RESEARCH ARTICLE Legume Research- An International Journal, Volume 49 Issue 3: 365-376 (March 2026)

Comparative Analysis of Mitochondrial and Chloroplast Genomes in Alfalfa (*Medicago sativa* L.)

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ABSTRACT

Background: Codon usage bias is a widespread phenomenon in many organisms, including higher plants and plays an important role in regulating gene expression efficiency and accuracy. As an important forage species with high nutritional value, Medicago sativa (alfalfa) has been extensively utilized not only in traditional breeding but also in molecular genetic improvement. Understanding the characteristics of codon preference in its organelle genomes can provide valuable guidance for optimizing gene design and enhancing the expression of exogenous genes in alfalfa or related species.

Methods: A total of 30 mitochondrial and 51 chloroplast high-quality coding sequences were obtained from the NCBI database. Codon usage patterns and preferences were analyzed using CodonW, R, MEGA and Excel.

Result: The effective number of codons (ENC) in mitochondria ranged from 44.73 to 61.0, with an average GC content of 0.42 and GC content at the third codon position (GC3s) of 0.37. In chloroplasts, the ENC ranged from 38.90 to 57.76, with an average GC content of 0.37 and GC3s of 0.27. Analyses using ENC-plot, neutrality plot and PR2 bias analysis suggested that codon usage bias in *Medicago sativa* is influenced by both natural selection and mutation pressure. Twelve optimal codons were identified in mitochondria (mostly ending in 'A' and 'U') and fourteen in chloroplasts, also showing a preference for 'A/U'-ending codons.

Key words: Chloroplast, Codon usage preference, *Medicago sativa,* Mitochondrial, Optimal codons.

INTRODUCTION

Mitochondria and chloroplasts are essential semiautonomous organelles in plant cells, each possessing distinct genomes and performing complementary physiological functions. The mitochondrial genome drives oxidative phosphorylation and energy metabolism, generating adenosine triphosphate (ATP) and maintaining redox homeostasis (Stefano *et al.*, 2015; Wang *et al.*, 2016). The chloroplast genome governs photosynthesis and carbon assimilation, converting light energy into chemical energy that sustains plant growth (Adamiec *et al.*, 2024). These two organelles not only underpin bioenergetic functions but also reflect ancient endosymbiotic origins and coevolution with the nuclear genome. Comparative analyses of organellar genomes reveal mechanisms of plant evolution, gene transfer and adaptation. Plant mitochondrial genomes are typically large and structurally dynamic, exhibiting frequent recombination, subgenomic circles and horizontal gene transfer (Kozik *et al.*, 2019; Gualberto *et al.*, 2014). In contrast, chloroplast genomes are more conserved and usually circular, encoding photosynthetic proteins but occasionally showing lineage-specific rearrangements and loss of the large inverted repeat (IR) region in some legumes (Palmer, 1985; Zhang *et al.*, 2024). Inter-organelle DNA exchange and differential selection pressures contribute to plant adaptation under environmental stress (Liu *et al.*, 2019; Morley *et al.*, 2017).

Medicago sativa L. (alfalfa) is a perennial forage legume of high agronomic and ecological importance, providing protein-rich feed and improving soil fertility *via* nitrogen fixation (Avci *et al.*, 2018). Despite its significance, few studies have examined evolutionary relationships between its mitochondrial and chloroplast genomes. The *M. sativa* chloroplast genome exhibits a typical IR-lacking structure (~125 kb) with variations in noncoding regions and adaptive signals in stress-related genes (Zhang *et al.*, 2024; Jia *et al.*, 2025). The mitochondrial genome shows high plasticity and extensive sequence exchange with the chloroplast genome in *Medicago* and related legumes (He *et al.*, 2023; Jo *et al.*, 2024). Organelle-level molecular adaptation has gained increasing attention in forage legumes. Codon-usage studies in *M. ruthenica* and *M. varia* revealed distinct synonymous codon preferences in genes linked to stress response and energy metabolism (Hao *et al.*, 2025; Wang *et al.*, 2020). DNA barcoding and transcriptomic approaches further distinguish *Medicago* species and

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How to cite this article: Xu, W., Ding, H., Mu, Y., Gao, C., Gao, Y., Wang, Q. and Shi, F. (2026). Comparative Analysis of Mitochondrial and Chloroplast Genomes in Alfalfa (*Medicago sativa* L.). *Legume Research*. **49(3):** 365-376. doi: 10.18805/LRF-882.

Submitted: 28-06-2025 **Accepted:** 24-11-2025 **Online:** 09-12-2025

identify functional loci within organellar genomes that contribute to adaptability and breeding improvement (Yang *et al*., 2023). These findings indicate that organelle genome variation drives evolutionary diversification and provides valuable cytoplasmic resources for enhancing stress tolerance, seed vigor and photosynthetic efficiency in legumes. Mitochondrial and chloroplast genomes also differ in codon-usage bias, nucleotide composition and synonymous substitution rates, all of which affect gene expression efficiency and adaptive evolution (Yang *et al*., 2024; Wang *et al*., 2020). Comparative investigation of these features can clarify organelle-specific selection mechanisms that shape energy metabolism and photosynthesis. Furthermore, organelle genomic variation offers cytoplasmic markers useful for breeding, since their maternal inheritance influences hybrid vigor, fertility restoration and stress resilience (Cauz-Santos *et al*., 2025; Zhang *et al*., 2025).

A systematic comparative analysis of *M. sativa* mitochondrial and chloroplast genomes is therefore essential for understanding organelle evolution, molecular adaptation and functional divergence. Integrating structural annotation, codon-usage bias, repeat-sequence characterization and selection-pressure analysis can elucidate evolutionary relationships and provide a theoretical basis for cytoplasmic inheritance and genetic improvement in alfalfa. Such insights will advance understanding of plant organelle evolution and support molecular breeding strategies to enhance stress tolerance, productivity and sustainability in forage crops.

MATERIALS AND METHODS

Materials for testing

The experiment was conducted in the Key Laboratory of Grassland Resources of Inner Mongolia Agricultural University from March to June 2025. Complete mitochondrial genome of *Medicago sativa* (GenBank accession number NC_068105.1) and chloroplast genome (GenBank accession number MZ983396.1) download in NCBI database. A total of 33 protein-coding gene sequences were obtained from the mitochondrial genome and 77 were obtained from the chloroplast genome. In order to analyze the codon bias more accurately, the gene coding sequences less than 300 bp were first removed and then the gene coding sequences with the start codon of ATG and the stop codons of TAA, TAG and TGA were selected. Finally, 30 mitochondrial genome sequences and 51 chloroplast genome sequences were obtained for subsequent data analysis (Yang *et al*., 2015).

Relative synonymous codon usage degree

Relative Synonymous Codon Usage Degree (RSCU) measures the ratio of a synonymous codon's observed usage in a gene to its expected average usage. It helps detect shifts in the usage pattern of all such codons within a gene (Grantham *et al*., 1980).

Relative codon fitness

Relative codon fitness is often evaluated through the codon adaptation index (CAI), a widely-adopted geometric approach. The CAI quantifies the relative adaptation levels of individual codons. This method has found extensive applications across diverse biological fields (Sharp *et al*., 1987).

ENC plot analysis of codon usage bias

The effective number of codons (ENC) measures the deviation of codon usage from randomness and indicates the bias in synonymous codon usage. High-expression genes, having fewer rare codons, show strong biases and lower ENC values; low-expression genes with weak biases have higher ENC values (Wright, 1990).

PR2-plot plot analysis

PR2 - plot analysis focuses on codon usage biases. It aims to precisely detect mutational imbalances between A/T and C/G at the third codon position. Analyzing A/T and C/G frequencies at this position reveals if mutational biases or natural selection shapes codon usage (Parvathy *et al*., 2022).

Neutral mapping analysis

correlates GC12 (first and second positions) with GC3 (third position) to infer evolutionary forces. A strong correlation suggests mutation-driven bias, while a weak or absent correlation indicates selection dominance (Sharp and Li, 1987; Sueoka *et al*., 1999).

Optimal codon analysis

Frequency of optimal codons (FOP), which is defined as the codons most frequently utilized in the highly expressed genes of a species. FOP is species - specific and the determination of optimal codons typically relies on a set of gene sequences and their corresponding expression data (dos Reis *et al*., 2003).

Analysis of evolutionary selection pressure

The nonsynonymous (Ka) and synonymous (Ks) substitution rates were analyzed using *Medicago sativa* mitochondrial and chloroplast protein-coding genes as references. Pairwise comparisons among species were conducted with MEGA11 (Kumar *et al*., 2016) for sequence alignment and DnaSP v5.10 (Rozas *et al*., 2017) to calculate Ka/Ks values using the MLWL method. The resulting Ka/Ks values for each gene were then summarized and visualized as a box plot.

Analysis of organelle fragment exchange

Horizontal gene transfer (HGT) between mitochondria and chloroplasts is frequent in higher plants, where 5-10% of mitochondrial sequences may occur in chloroplast genomes (Smith, 2011). Homologous regions between the mitochondrial and chloroplast genomes of *Medicago sativa* were identified and visualized using TBtools to reveal organelle-level genetic exchange and evolutionary patterns.

Data processing

CodonW0R0EMBOSS0MEGA and other software tools were used for basic codon characterization, codon bias analysis neutral mapping analysis, *etc*. in R. The codon usage preference of individual genes was also analyzed.

RESULTS AND DISCUSSION

Genome structure and characteristics of *Medicago sativa*

The mitochondrial genome of *Medicago sativa* spans 290,285 bp, encoding 55 genes-18 tRNAs, 3 rRNAs and 34 protein-coding genes-with a GC content of 45.34% (Fig 1). The chloroplast genome measures 125,637 bp, comprising 111 genes-30 tRNAs, 4 rRNAs and 77 protein-coding genes (Table 1) and a GC content of 33.82%.GC content influences genome stability and gene expression (Du *et al*., 2018; Zhou *et al*., 2014). In *M. sativa*, mitochondrial coding sequences averaged 42.47% GC (GC_1 = 47.95% > GC_2 = 42.21% > GC_3 = 37.02%), while chloroplast sequences averaged 37.15% GC (GC_1 = 46.38% >GC_2 = 38.54% > GC_3 = 26.54%).

The lower GC*f* values in both organelles indicate weak codon-usage bias and an AT-rich preference, typical of plant genomes (Zhao *et al*., 2021). Overall, the nucleotide composition of *M. sativa* organellar genomes reflects structural and evolutionary features that may affect molecular breeding and transgene design.

GC content analysis

CodonW (Fig 2) analyzed 30 mitochondrial protein-coding genes of *Medicago sativa*, revealing a mean GC content of 42.47% (33.90-51.80%) and GC_3 of 37.00% (26.01-57.40%). Similarly, 51 chloroplast genes showed an average GC content of 37.25% (30.40-43.70%) and GC_3 of 26.54% (19.78-33.46%). GC content affects genome stability and expression because G-C pairs form three hydrogen bonds, enhancing structural and thermal stability (Du *et al*., 2018; Zhou *et al*., 2014). Both organelles exhibited the typical GC_1 > GC_2 > GC_3 pattern-mitochondria: 47.95%,

Table 1: Detailed characteristics of *Medicago sativa* mitochondrial and chloroplast genome.

Gene type	Count	Specific gene names
Protein-coding genes	34	
-NADH dehydrogenase complex	9	nad1, nad2, nad3, nad4, nad4L, nad5, nad6, nad7, nad9
-Cytochrome c oxidase	3	cox1, cox2, cox3
-Cytochrome b	1	cob
-ATP synthase	5	atp1, atp4, atp6, atp8, atp9
-Cytochrome c maturation enzymes	4	ccmB, ccmC, ccmFc, ccmFn
-Ribosomal proteins (Small subunit)	6	rps1, rps3, rps4, rps10, rps12, rps14
-Ribosomal proteins (Large subunit)	2	rpl5, rpl16
-Other functional proteins	4	matR (Maturase), mttB (Transport Protein)
tRNA genes	18	
-Standard tRNAs	18	trnD-GUC, trnF-GAA, trnP-UGG, trnK-UUU, trnM-CAU (2 copies), trnE-UUC, trnT-UGU, trnI-CAU, trnG-GCC, trnQ-UUG, trnW-CCA, trnC-GCA, trnN-GUU, trnY-GUA, trnH-GUG
rRNA genes	3	
-Ribosomal RNAs	3	rrn5 (5S rRNA), rrn18 (18S rRNA), rrn26 (26S rRNA)
Total coding genes	55	34 (Proteins) + 18 (tRNAs) + 3 (rRNAs)
Photosynthesis-related genes	21	psaA, psaB, psaC, psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, psbZ, petA, petB, petD
ATP synthase subunits	6	atpA, atpB, atpE, atpF, atpH, atpI
NADH dehydrogenase complex	11	ndhA, ndhB, ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK
Ribosomal proteins (Large Subunit)	9	rpl2, rpl14, rpl16, rpl20, rpl22, rpl23, rpl32, rpl33, rpl36
Ribosomal proteins (Small Subunit)	11	rps2, rps3, rps4, rps7, rps8, rps11, rps12, rps14, rps15, rps16, rps18
rRNA genes	4	rrn16, rrn23, rrn4.5, rrn5
tRNA genes	30	trnH-GUG, trnL-UAA, trnK-UUU, trnY-GUA, trnE-UUC, ...
RNA polymerase genes	4	rpoA, rpoB, rpoC1, rpoC2
Hypothetical/Putative genes	3	ycf1, ycf2, ycf3
Other functional genes	3	matK, ccsA, clpP
Total coding genes	111	77 (Proteins) + 30(tRNAs) + 4(rRNAs)

42.21%, 37.02%; chloroplasts: 46.38%, 38.54%, 26.54% indicating an AT-rich bias at the third position and weak codon usage bias (Table 2). This conserved A/U-ending preference, consistent with *Arabidopsis* and soybean (Zhao *et al.*, 2021), reflects the combined effects of translational selection and mutational pressure in *M. sativa* organelles.

Neutral plot analysis

In *Medicago sativa* mitochondria,GC_3 ranged from 26.01-57.40% and GC_{12} from 37.89-50.39%. The regression y = -0.0275x + 0.468 (R^2 = 0.0023) showed no correlation between GC_{12} and GC_3, indicating nonuniform base composition and strong selective influence on codon bias.For chloroplast genes (Fig 3), GC_3 varied from 19.78-33.46% and GC12 from 32.17-53.24%, with y = 0.1375x + 0.3881 (R^2 = 0.0075) revealing a weak positive correlation, suggesting joint effects of selection and mutation pressure. Following Sharp and Li (1987), low GC_{12}-GC_3 correlations indicate selection dominance. Thus, in both organelles, codon bias is mainly shaped by selection rather than mutation, consistent with translational efficiency models (Sueoka, 1999; Bhattacharyya *et al.*, 2019). Similar selection-driven patterns occur in *Arabidopsis*, maize and soybean (Smith *et al.*, 2011; Wang *et al.*, 2020).

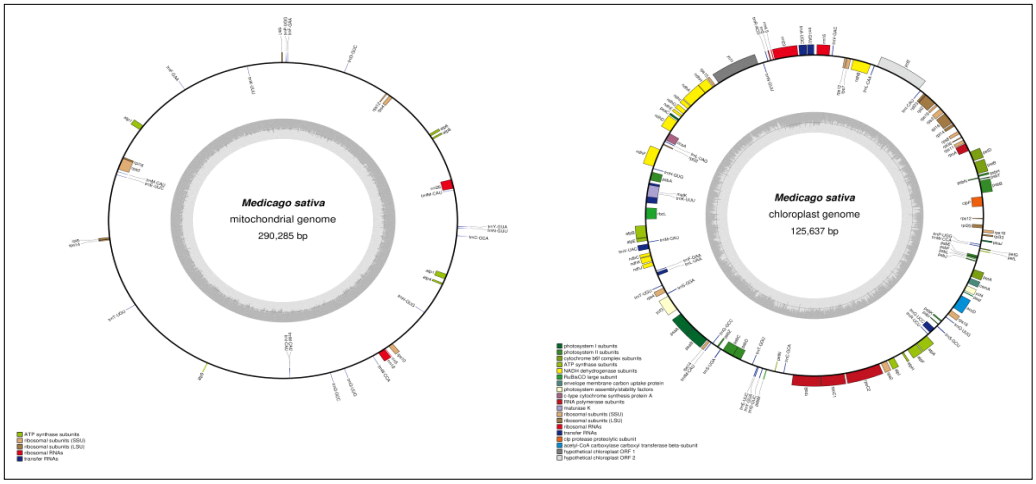


Fig 1: Circular gene map of the mitochondrial mitochondrial and chloroplasts in *Medicago sativa.*

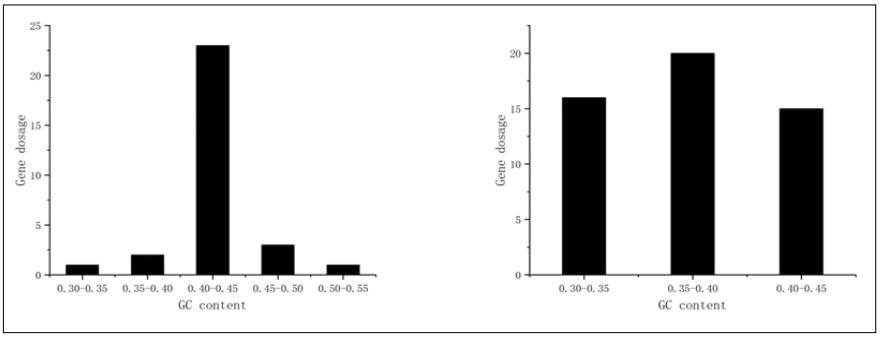


Fig 2: Mitochondrial and chloroplasts GC content.

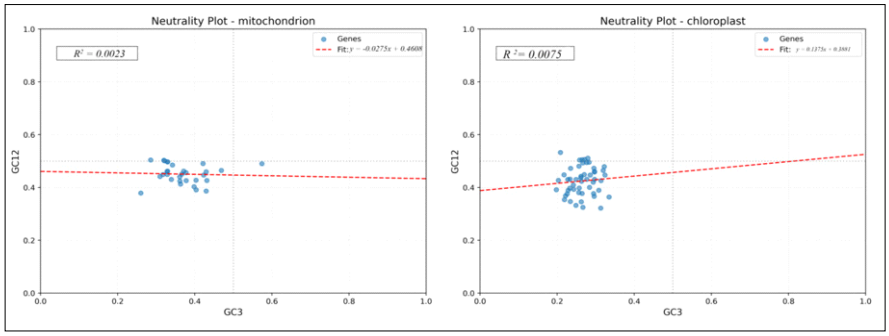


Fig 3: Neutral graph analysis of mitochondrial and chloroplasts codons.

Relative codon adaptation

The codon adaptation index (CAI) measures how closely gene codon usage matches that of highly expressed genes, reflecting expression efficiency (Sharp and Li, 1987). In *Medicago sativa* mitochondria, CAI values ranged from 0.123 to 0.234 (Fig 4), indicating low expression potential. CAI correlated positively with ENC and GC content, suggesting codon bias is mainly shaped by nucleotide composition rather than translational selection.In chloroplasts, CAI values ranged from 0.119 to 0.305 (Fig 5), showing low–moderate expression potential. Here, CAI correlated negatively with ENC and positively with GC content, implying greater influence of gene expression.Thus, mitochondrial codon usage is composition-driven, whereas chloroplast codon usage reflects both compositional and translational selection. These trends agree with broader plant organelle patterns, where CAI effectively predicts expression potential (dos Reis *et al.*, 2003).

ENC plot analysis of codon usage bias

The effective number of codons (ENC) quantifies codon usage bias, where values near 20 indicate strong bias and values close to 61 suggest random codon use (Wright, 1990).

In *Medicago sativa* mitochondria, ENC values ranged from 44.73-61.00, all above 28, revealing weak codon bias.

The ENC-GC3 plot (Fig 6) showed most genes close to the expected curve, suggesting that mutational pressure predominates but natural selection also contributes.

Similarly, chloroplast genes displayed ENC values of 38.90-57.76, indicating weak bias. The ENC-GC3 relationship mirrored the mitochondrial pattern, implying joint influences of mutation and selection on codon preference.

These findings align with neutral and PR2-plot analyses, indicating dual constraints: background nucleotide composition shaped by mutation and fine-tuning by translational selection for expression efficiency. Comparable trends were observed in other species-soybean (Gualberto *et al.*, 2014) and maize-where selection intensified in highly expressed or domestication-related genes.

PR2-plot bias analysis

The PR2 (Parity Rule 2) plot assesses nucleotide asymmetry (A vs. T, G vs. C) at the third codon position, where (0.5, 0.5) indicates no bias (Parvathy *et al.*, 2022). In *Medicago sativa* mitochondria (Fig 7), cytosine occurred less often than guanine and thymine more than adenine, with most genes below the midline (y<0.5), showing preference for G/T-ending codons-implying effects of both mutation and selection. Similarly, chloroplast genes favored G and T at the third position, also clustering below the center.

Combined with ENC and neutrality analyses, these findings suggest that mutational bias, natural selection and organelle-specific evolution jointly shape codon usage in *M. sativa*, consistent with reports for *Miscanthus* and *Arachis* (Sheng *et al.*, 2021; Yang *et al.*, 2023; Shen *et al.*, 2025).

Table 2: Codon preference parameters of mitochondrial and chloroplast genome coding genes in *Medicago sativa*.

Species	CDS no.	Codon no.	T3%	C3%	A3%	G3%	GC1/%	GC2/%	GC3/%	GC12/%	GC-all/%	CAI	ENC
NC_068105	30	9479	33.45	19.12	28.65	18.78	47.95	42.21	37.02	45.06	42.47	16.36	53.16
MZ983396	51	20704	39.59	12.05	33.86	14.49	46.38	38.54	26.54	42.46	37.15	17.12	45.55

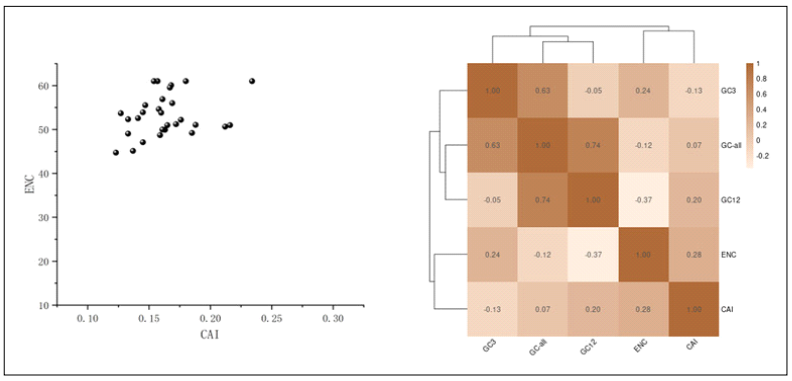


Fig 4: Relative adaptability of mitochondrial codons.

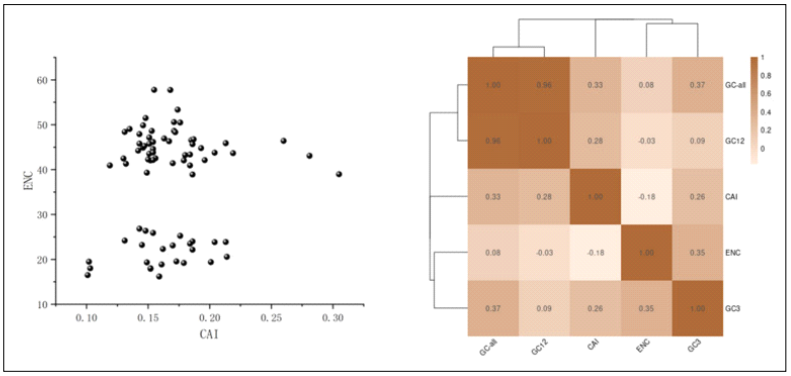


Fig 5: Relative adaptability of chloroplast codons.

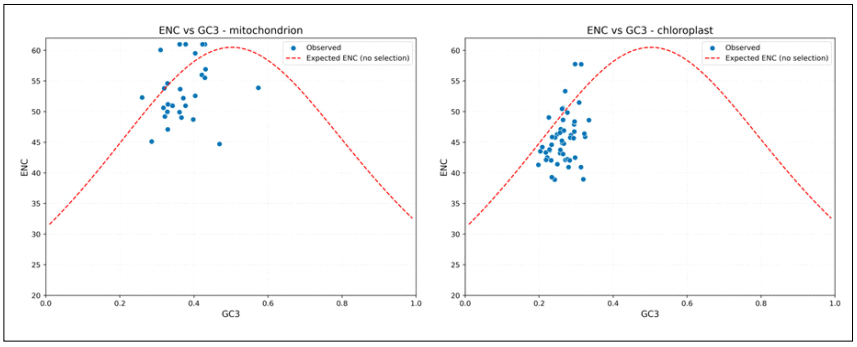


Fig 6: Association analysis mitochondrial and chloroplasts between ENC and GC3.

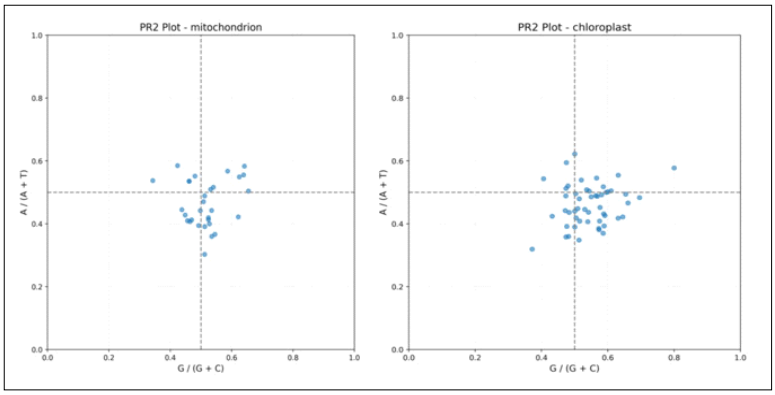


Fig 7: Mitochondrial and chloroplasts PR2-plot bias analysis.

Relative synonymous codon usage analysis

Relative synonymous codon usage (RSCU) quantifies the frequency of a codon relative to its expected occurrence under equal usage. Values greater than 1 indicate codon preference, while those below 1 indicate avoidance.

In the *Medicago sativa* mitochondrial genome, 32 codons had RSCU>1, with 71.88% ending in A or U-particularly favoring U-ending codons-revealing a strong AU-rich bias (Fig 8). Similarly, 31 codons in the chloroplast genome showed RSCU>1 and 96.55% of these ended in A or U, confirming an AT-rich trend (Fig 9). This pattern corresponds to the low GC3 content and suggests that both translational selection and mutational pressure shape codon usage. Comparable A/U-ending preferences have been reported in *Arachis* and *Miscanthus* chloroplast genomes, especially in photosynthesis-and stress-related genes (Yang *et al.*, 2023; Sheng *et al.*, 2021). Such compositional and selective influences ensure efficient translation and evolutionary stability.

Overall, codon usage in *M. sativa* is clearly nonrandom, reflecting the combined effects of nucleotide composition and evolutionary selection, consistent with patterns observed in other higher plants.

Optimal codon analysis

Optimal codons, defined as those most frequent in highly expressed genes, reflect translational efficiency and tRNA abundance (dos Reis *et al.*, 2003). In the *Medicago sativa* mitochondrial genome (Table 3), 13 optimal codons were identified (ΔRSCU≥0.08, RSCU≥1), six ending with U and five with A, showing a clear A/U-ending bias consistent with its AT-rich composition. Similarly, 14 optimal codons were detected in the chloroplast genome, mainly A-or U-ending, again indicating preference for A/U codons.

This pattern agrees with previous findings that organelle genomes favor codons matching abundant tRNAs for efficient translation (Zhao *et al.*, 2021). Comparable A/U-ending preferences have also been reported in *Arachis* and *Miscanthus* chloroplasts (Yang *et al.*, 2023; Sheng *et al.*, 2021). Such organelle-specific codon usage provides valuable references for transgene optimization and synthetic biology applications in *M. sativa* and related species.

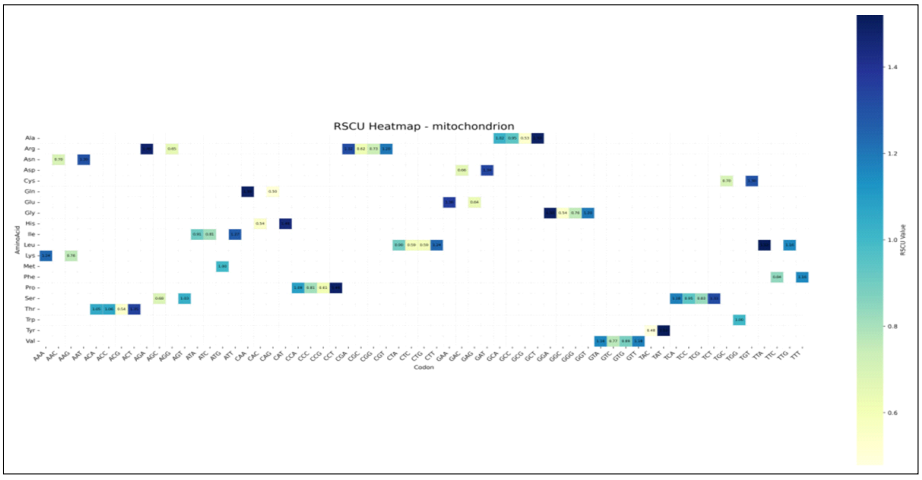


Fig 8: RSCU analysis of amino acids in *Medicago sativa* mitochondrial genome.

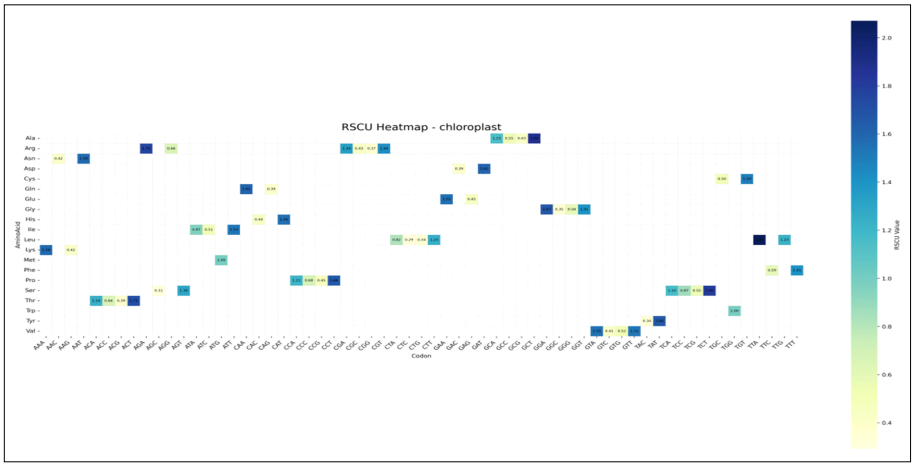


Fig 9: RSCU analysis of amino acids in the chloroplast genome of *Medicago sativa.*

Table 3: Relative frequency of synonymous codons used in *Medicago sativa* mitochondria and chloroplasts.

Amino acids	Codon	High expression		Low expression		ΔRSCU	Amino acids	Codon	High expression		Low expression		ΔRSC
		RSCU	Amount	RSCU	Amount				RSCU	Amount	RSCU	Amount	
Phe	UUU	1.28	48	1.39	16	-0.11	Ser	UCU*	1.67	22	1.58	10	0.09
	UUC*	0.72	27	0.61	7	0.11		UCC*	0.84	11	0.63	4	0.21
Leu	UUA	1.72	27	1.7	17	0.02		UCA	1.14	15	1.58	10	-0.44
	UUG	0.96	15	1.4	14	-0.44		UCG	0.76	10	0.95	6	-0.19
	CUU	1.28	20	1.5	15	-0.22	Pro	CCU	1.19	17	1.53	13	-0.34
	CUC*	0.7	11	0.3	3	0.4		CCC	0.84	12	0.82	7	0.02
	CUA*	0.77	12	0.5	5	0.27		CCA*	1.26	18	0.82	7	0.44
	CUG	0.57	9	0.6	6	-0.03		CCG	0.7	10	0.82	7	-0.12
Ile	AUU	1.29	33	1.23	16	0.06	Thr	ACU*	1.26	18	0.44	3	0.82
	AUC*	0.94	24	0.77	10	0.17		ACC*	1.4	20	1.19	8	0.21
	AUA	0.78	20	1	13	-0.22		ACA	0.91	13	1.63	11	-0.72
Met	AUG	1	30	1	11	0		ACG	0.42	6	0.74	5	-0.32
Val	GUU*	1.47	25	1.04	7	0.43	Ala	GCU	1.65	26	2	15	-0.35
	GUC	0.47	8	0.59	4	-0.12		GCC	0.63	10	0.8	6	-0.17
	GUA*	1.29	22	1.04	7	0.25		GCA*	1.52	24	0.8	6	0.72
	GUG	0.76	13	1.33	9	-0.57		GCG	0.19	3	0.4	3	-0.21
Tyr	UAU	1.47	25	1.67	5	-0.2	Cys	UGU	1.5	3	2	4	-0.5
	UAC	0.53	9	0.33	1	0.2		UGC*	0.5	1	0	0	0.5
TER	UAA*	2	2	1	1	1	TER	UGA	1	1	2	2	-1
	UAG	0	0	0	0	0	Trp	UGG	1	26	1	7	0
His	CAU	1.69	27	1.82	10	-0.13	Arg	CGU*	1.66	8	1.33	10	0.33
	CAC*	0.31	5	0.18	1	0.13		CGC*	0.62	3	0.4	3	0.22
Gln	CAA	1.69	11	1.67	10	0.02		CGA	1.45	7	1.47	11	-0.02
	CAG	0.31	2	0.33	2	-0.02		CGG*	1.24	6	0.8	6	0.44
Asn	AAU*	1.43	15	1.18	10	0.25	Ser	AGU*	1.14	15	0.47	3	0.67
	AAC	0.57	6	0.82	7	-0.25		AGC	0.46	6	0.79	5	-0.33
Lys	AAA*	1.25	10	1.08	14	0.17	Arg	AGA	0.41	2	1.47	11	-1.06
	AAG	0.75	6	0.92	12	-0.17		AGG*	0.62	3	0.53	4	0.09
Asp	GAU	1.58	19	1.87	14	-0.29	Gly	GGU	1.47	32	1.41	12	0.06
	GAC*	0.42	5	0.13	1	0.29		GGC	0.46	10	0.47	4	-0.01
Glu	GAA	1.42	17	1.58	15	-0.16		GGA	1.33	29	1.65	14	-0.32
	GAG*	0.58	7	0.42	4	0.16		GGG*	0.74	16	0.47	4	0.27
Phe	UUU*	1.49	29	1.06	36	0.43	Ser	UCU	2	18	2.05	28	-0.05
	UUC	0.51	10	0.94	32	-0.43		UCC	0.56	5	0.66	9	-0.1
Leu	UUA*	2.31	25	2.11	40	0.2		UCA*	1.44	13	1.17	16	0.27
	UUG	0.79	12	0.95	18	-0.16		UCG	0.22	2	0.59	8	-0.37

Table 3: Continue....

Table 3: Continue....

	CUU	1.32	20	1.63	31	-0.31	Pro	CCU	1.55	12	1.79	21	-0.24
	CUC*	0.26	4	0.16	3	0.1		CCC	0.65	5	0.68	8	-0.03
	CUA	0.73	11	0.95	18	-0.22		CCA*	1.29	10	0.85	10	0.44
	CUG*	0.59	9	0.21	4	0.38		CCG	0.52	4	0.68	8	-0.16
Ile	AUU	1.61	38	1.57	56	0.04	Thr	ACU	1.4	20	1.63	24	-0.23
	AUC	0.42	10	0.67	24	-0.25		ACC	0.91	13	0.88	13	0.03
	AUA*	0.97	23	0.76	27	0.21		ACA*	1.26	18	0.95	14	0.31
Met	AUG	1	17	1	28	0		ACG	0.42	6	0.54	8	-0.12
Val	GUU	1.16	13	1.76	29	-0.6	Ala	GCU	1.53	26	2.47	42	-0.94
	GUC	0.36	4	0.48	8	-0.12		GCC*	0.88	15	0.24	4	0.64
	GUA*	1.69	19	1.45	24	0.24		GCA*	1.06	18	0.94	16	0.12
	GUG*	0.8	9	0.3	5	0.5		GCG*	0.53	9	0.35	6	0.18
Tyr	UAU*	1.6	28	1.35	25	0.25	Cys	UGU	1.33	8	1.75	7	-0.42
	UAC	0.4	7	0.65	12	-0.25		UGC*	0.67	4	0.25	1	0.42
TER	UAA*	1.8	3	1.2	2	0.6	TER	UGA	0.6	1	1.2	2	-0.6
	UAG	0.6	1	0.6	1	0	Trp	UGG	1	10	1	25	0
His	CAU	1.38	9	1.64	18	-0.26	Arg	CGU	0.57	5	1.85	16	-1.28
	CAC*	0.62	4	0.36	4	0.26		CGC	0.45	4	0.69	6	-0.24
Gln	CAA	1.58	34	1.71	30	-0.13		CGA*	2.04	18	1.5	13	0.54
	CAG*	0.42	9	0.29	5	0.13		CGG*	0.68	6	0.12	1	0.56
Asn	AAU*	1.52	44	1.2	33	0.32	Ser	AGU*	1.67	15	0.95	13	0.72
	AAC	0.48	14	0.8	22	-0.32		AGC	0.11	1	0.59	8	-0.48
Lys	AAA	1.55	34	1.67	41	-0.12	Arg	AGA	1.13	10	1.5	13	-0.37
	AAG*	0.45	10	0.33	8	0.12		AGG*	1.13	10	0.35	3	0.78
Asp	GAU	1.65	33	1.62	21	0.03	Gly	GGU	0.75	10	2.2	39	-1.45
	GAC	0.35	7	0.38	5	-0.03		GGC*	0.68	9	0.23	4	0.45
Glu	GAA	1.49	50	1.6	44	-0.11		GGA*	2.04	27	1.18	21	0.86
	GAG*	0.51	17	0.4	11	0.11		GGG*	0.53	7	0.39	7	0.14

Table 4: Mitochondria and chloroplast codon Ka/Ks analysis.

Gene pairs	Ka	Ks	Ka/Ks
nad2/rps4	2.0042	2.4495	0.818207798
nad2/rps12	3.9395	0	0
nad2/rpl16	0	2.8804	0
nad2/rps3	1.88	2.0245	0.928624352
nad2/rps14	2.1887	2.2186	0.986523033
ccmB/rps4	0	1.2437	0
ccmB/rps12	2.7599	3.8693	0.713281472
ccmB/rpl16	0	2.6288	0
ccmB/rps3	2.4993	0	0
ccmB/rps14	2.0549	1.4015	1.46621477
atp8/rps4	0	1.0483	0
atp8/rps12	1.534	1.579	0.97150095
atp8/rpl16	2.0567	1.1111	1.85104851
atp8/rps3	3.0088	2.038	1.476349362
atp8/rps14	1.1682	0	0
atp6/rps4	0	0	0
atp6/rps12	0	1.9645	0
atp6/rpl16	2.248	2.0477	1.097817063
atp6/rps3	2.4819	0	0
atp6/rps14	2.6044	2.1581	1.20680228
rps4/rps12	2.0872	1.7485	1.193708893
rps4/rpl16	0	0	0
rps4/rps3	0	0	0
rps4/rps14	3.0652	2.0147	1.521417581
rps12/rpl16	1.3318	2.0709	0.643102033
rps12/rps3	1.6952	1.3253	1.279106617
rps12/rps14	0	1.9496	0
nad3/rpl16	0	1.8201	0
nad3/rps3	2.8333	0	0
nad3/rps14	2.0179	3.5227	0.572827661
nad4/rpl16	0	1.3231	0
nad4/rps3	2.6957	0	0

Analysis of evolutionary selection pressure

The Ka/Ks ratio indicates selective pressure on protein-coding genes: values >1, <1 and ≈1 represent positive, purifying and neutral selection, respectively (Nei and Gojobori, 1986). Pairwise Ka/Ks analyses of *Medicago sativa* mitochondrial and chloroplast genes were conducted using MEGA11 (Kumar *et al.*, 2016) and DnaSP v5.10 (Rozas *et al.*, 2017). Mitochondrial genes showed a mean Ka/Ks of 0.52, indicating predominant purifying selection, though several pairs (*e.g.*, ccmB/rps14, atp8/rps3, rps4/rps14) exhibited Ka/Ks>1, suggesting adaptive evolution in respiration-related genes (Table 4). Chloroplast genes had a mean Ka/Ks of 0.70, also under purifying selection, with some pairs (*petD/rps3*, *rpl14/rpl16*, *rps11/rps3*) showing signs of positive selection in photosynthetic or ribosomal functions (Table 5).

These patterns align with previous reports that photosynthetic genes frequently experience adaptive evolution, whereas core metabolic genes remain conserved (Yang, 2007; Sloan *et al.*, 2017), reflecting a balance between conservation and adaptation in *M. sativa* organelles.

Analysis of organelle fragment exchange

Horizontal gene transfer (HGT) between mitochondria and chloroplasts is common in plants. In *Medicago sativa*, multiple collinear regions between mitochondrial and chloroplast genes indicate inter-organelle exchange. Several tRNA genes (*e.g.*, *trnW-CCA*, *trnN-GUU*, *trnD-GUC*) were shared between both genomes (Fig 10), suggesting ancient transfer events and functional conservation (Smith, 2011; Morley *et al.*, 2017). Similarly, collinearity between mitochondrial 18S/26S rRNAs and chloroplast 16S/23S rRNAs implies coordinated ribosomal evolution supporting translational compatibility. Fragments of *nad7* also aligned with chloroplast *23S rRNA*, reflecting co-evolution between respiration and translation systems (Gualberto and Newton, 2017). Overall, HGT between the two organelles in *M. sativa* likely promotes genome stability, redundancy and adaptive

Table 5: Ka/Ks analysis of 22 pairs of repeat gene pairs in chloroplasts codon.

Gene pairs	Ka	Ks	Ka/Ks
clpP/rpl16	0	1.652	0
clpP/rps3	0	0	0
clpP/rps12	0	1.843	0
clpP/rps4	2.4027	0	0
clpP/rps14	0	1.49	0
psbB/rpl16	1.7729	1.2693	1.396754116
psbB/rps3	0	1.7641	0
psbB/rps12	1.8392	1.4641	1.256198347
psbB/rps4	2.3558	2.171	1.085122064
psbB/rps14	0	1.4523	0
petB/rpl16	0	1.76	0
petB/rps3	1.8217	4.2874	0.424896207
petB/rps12	0	0	0
petB/rps4	2.1789	2.0637	1.055822067
petB/rps14	4.8834	1.5949	3.061884758
petD/rpl16	2.0008	0	0
petD/rps3	2.055	0.9667	2.125788766
petD/rps12	2.4077	2.0849	1.15482757
petD/rps4	3.5478	0	0
petD/rps14	0	2.4087	0
rpoA/rpl16	0	1.5286	0
rpoA/rps3	1.5263	1.6134	0.946014627
rpoA/rps12	1.9143	1.5627	1.224995201
rpoA/rps4	1.6802	1.2145	1.383449979
rpoA/rps14	0	2.2322	0
rps11/rpl16	1.9493	0	0
rps11/rps3	3.1223	2.2237	1.404101273
rps11/rps12	1.921	1.5674	1.225596529
rps11/rps4	2.1133	1.7847	1.18412058
rps11/rps14	1.8633	0	0
rpl14/rpl16	3.5771	1.9846	1.802428701
rpl14/rps3	2.2119	1.3695	1.615115005

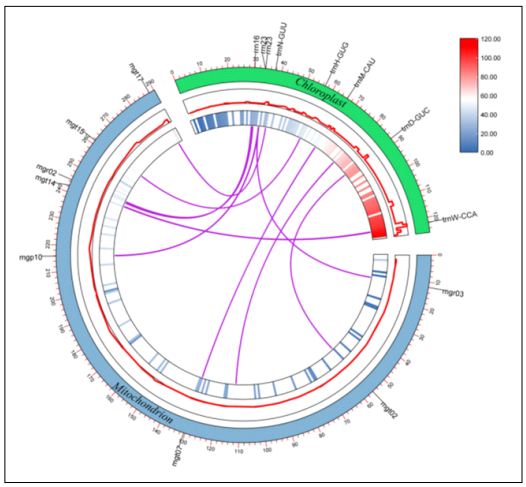


Fig 10: Analysis of organelle fragment exchange.

flexibility through mutation, recombination and gene transfer (Hao and Palmer, 2009; Rice *et al*., 2013).

CONCLUSION

This study comparatively analyzed the mitochondrial and chloroplast genomes of *Medicago sativa*, focusing on codon usage bias, nucleotide composition and selection pressure. Results showed that codon usage in both organelles is mainly shaped by natural selection and mutation pressure, with a preference for A/U-ending codons and generally AT-rich composition, especially at the third codon position.Thirteen and fourteen optimal codons were identified in the mitochondrial and chloroplast genomes, respectively, most ending in A or U, indicating translational selection. Ka/Ks analysis revealed that most genes are under purifying selection, while a few show positive selection, suggesting adaptive evolution. Gene fragment exchange between organelles further reflects their evolutionary interaction. Overall, the findings clarify organellar genome evolution in *M. sativa* and provide guidance for transgenic improvement-organelle-specific codon optimization could enhance gene expression and support breeding of more resilient, high-yield cultivars.

ACKNOWLEDGEMENT

The present study was supported by the Major Demonstration Program for Seed Industry Science and Technology Innovation of the Inner Mongolia Autonomous Region (Grant No. 2022JBGS0040) and Key Laboratory of Grassland Germplasm Innovation and Sustainable Utilization of Grassland Resources in Inner Mongolia Autonomous Region-Research on High-Quality herbage breeding and High-Yield, Stress-Resistant Cultivation Techniques (2025KYPT0033).

Disclaimers

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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