza* (Hu et al., 2020), *S. yangii, S. miltiorrhiza* f. *alba* (Gao et al., 2020), *S. chienii* (Cheng et al., 2023), *S. azurea*, *S. iodantha*, *S. microphylla*, *S. nipponica*, and *S. umbratical* (Yu et al., 2023). The previous results show that the circular plastome length of the *Salvia* species varies from 150,814- 153,995 bp. The shortest large single-copy region (LSC) length was 82,102 bp in *S. iodantha* whereas the longest was 84,573 bp in *S. japonica*. Similarly, the small single-copy region (SSC) was varied from 17,432 bp (*S. microphylla*) to 17,965 bp (*S. rosmarinus*). The length of the inverted repeat (IR) regions varied 25,469 (*S. miltiorrhiza*) to 25,916 (*S. japonica*). The GC content is slightly constant in all *Salvia* species which approximately is 38 % with minor variation. The numbers of coding genes of *Salvia* taxa ranged from 133 to 134 which comprises of 8 ribosomal RNA genes, 37 transfer RNA genes, 88-89 protein coding genes. Many of the Angiosperm plants inherit their chloroplast genomes maternally, and most of the genes are crucially conserved. Thus, the complete chloroplast genome sequences consider a functional tool for species and genera Identifications (Nock et al., 2011). A couple of phylogenetic studies were conducted for various *Salvia* species to know the taxonomic position as well as evolutionary relationships of these species (Liang et al., 2019; Gao et al., 2020; Hu et al., 2020; Cheng et al., 2023; Yu et al., 2023). Some of the mentioned studies were demonstrated that the *S. miltiorrhiza* is cluster with S. *przewalskii* and *S. chienii* on one clade (Cheng et al., 2023), whereas *S. yangii, S. officinalis* and *S. rosmarinus* nested together on another clade (Yu et al., 2023). In current study, the *S. multicaulis* Vahl. is sequenced, using Next Generation Sequencing Techniques to help us to understand the complete chloroplast genome structure, composition, and to reconstruct the phylogenetic tree to clarify the evolutionary relationships among *Salvia* species.

MATERIALS AND METHODS

Sample collection, DNA extraction and sequencing technique

In the middle of spring 2023, fresh samples of *S. multicaulis* Vahl. was collected from the Bekhair mountain-Duhok, Kurdistan Region of Iraq and then the herbarium specimen was prepared and deposit at the Duhok Province University Herbarium (DPUH), University of Duhok. The total genomic DNA was extracted using a modified CTAB method (Doyle, 1987). The isolated purified DNA samples were sent to the DNA Link Inc. company, Republic of Korea. A 350-bp paired-end library was constructed using Illumina's TruSeq, Nano DNA Library Preparation kit, following the manufacturer's instructions.

Complete chloroplast assembly and annotation

The complete chloroplast genome (cp genome) was assembled using *s. officinalis* (NC_023209) as reference genome. The consensus aligned sequences was annotated

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with *Geneious Prime* software (Kearse et al., 2012) (https://www.geneious.com) as follows; 20% of the total genomic DNA, the bi-direction reads (forwards and reverse), 151 bp each were paired to get 193,967,238 raw read sequences, then these data were mapped to the reference genome. In total, 9,834,002 reads were assembled. On other way, de novo assembly applied to the later assembled read to generate 10 contigs. Later, each contig compared separately with the consensus sequence that generate from the reference genome. Consequently, the circular complete chloroplast genome was produced. To annotate the generated plastome, complete annotation transferred from the reference genome *S. officinalis* (NC_023209). The latest annotated chloroplast genome submitted to the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov). The approved plastome was drawn using Draw Organelle Genome Maps (OGDRAW) (Greiner et al., 2019).

Phylogenetic analysis

Based on the recently published paper, 15 complete chloroplast genomes of *Salvia* species plus *Mentha longifolia* were downloaded from NCBI (Table 1). To construct the phylogenetic tree including *S. multicaulis* the following datasets were applied; *MAFFT* v7.486 (Katoh and Standley, 2013), for alignment; Mega 11 (Tamura et al., 2021) and Mrbayes (v 3.2.7) (Ronquist et al., 2012) for conducting the maximum Bayesian reference trees respectively.

Comparative analysis

Five closest species; *S.* multicaulis, *S. officinalis* (ON641364), *S. yangii* (MT537168), *S. rosmarinus* (MT634145), *S. chanryoenica* (MH261357) were compared using mVISTA program sets (Frazer et al., 2004) with multi-LAGAN (Brudno et al., 2003) to visualize the similarity and differences.

Table 1. The plant materials used in this study

Taxa	Accession No. (NCBI)	Taxa	Accession No. (NCBI)
Salvia multicaulis	**OR684571**	***Salvia mairei***	**NC_053378**
Salvia officinalis	**ON641364**	***Salvia yunnanensis***	**MK944405**
Salvia yangii	**MT537168**	***Salvia meiliensis***	**MN520018**
Salvia Rosmarinus	**MT634145**	***Salvia honania***	**NC_058852**
Salvia chanryoenica	**MH261357**	***Salvia daiguii***	**NC_059718**
Salvia przewalskii	**MK344723**	***Salvia nanchuanensis***	**MW43540**
Salvia prattii	**MK944407**	***Salvia miltiorrhiza***	**JX312195**
Salvia umbratical	**MW752208**	***Salvia chienii***	**OK094518**

RESULTS AND DISCUSSION

Plastome features of *S. multicaulis*

In the current study, 20 % of *S. multicaulis* genome were sequenced to get complete chloroplast genomes (plastome). The Illumina pair-end of (193,967,238) raw reads sequences was de novo assembled to generate circular quadripartite plastome with 151,373 base pair (bp) length. This quadripartite genome comprises of a large single-copy region (LSC) of 82682 bp, a small single-copy region (SSC) of 17,682 bp, and two inverted repeat (IR) regions of 25,592 bp. The GC content of the entire plastome is 38 % (Table 2). The total number of generated genes from the assembled sequences are 133; 88 protein-coding (CDS), transfer-RNA (tRNA), ribosomal-RNA (rRNA) genes. The genes length varies from 90-5532 bp in CDS, 71-93 in tRNA, and 103-2811 in rRNA genes. In this quadripartite structure, there were 19 duplicated genes; 8 CDs, 7 tRNA and 4 rRNA (ndhB, rpl2, rpl23, rps7, rps12, ycf1, ycf2, ycf15, trnA-UGC, trnI-CAU, trnI-GAU,trnL-CAA, trnN-GUU,trnR-ACG, trnV-GAC rrn4.5, rrn5, rrn16, rrn23) respectively (Figure 1 & Table 3). There are 18 different genes with introns (atpF, clpP1, ndhA, ndhB, pafl, petB, petD, rpl16, rpl2, rpoC1, rps12, rps16, trnA-UGC, trnG-UCC, trnI-GAU, trnK-UUU, trnL-UAA, trnV-UAC) (Figure 1 & table 2). Among these genes, the clpP1 and pafl have double introns whereas rps12 is trans-spliced all around the plastome. The first exon positioned in LSC region, and

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the two others are localized in IR region (Figure 1). The *S. multicaulis* plastome sequence considers as first complete chloroplast genome in the NCBI. Although, the cp genome is stable in most cases of the land plant (Mower and Vickrey, 2018), but still there is notable variation in their length due to the repeat sequences, and the copy numbers variation in the IR, SSC, and LSC gene regions (Li et al., 2022). The total length of *S. multicaulis* plastome, 151373 bp, is relatively close to S. *chienii* (Cheng et al., 2023), *S. nipponica*, (Yu et al., 2023). Even though, the 38% of GC content of the total chloroplast genome in *S. multicaulis* is qualified as AT-rich. This proportion is entirely like the other *Salvia* species, *S. miltiorrhiza* f. *alba*, and *S. miltiorrhiza* (Gao et al., 2020). The complete annotated *S. multicaulis* plastome comprises 88 protein-coding and 37 transfer-RNA genes which are almost identical to *S. yangii* (Gao et al., 2020), *S. iodantha*, S., *S. nipponica*, and *S. umbratical* (Yu et al., 2023).

Table 2. The *S. multicaulis* plastome features

Features	Chloroplast
Genome size (bp)	151,373
large single copy (bp)	82682
small single copy (bp)	17507
inverted repeat A (bp)	25592
inverted repeat B (bp)	25592
GC content (%)	38
No. of protein-coding genes	88
No. of transfer-RNA genes	37
No. ribosomal-RNA genes	8
No. of genes with introns	18

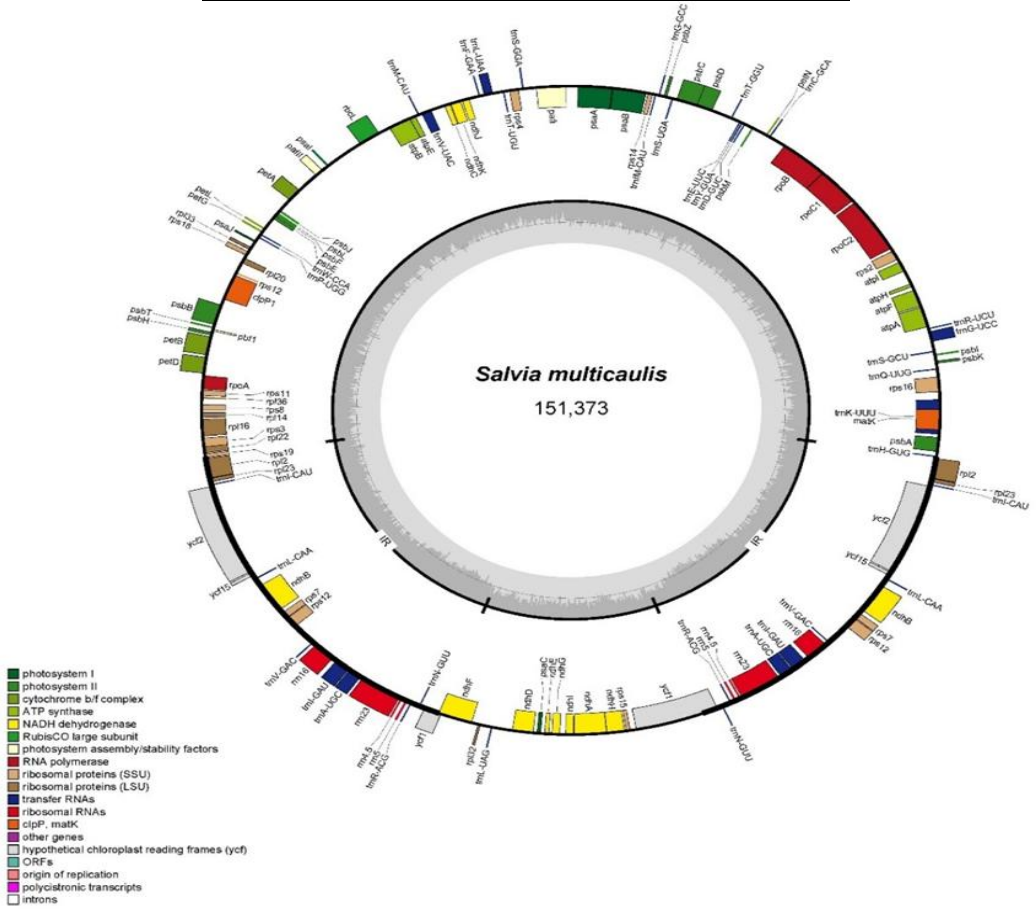

Figure 1. The circular complete chloroplast genome of *S. multicaulis*. The outside genes are clockwise while the inside genes are counterclockwise directions. The colour of each gene refers to their function in the genome.

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Table 3. The complete chloroplast genes of the *S. multucaulis* include start, end, and length

Name	Start	End	Length	Name	Start	End	Length	Name	Start	End	Length
trnH	11	85	75	trnM	51537	51609	73	trnL	92330	92410	81
psbA	414	1472	1059	atpE	51820	52221	402	ndhB	92963	95170	2208
trnK	1709	4297	2589	atpB	52218	53714	1497	rps7	95445	95912	468
matK	2029	3558	1530	rbcL	54501	55946	1446	trnV	98375	98446	72
rps16	4868	5977	1110	accD	56621	58099	1479	rrn16	98673	100163	1491
trnQ	6531	6602	72	psaI	58567	58677	111	trnI	100462	101483	1022
psbK	6989	7168	180	pafII	59110	59664	555	trnA	101548	102425	878
psbI	7567	7677	111	cemA	60319	61008	690	rrn23	102591	105401	2811
trnS	7841	7928	88	petA	61211	62173	963	rrn4.5	105500	105602	103
trnG	8646	9400	755	psbJ	63162	63284	123	rrn5	105852	105972	121
trnR	9580	9651	72	psbL	63414	63530	117	trnR	106216	106289	74
atpA	9758	11281	1524	psbF	63554	63673	120	trnN	106814	106885	72
atpF	11377	12636	1260	psbE	63688	63939	252	ycf1	107215	108372	1158
atpH	12916	13161	246	petL	64833	64928	96	ndhF	108212	110470	2259
atpI	14190	14933	744	petG	65108	65221	114	rpl32	110916	111077	162
rps2	15159	15869	711	trnW	65337	65410	74	trnL	111789	111868	80
rpoC2	16079	20248	4170	trnP	65585	65658	74	ccsA	111965	112936	972
rpoC1	20425	23241	2817	psaJ	65937	66071	135	ndhD	113171	114571	1401
rpoB	23268	26480	3213	rpl33	66516	66716	201	psaC	114803	115048	246
trnC	27619	27689	71	rps18	66887	67192	306	ndhE	115299	115604	306
petN	27853	27942	90	rpl20	67426	67812	387	ndhG	115784	116314	531
psbM	28885	28989	105	rps12	68607	138090	905	ndhI	116688	117194	507
trnD	29509	29582	74	rps12	68607	96759	908	ndhA	117275	119373	2099
trnY	29693	29776	84	clpP1	68843	70770	1928	ndhH	119375	120556	1182
trnE	29847	29919	73	psbB	71224	72750	1527	rps15	120656	120928	273
trnT	30488	30559	72	psbT	72933	73040	108	ycf1	121310	126841	5532
psbD	31911	32972	1062	pbf1	73101	73232	132	trnN	127171	127242	72
psbC	32956	34341	1386	psbH	73338	73559	222	trnR	127767	127840	74
trnS	34586	34678	93	petB	73685	75068	1384	rrn5	128084	128204	121
psbZ	35024	35212	189	petD	75252	76429	1178	rrn4.5	128454	128556	103
trnG	35490	35560	71	rpoA	76606	77613	1008	rrn23	128655	131465	2811
trnfM	35734	35807	74	rps11	77684	78100	417	trnA	131631	132508	878
rps14	35966	36268	303	rpl36	78210	78323	114	trnI	132573	133594	1022
psaB	36391	38595	2205	infA	78419	78652	234	rrn16	133893	135383	1491
psaA	38621	40873	2253	rps8	78773	79177	405	trnV	135610	135681	72
pafI	41653	43605	1953	rpl14	79355	79723	369	rps7	138144	138611	468
trnS	44431	44517	87	rpl16	79853	81120	1268	ndhB	138886	141093	2208
rps4	44775	45380	606	rps3	81263	81925	663	trnL	141646	141726	81
trnT	45722	45794	73	rpl22	81910	82377	468	ycf15	141987	142235	249
trnL	46474	47047	574	rps19	82447	82725	279	ycf2	142326	149156	6831
trnF	47331	47403	73	rpl2	82785	84272	1488	trnI	149245	149318	74
ndhJ	48072	48548	477	rpl23	84291	84572	282	rpl23	149484	149765	282
ndhK	48659	49336	678	trnI	84738	84811	74	rpl2	149784	151271	1488
ndhC	49388	49750	363	ycf2	84900	91730	6831				
trnV	50709	51356	648	ycf15	91821	92069	249				

Phylogeny of *Salvia* species

Based on the recently published papers, 16 complete chloroplast genomes of *Salvia* species including *S. multicaulis* plus *Mentha longifolia* as an outgroup were used to conduct the phylogenetic tree applying Bayesian analysis. The results showed that the dendrogram is considerably separated into two robust (posterior probability (pp) = 1) clades. Clade 1 comprises *S. rosmarinus*, *S.*

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officinalis, S. multicaulis, S. yangii, whereas the Clade 2 includes 14 species to form two subclades (Figure 2). Firstly, clade 1 proposed that all four species were diversified from the outgroup earlier. Then individually, *S. Rosmarinus* isolated from the group, later *S. yangii* separated from *S. officinalis, S. multicaulis*. Moreover, the S. *chanryoenica* early diversified from the second clade. Continuedly, *S. Przewalskii, S. prattii, S. umbratical* were clustered to submerge the first sub-clade then *S. mairei. S. yunnanensis, S. meiliensis, S. honania, S. daiguii, S. nanchuanensis, S. miltiorrhiza S. chienii* to produce the second subclade. Consequently, the Bayesian analysis demonstrated that there is a great extent of convergence within genus *salvia*. The two distinct clades were identified and isolated geographically. The basal clade was Central and Western Asia (CAM) including Mediterranean region and second clade was the East Aisa (EA). There was considerable connection between CAM and EA regions, suggesting that belongs to the early floral diversity of Iran and Turkey native species (Esra et al., 2011; Askari et al., 2021), and the later subsequent diversification in South Asia (Kriebel et al., 2019). Although the divergence time was not estimated in this study, while tree topology indicates that the first clade is regeared as the oldest compared to other clades. This result was confirmed by Yu et al., (2023) when analyzed 58 *Salvia* taxa phylogenetically. Thus, this study supports the previous suggesting results of *salvia* diversion and speciation, starting from West Asia to America went-through East Asia and Europe (Walker et al., 2015; Drew et al., 2017).

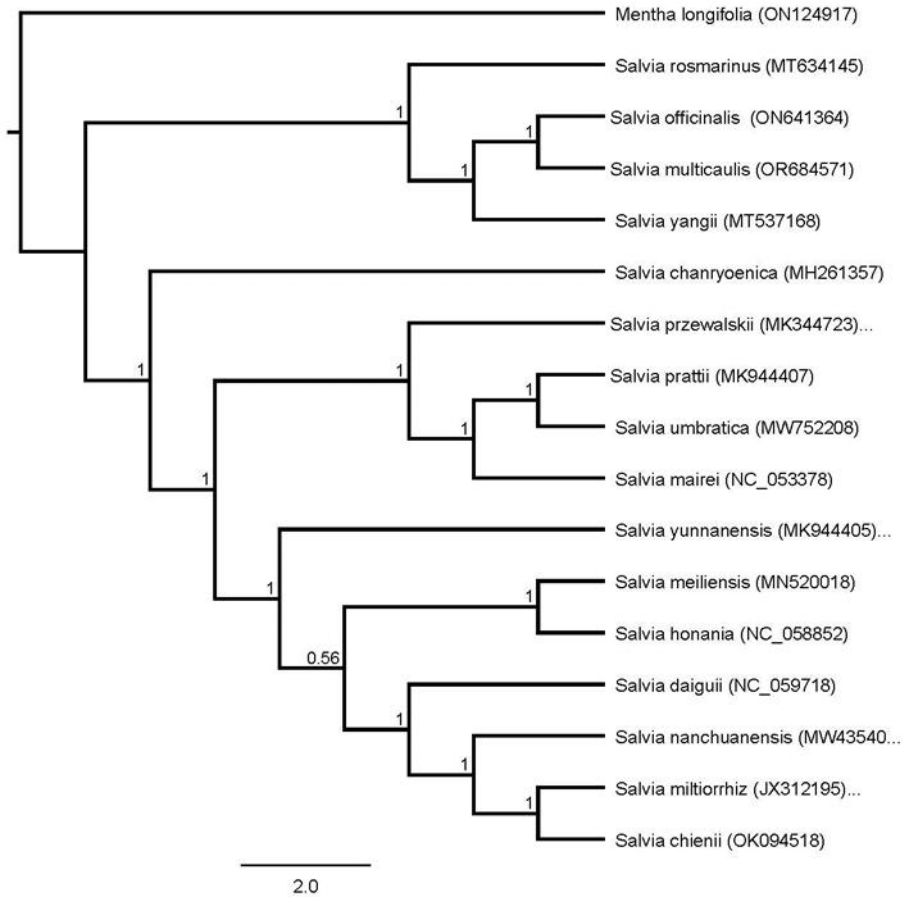

Figure 2. The Bayesian Phylogenetic Tree of 16 *salvia* species including *salvia multicaulis* plus *Mentha longifolia* as an outgroup. Comprise 133 gene sequences with introns and spacers. The number above each branch is the posterior probability › 0.5 support.

Complete chloroplast genome comparison of the closely related Salvia species

Accordingly, the five closely related species of *Salvia* including *S. multicaulis* were aligned and compared using mVISTA program data sets. The result shows that chloroplast genomes among these taxa are significantly conserved. The coding regions expressed low

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conservation when compared to the non-coding regions (Figure 3). Despite that there are 133 genes and 151373 bp length all around the complete chloroplast genome, only few of them are variable with little informative characters. The preliminarily results demonstrated that majority of the spacers among protein-coding, ribosomal RNA, transfer RNA regions were variable (rps16-trnQ, atpF-atpl, petN-psbm, pafl- trnS, trnL-trnF). On the other hand, *Maturase K* (matK) gene as protein-coding region shows significant variation. Consequently, the above-mentioned genes and spacers are considerable useful for applying as a barcode to delimit the evolutionary relationships among deferent *Salvia* species. Among the five completely aligned species, the DNA sequences in coding gene regions are highly conserved compared to those in interspecific spacer regions. Despite that, the spacer is variable and informative, the variability has been seen much higher in protein coding gene when compared to transfer gene regions. Similar results have been recorded by Yu et al., (2023) when comparative analysis was conducted to many *Salvia* species.

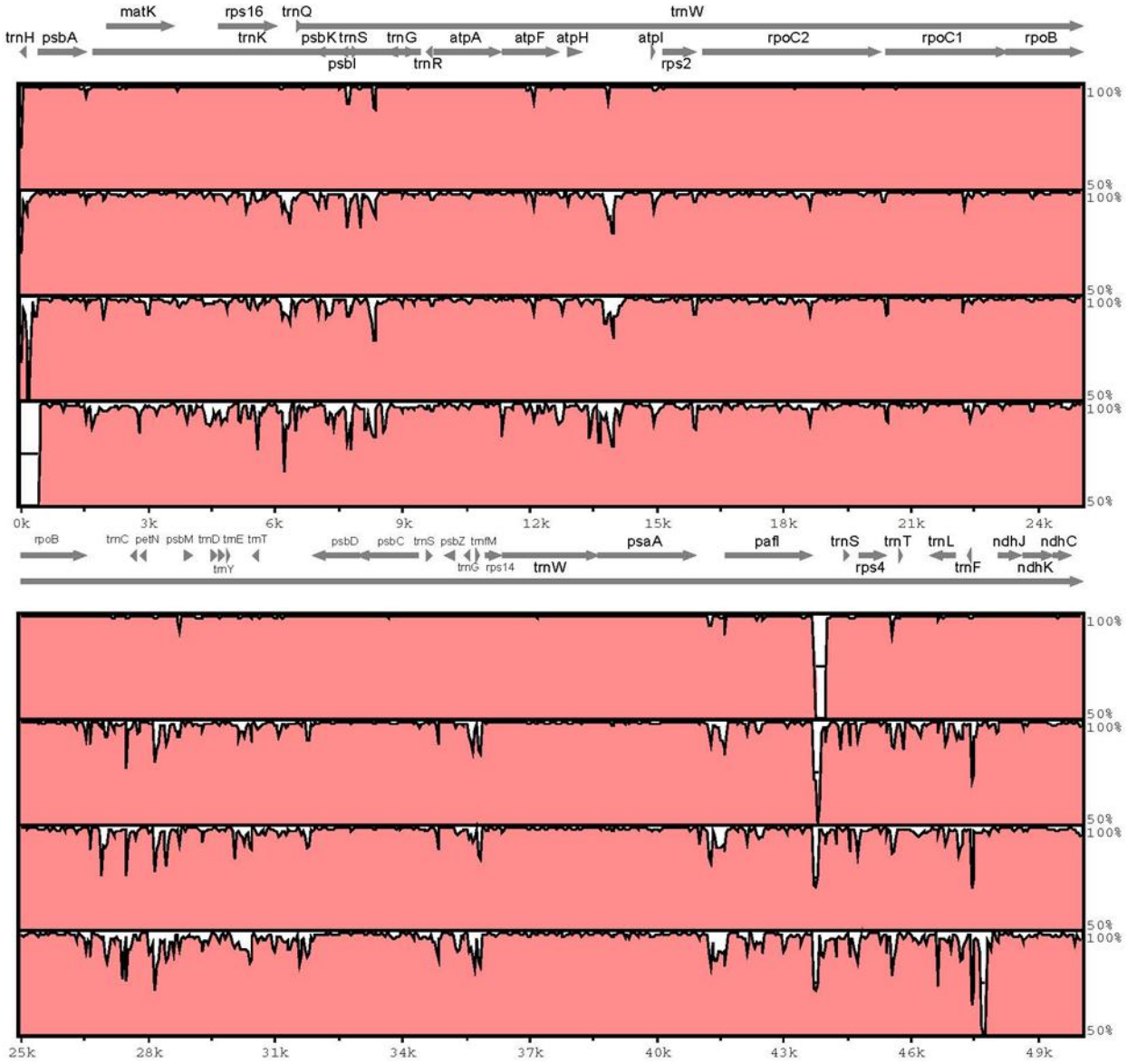

Figure 3. The graphic view of the partial chloroplast genomes of salvia (1-50k). Comparison between The *Salvia multicaulis* (OR684571), *Salvia officinalis* (ON641364), *Salvia yangii* (MT537168), *Salvia rosmarinus* (MT634145), *Salvia chanryoenica* (MH261357) plastomes were appied using mVISTA program sets. The X axis is partial chloroplast genome of *salvia multicaulis* whereas, the Y axis is the sequences variation percentage. The above grey arrows are the genes indication directions.

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CONCLUSION

In this research paper, we submitted the *S. multcaulis* complete chloroplast genome to NCBI for the first time in Iraq and surrounded area. The genomic structure of this species was recognized and compared to other closely related taxa. The circular plastome length and genes arrangement in most cases were either similar or slightly different when compared to other species. The taxonomic position of this taxon and evolutionary relationships with another taxon was determined. *S. multicaulis* was located on Clade one as a sister to *S. officinalis*. Consequently, the five closely related species were aligned and compared. The results expressed that the *S. multicaulis* is practically indistinguishable from *S. officinalis*, except for some variation on interspecific spacer. Thus, the variation between these regions could be used as a barcode to identify the inter and intraspecific variations.

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CONFLICT OF INTEREST

The author declares that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

AUTHOR/S DECLARATION

We confirm that all Figures and Tables in the manuscript are original to us. Additionally, any Figures and images that do not belong to us have been incorporated with the required permissions for re-publication, which are included with the manuscript.

Author/s signature on Ethical Approval Statement

Ethical Clearance and Animal welfare

Funds: The author received no specific funding for this work

AUTHOR'S CONTRIBUTION STATEMENT

J M was responsible for designing the study. The experiment and data analysis were carried out and performed by an author.

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تحديد جينوم البلاستيدات الخضراء لنبات .*Salvia multicaulis* Vahl (Lamiaceae)

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المستخلص

اجريت هذه الدراسة لتحديد جينوم البلاستيدات الخضراء (البلاستوم) الكامل لـ *S. multicaulis* لأول مرة في العراق والبلدان المحيطة به. في هذه الدراسة، تم تسلسل جينوم البلاستيدات الخضراء الكامل وتجميعه وتشخيص المناطق المشفره به. ثم تم إجراء تحليل شجرة النشوء والتطور له. وبناءً على ذلك، تمت محاذاة الأنواع المرتبطة ارتباطًا وثيقًا ومقارنتها. كان طول البلاستوم الرباعي الدائري 373 151 زوج قاعده (bp) يشمل منطقة كبيرة أحادية النسخ (LSC) مع 82682 زوج قاعده، ومنطقة صغيرة أحادية النسخ (SSC) مع 17682زوج قاعده، ومنطقة تكرار مقلوبة (IR) من 25592 زوج قاعده. كان محتوى GC الكامل للبلاستوم 38٪. يُظهر تحليل الأشجار الوراثية أن هناك سلالتين متميزتين. تتكون المجموعة الأولى من الأصناف من وسط غرب آسيا/منطقة البحر الأبيض المتوسط. وتشمل الفئة الثانية أصنوفة شرق آسيا. كان *S. multicaulis* يقع على أول clade كمجموعة شقيقة لـ *S. officinalis* و *S. yangii* وبالتالي، أظهر تحليل الأنواع الخمسة المتجمعة أن مناطق الفضاءات بين الجينات (الانترونات) متغيرة للغاية مقارنة بمنطقة الترميز (الاكسونات).

الكلمات المفتاحية: البلاستوم:, تسلسل الجيل التالي , شجرة النشوء والتطور, *Salvia*