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Complete Mitochondrial Genome Characterization of *Saga hakkarica* Şirin & Taylan, 2019 (Orthoptera: Tettigoniidae, Saginae): with Notes on Intrageneric Mitogenomics

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ABSTRACT

In this study, the complete mitochondrial genome (mitogenome) of *Saga hakkarica* Şirin & Taylan, 2019, an Anatolian endemic species belonging to the order Orthoptera, family Tettigoniidae, subfamily Saginae, was characterized and genetically analysed. Next-generation sequencing technologies were employed to obtain the mitogenome sequences of *S. hakkarica*. With a total of 14,508,962 reads were generated, with an overall sequence length of approximately 2.19 Gbp. The mitogenome exhibited a GC content of 46.8% and an AT content of 53.2%. The analyses showed that the mitogenome of *S. hakkarica* is 15,755 bp in length and comprises 37 genes. These include 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes, 13 protein-coding genes (PCGs), along with a control region. Furthermore, it was determined that 23 of the 37 genes are located on the forward strand, while the remaining 14 genes are situated on the reverse strand. The mitogenome of *S. hakkarica* shows a high degree of similarity to Orthoptera mitogenomes reported in the literature with respect to the number of PCG, tRNA, and rRNA genes. Although the start and stop codons exhibit overall similarity, specific at the sequence-level differences were identified. In conclusion, this study presents, for the first time, the structural and functional characteristics of the complete mitogenome of *S. hakkarica*. As the second mitogenome dataset obtained from the subfamily Saginae, it provides an intrageneric evaluation and an important resource for future genetic and phylogenetic studies.

Keywords: Insecta, next-generation sequencing, genomic library, Anatolian endemic species, molecular evolution.

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INTRODUCTION

The use of mitochondrial DNA (mtDNA) sequences in phylogenetic studies offers several advantages, including the absence of introns and the compact, conserved structure of mitochondrial genomes, which simplifies sequencing efforts. In Orthoptera, interest in complete mitochondrial genome studies has grown since the mitogenome of *Locusta migratoria* was published (Flook, Rowell, & Gellissen, 1995). In Tettigoniidae, mitogenome-based studies have become a useful tool for clarifying evolutionary relationships (Zhou, Ye, Huang, & Shi, 2010; Zhou et al., 2017; Mugleston, Naegle, Song, & Whiting, 2018; Zhao et al., 2025). Collectively, these studies have clarified phylogenetic patterns within Tettigoniidae, with many supporting the monophyly or close relationships of subfamilies such as Phaneropterinae, Meconematinae, Mecopodinae, Conocephalinae, and Tettigoniinae. Insects typically possess a circular mitochondrial genome of 14-20 kb, containing 13 protein-coding genes (PCGs), 22 tRNAs, and two rRNAs, with conserved gene content and order (Wolstenholme, 1992; Zhang, Szymura, & Hewitt, 1995; Zhao et al., 2025). Such conservation enhances their utility for evolutionary studies. In mitogenomic studies, features such as gene order, base composition, and protein-coding regions have been commonly used to infer phylogenetic relationships (Cameron, 2014; Wang, Huang, & Qiao, 2014; Zhao et al., 2025). The maternal inheritance and high mutation rate (~2% per million years) of mtDNA enhance its inability to reconstructing maternal lineages (Brown, George, & Wilson, 1979; Moritz, Dowling, & Brown, 1987; Avdeyev Jiang, Aganezov, Hu, & Alekseyev, 2016). Genes such as *cox1*, *cytb*, and *nad* are frequently used in phylogenetics because of their high substitution sensitivity. While mtDNA lacks recombination, which benefits phylogenetic clarity, evolutionary rates may vary depending on environmental and biological factors (Rand, 2001; Nabholz, Glemin, & Galtier, 2008). Marker-based approaches have limitations such as heteroplasmy, NUMTs, and rare recombination events, which can affect accuracy (Parr et al., 2006; Wang, Xiang, Ning, Liu, Liu, & Zhao, 2020). Challenges also include low DNA quality, GC-rich region biases, and PCR efficiency. Sanger sequencing, though useful for targeted regions such as *cox1*, *cytb*, 12S rRNA, and 16S rRNA, is limited by low throughput and cost (Kircher & Kelso, 2010; Stewart & Chinnery, 2015). Next-generation sequencing (NGS) platforms like Illumina and long-read technologies now offer high-throughput, cost-effective solutions for complete mitogenome studies (Crampton-Platt, Yu, Zhou, & Vogler, 2016; Wang et al., 2020), improving phylogenetic resolution.

According to GenBank, approximately 8,725 mitogenomes from 5,831 species across 28 insect orders have been sequenced to date (Cameron, 2025). Diptera, Lepidoptera, Hemiptera, and Blattodea dominate the dataset, while Orthoptera accounts for only 383 mitogenomes from 281 species (Cameron, 2025). Orthoptera remains a promising group for the study of mitogenomic evolution (Fenn, Song, Cameron, & Whiting, 2008). Gaugel, Hawlitschek, Dey, & Husemann (2023) identified major mitochondrial rearrangements in Orthoptera, including inversions, translocations, and tandem duplication/random loss events. The mitogenome contains 13 PCGs vital for oxidative phosphorylation (OXPHOS), 22 tRNAs, two rRNAs, and an A+T-rich control region with regulatory motifs (Wolstenholme, 1992; Boore, 1999; Crozier & Crozier, 1993; Chen et al., 2019). The *cox1*, *cox2*, *cox3*, and *cytb* loci encode parts of the cytochrome c oxidase complex and cytochrome b; *nad1-nad6*

encode NADH dehydrogenase subunits. Mitochondrial transcripts are processed according to the tRNA punctuation model (Xu et al., 2023). The considerable variation observed in gene arrangement and transcriptional patterns among insects (Cameron, 2014; Song et al., 2015, 2016) is considered valuable for identifying lineage-specific evolutionary signals. Research focusing on the *cox1* gene in particular has facilitated the development of DNA barcoding techniques (Hebert, Cywinska, Ball, & deWaard, 2003). Given that the evolutionary rate of mitochondrial DNA is generally faster than that of nuclear DNA, mtDNA is considered a key molecular marker for phylogenetic studies (Avise et al., 1987).

Saga is among the orthopteran genera for which hardly any mtDNA data exists to date. It comprises 17 species across the Palearctic, with primary distributions in Anatolia and the Balkans (Kaltenbach, 1970, 1974; Şirin, Taylan, Sevgili, & Mol, 2019; Cigliano, Braun, Eades, Otte, 2025). In Anatolia, 10 species are recorded, including the endemic *S. hakkarica* (Karabağ, 1958; Şirin et al., 2019; Cigliano et al., 2025). The other *Saga* species have been reported from the Balkans (Kaltenbach, 1970; Lemonnier-Darcemont et al., 2009; Kolics et al., 2012). In contrast, *S. pedo* shows a broad distribution range extending from Western Europe to Central Asia (Kolics et al., 2008).

Only three molecular studies on *Saga* exist (Giannoulis et al., 2011; Kolics et al., 2012; Binici, Şirin, & Taylan, 2021), primarily using partial mitochondrial and nuclear gene sequences. To date, the only available mitogenomic study within the genus *Saga* is on the mitogenome of *S. natoliae* (Karakaş, Uluar, Yartaş, & Çıplak, 2025). In Anatolian Orthoptera, most mtDNA studies focus on specific genes like 16S rDNA, cox1, and ITS (Taylan, Di Russo, Rampini, & Ketmaier, 2013; Kaya, Boztepe, & Çıplak, 2015; Taylan & Şirin, 2016; Kaya, 2018; Şirin, Taylan, Bircan, Akyıldız, & Can, 2021), and only six species have fully sequenced mitogenomes (Öztürk & Çıplak, 2019; Karşı & Çıplak, 2019 a,b; Karakaş, Yahyaoğlu, Uluar, Budak, & Çıplak, 2023; Karakaş et al., 2025). This study presents the complete mitochondrial genome of *S. hakkarica*, an Anatolian endemic.

In phylogenetic studies of the subfamily Saginae, *cox2* and four nuclear genes have been used to infer evolutionary relationships, revealing that Saginae forms a sister group to other Tettigoniidae members (Mugleston et al, 2018). NGS technologies offer higher data throughput and precision than traditional Sanger methods, enabling full mitogenome characterization even in rare or endemic taxa. This study presents the complete mitochondrial genome of *S. hakkarica*, an endemic species from Hakkari Province (Turkey). It focuses on genome size, protein-coding genes, tRNA and rRNA content, and the non-coding A+T-rich region. In the present study, we aimed to generate and comprehensively characterize the complete mitochondrial genome of *Saga hakkarica*, an Anatolian-endemic species for which genomic resources are currently lacking. As this represents only the second mitogenome available for the subfamily Saginae, one of the principal aims of the study was to expand the existing mitogenomic dataset and provide a reliable framework for downstream comparative and evolutionary analyses. The newly assembled mitogenome is intended to serve as a reference sequence for future studies focusing on species delimitation, population genetics, and molecular systematics of *S. hakkarica* and related species. Additionally, the dataset contributes essential markers for improving phylogenetic resolution within Orthoptera, particularly across Tettigoniidae and

Saginae. Collectively, the study aims to enhance the molecular baseline necessary for elucidating the evolutionary history and systematic affinities of this understudied lineage.

MATERIALS AND METHODS

Taxon sampling and studying methods

The specimens of *S. hakkarica* analysed in this study were collected using an insect net from the type locality of the species: Hakkari, Bayköy, 2035 m, N: 37°32.89 E: 43°42.70, 09.VII.2024, 2♀♀, (leg.: M.S. Taylan & Ş. Beyter) (Fig. 1). The identification of the collected specimens was carried out based on relevant taxonomic literature (Harz, 1969; Kaltenbach, 1970; Şirin et al., 2019). Following identification, the specimens were preserved in 96% ethanol and transported to the laboratory of the Hakkari University Center for Biodiversity Application and Research (HAKBİYOM) for further laboratory analyses. The total and validated mitogenome sequence of *Saga hakkarica* was deposited in GenBank under the accession number *PX125591*.

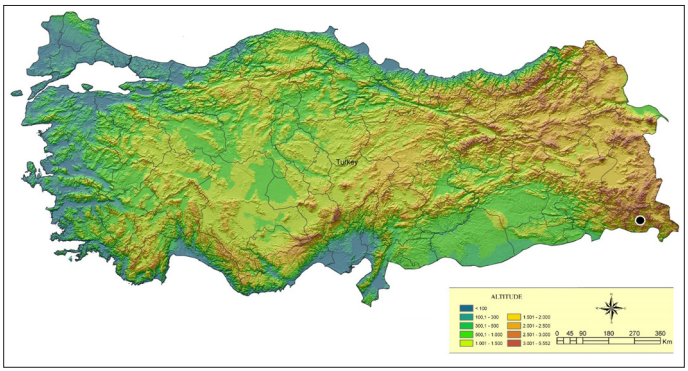

Figure 1. Sampling locality of *Saga hakkarica* (black circle with a white outline indicates the type and sampling locality).

Mitochondrial DNA isolation

From two individuals of *S. hakkarica*, the chitin layer of the right hind femur was carefully incised with a scalpel, and approximately 150 mg of underlying muscle tissue was extracted mtDNA-was then isolated from the obtained tissue samples using the Abcam mitochondrial DNA isolation kit (ab65321) and the related process.

DNA quality control, genomic library preparation, and sequencing process

DNA quantity and quality were determined with the QuantiFluor® dsDNA System (Promega) using a Victor Nivo Multimode Microplate Reader. Integrity was assessed with an Agilent 4200 TapeStation System, and purity (260/280 and 260/230) was measured using a NanoPhotometer N120 (IMPLEN). For NGS analysis, DNA libraries were prepared using the Nextera DNA XT Kit (Illumina). Fragment size distribution was determined by the Agilent 2100 Bioanalyzer. Library concentrations were quantified following the

Illumina qPCR Quantification protocol using the LightCycler qPCR instrument. The sequencing process was carried out by an external provider (Macrogen) on the Illumina platform, targeting paired-end reads with a length of 150 bp. The obtained sequence data were provided in FASTQ format. As part of the quality control, base-by-base quality assessments were performed and Q20/Q30 scores were calculated.

Mitochondrial genome assembly process

Raw sequencing data were provided as two files in fastq.gz format and uploaded to the system via the Galaxy Tools platform (https://usegalaxy.eu/). For quality control and trimming, the Trimmomatic tool (Galaxy version 0.39+galaxy2), widely used for Illumina platform data, was applied; low-quality bases were cleaned and paired-end reads were aligned (Bolger, Lohse, & Usadel, 2014). The trimmed data were converted from FASTQ to FASTA format using FASTX-toolkit (Galaxy version 1.0.2+galaxy2) to prepare them for genome assembly. The mitochondrial genome assembly was performed using NOVOPlasty (Galaxy version 4.3.1+galaxy0), which achieves high accuracy on circular genomes (Dierckxsens, Mardulyn, & Smits, 2017). During the assembly, forward and reverse FASTA files were specified; the seed sequence was set as the Cox1 gene of *S. hakkarica* (Accession No: PQ628100) obtained from the NCBI GenBank database. The reference genome option was marked "Yes," and the mitogenome of *S. natoliae* (Orthoptera; Tettigoniidae; Saginae) (Accession No: PQ636017.1) was used as the reference genome. The platform was set to "Illumina," read length was 151 bp, and total paired-end length was set to 300 bp. Assembly parameters included the mitogenome as the genome type, target genome length ranges of 15,000-17,000 bp, and read overlap length of 39 bp. The "Extend seed" option was disabled. At the end of the process, a "circular assembly" output was obtained, and the complete mito-genome of *S. hakkarica* was assembled in FASTA format.

Mitochondrial genome characterization and statistics

The general features of the mitogenome of *S. hakkarica* were determined using the MITOS analysis module via the PROKSEE.CA web application (Donath et al, 2019; Grant et al., 2023). In this context, the genome data in FASTA format was uploaded to the system, and the annotation process was carried out by selecting the "Invertebrate mitochondrial" genetic code parameter. As a result of the analysis, a circular map of the complete mtDNA was obtained and used for visualization purposes in the study. Through the annotation data, protein-coding genes (PCGs), tRNA and rRNA genes, as well as non-coding regions, were systematically evaluated. The positions, lengths, start and stop codons, strand orientations (forward-reverse), and amino acid sequences of the protein-coding genes were identified; these data were validated by comparison with mitogenome data of grasshopper species available in the NCBI database. Similarly, the number, positions, lengths, anticodon types, and strand orientations of tRNA genes were characterized. For determining the start and end positions of rRNA genes, tRNA genes were used as references, and these locations were verified by comparison with mitogenomes of grasshopper species in the NCBI database. Additionally, the non-coding regions present on the mtDNA were identified, their sizes calculated, and A+T richness

ratios examined. To detect gene overlaps and intersecting regions, annotation data obtained in GFF format were analyzed using the Google Colab platform. During this process, the "pandas" (McKinney, 2010) and "re" libraries were utilized for data processing. Nucleotide and amino acid level analyses of the mitochondrial gene regions belonging to *S. hakkarica* were carried out using the Python programming language within the Google Colab environment. The analyses employed Biopython, pandas, matplotlib, and collections libraries. During the analysis, the *S. hakkarica* mitochondrial genome sequence in FASTA format and the annotation data in GFF3 format were uploaded to the Google Colab platform, and the full genome was read with Bio.SeqIO module. Only the 13 protein-coding gene regions defined as "CDS" in the GFF3 file were filtered. For genes located on the negative strand, the complementary sequences were reverse-complemented to obtain the correct sequences. For each gene region, total nucleotide percentages (%A, %T, %G, %C), as well as nucleotide percentages at the first, second, and third codon positions within coding sequences, were calculated. Amino acid-level analyses were performed through translation of the previously identified mitochondrial protein-coding gene sequences. Genetic translation was conducted using the Seq.translate function within the Biopython library based on NCBI Translation Table 1 (The Invertebrate Mitochondrial Code). The percentage of each amino acid in the resulting protein sequences was calculated. For each gene, the obtained protein sequence was analysed with the "collections.Counter" module to determine amino acid composition; these calculated percentages were tabulated using the pandas library. Amino acids were converted to the standard three-letter code for comparative analyses. Additionally, the amino acid percentage data were visualized in heatmap format for each gene and amino acid. Visualization was performed using the Seaborn and Matplotlib libraries within the Google Colab environment (Hunter, 2007).

Table 1. Amino Acid Composition (%) of Protein-Coding Genes (PCGs) in *Saga hakkarica*.

	nad1 (%)	nad2 (%)	nad3 (%)	nad4 (%)	nad4L (%)	nad5 (%)	nad6 (%)	cox1 (%)	cox2 (%)	cox3 (%)	atp6 (%)	atp8 (%)	Cytb (%)	Mean
Ala	6.33	4.39	5.13	4.71	3.06	5.31	2.29	7.80	4.85	6.87	6.25	5.66	6.61	5.33
Cys	1.58	1.17	0.85	2.02	3.06	1.66	0.57	0.00	0.88	0.38	0.45	0.00	0.79	1.03
Asp	1.58	0.29	2.56	1.12	1.02	2.49	1.71	2.92	3.96	1.15	0.45	1.89	2.91	1.85
Glu	3.16	2.34	4.27	2.02	4.08	2.49	1.14	1.75	3.96	2.67	1.79	0.00	0.79	2.34
Phe	11.71	7.89	9.40	7.17	12.24	6.47	7.43	7.60	6.17	9.92	8.48	13.21	8.47	8.94
Gly	7.59	4.68	2.56	8.74	6.12	7.30	2.29	8.97	3.52	7.25	4.91	0.00	6.35	5.41
His	0.00	1.75	0.85	1.35	2.04	0.83	1.14	2.92	3.08	5.73	2.68	5.66	3.17	2.40
Ile	7.28	10.23	14.53	7.40	4.08	7.96	13.71	8.97	8.37	8.02	9.82	1.89	11.11	8.72
Lys	2.22	2.63	3.42	2.69	1.02	2.32	3.43	1.17	1.76	0.38	0.89	3.77	1.85	2.12
Leu	17.41	18.71	16.24	20.40	17.35	15.92	17.71	13.45	12.78	12.60	17.86	16.98	14.55	16.30
Met	3.48	6.14	4.27	6.95	8.16	8.29	5.14	4.87	4.85	2.29	5.80	9.43	4.23	5.69
Asn	2.53	5.85	0.85	2.02	3.06	3.81	6.29	3.12	7.05	3.05	5.36	5.66	6.08	4.21
Pro	3.16	2.63	3.42	2.47	0.00	2.49	4.57	5.46	4.85	4.96	4.91	7.55	5.82	4.02
Gln	1.58	2.05	1.71	1.12	4.08	1.99	2.29	2.34	3.08	3.05	1.79	7.55	2.12	2.67
Arg	2.22	0.58	2.56	1.35	2.04	0.83	0.57	1.56	3.08	1.53	1.34	0.00	2.12	1.52
Ser	8.54	13.74	11.97	10.54	8.16	12.11	9.14	7.60	6.61	6.87	8.93	5.66	5.03	8.84
Thr	4.11	5.85	6.84	2.69	3.06	3.48	11.43	6.82	8.37	8.78	9.38	7.55	5.29	6.43
Val	6.65	2.92	4.27	6.05	13.27	6.97	3.43	6.24	6.17	4.96	4.46	0.00	4.76	5.40
Trp	2.22	2.63	4.27	2.91	2.04	2.49	1.14	2.92	2.64	4.96	2.23	5.66	3.17	3.02
Tyr	6.65	3.51	0.00	6.28	2.04	4.81	4.57	3.51	3.96	4.58	2.23	1.89	4.76	3.75

RESULTS

Mitochondrial genome characterization

The total mitogenome length was determined to be 15,755 bp. The mitogenome sequence and its corresponding amino acid translations were validated by GenBank curators and have been deposited in the GenBank database under accession number PX125591. The mitogenome of *S. hakkarica* contains total of 37 gene regions. These include 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes, and 13 protein-coding genes. The protein-coding genes comprise 7 NADH dehydrogenase subunit genes (*nad1-nad6* and *nad4L*), three cytochrome c oxidase subunit genes (*cox1-cox3*), one cytochrome *b* gene (*cob*), and two ATP synthase subunit genes (*atp6 and atp8*). In addition, a putative control region (origin of replication) was identified (Fig. 2). Strand orientation analysis revealed that 23 of the 37 annotated genes are located on the forward (F) strand, while the remaining 14 genes are encoded on the reverse (R) strand.

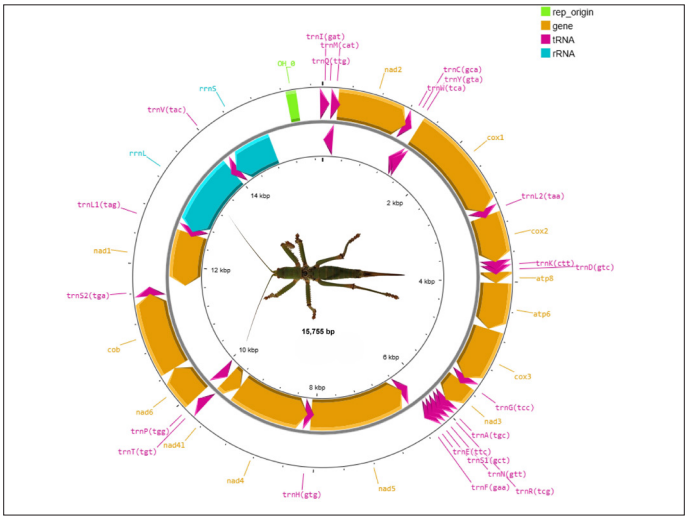

Figure 2. Mitogenome of *Saga hakkarica*, showing the arrangement of protein-coding genes, tRNAs, rRNAs, and the control region.

Characterization of protein-coding genes

The total lengths of PCGs were calculated and are presented as follows: *nad2* (1,029 bp), *cox1* (1,540 bp), *cox2* (682 bp), *atp8* (162 bp), *atp6* (673 bp), *cox3* (787 bp), *nad3* (354 bp), *nad6* (528 bp), and *cob* (1,137 bp). Most of these genes are located on the F strand of the mitochondrial genome. The remaining PCGs, including *nad5* (1,740 bp), *nad4* (1,339 bp), *nad4L* (297 bp), and *nad1* (951 bp), are located on the R strand (Table 2). Within the forward strand, the longest gene is *cox1* (1,540 bp), while the shortest is *atp8* (162 bp). On the reverse strand, the longest gene is *nad5* (1,740 bp), and the shortest is *nad4L* (297 bp) (Table 2). Further analysis identified the start and stop codons of all PCGs. The identified start codons include: ATT (*atp8, nad3, nad5,* and *nad6*), ATA (*atp6),* ATG (*cob, cox2, cox3, nad4,* and *nad4L*), GTG (*nad2*), TTG (*nad1*), and ATC (*cox1*). Stop codons were found to

be TAA for *atp8*, *nad1*, *nad2*, *nad3*, *nad4L*, *nad5*, *nad6,* and *cob,* whereas incomplete stop codons represented by a single T were identified in *cox1*, *cox2*, *cox3*, *nad4*, and *atp6*. The nucleotide sequences of the 13 PCGs in the mitogenome of *S. hakkarica* were analysed according to codon positions (1st, 2nd, and 3rd positions) separately (Table 3). The overall nucleotide composition was determined as follows: 28.61% A, 19.24% C, 38.10% T, and 14.05% G. Base composition by codon position was calculated as: 1st position: 30.21% A, 18.29% C, 31.27% T, and 20.23% G, 2nd position*:* 19.69% A, 21.32% C, 44.85% T, and 14.14% G, 3rd position: 35.91% A, 18.12% C, 38.21% T, and 7.76% G. At the amino acid level, each protein-coding gene was translated and the amino acid composition was calculated (Table 1 and Fig. 3). Leucine (Leu) was the most frequently represented amino acid, accounting for 16.30% of the total, followed by Phenylalanine (Phe) at 8.94% and Serine (Ser) at 8.84%. The least represented amino acids were Lysine (Lys) at 2.12%, Arginine (Arg) at 1.52%, and Cysteine (Cys) at 1.03%.

Table 2. Gene regions and order in the mitochondrial genome (mtDNA) of *Saga hakkarica*.

Gene	Start Position (bp)	End Position (bp)	Length (bp)	Start Codon	Stop Codon	Anti codon	Strand
trnI	1	65	65			GAU	F
trnQ	66	134	69			UUG	R
trnM	156	222	67			CAU	F
nad2	223	1251	1029	GTG	TAA		F
trnW	1250	1315	66			UCA	F
trnC	1308	1370	63			GCA	R
trnY	1371	1436	66			GUA	R
cox1	1429	2968	1540	ATC	T--		F
trnL2	2969	3033	65			UAA	F
cox2	3039	3720	682	ATG	T--		F
trnK	3730	3799	70			CUU	F
trnD	3799	3864	66				F
atp8	3865	4026	162	ATT	TAA		F
atp6	4023	4695	673	ATA	T--		F
cox3	4696	5482	787	ATG	T--		F
trnG	5483	5547	65			UCC	F
nad3	5548	5901	354	ATT	TAA		F
trnA	5915	5979	65			UGC	F
trnR	5980	6043	64			UCG	F
trnN	6065	6130	66			GUU	F
trnS1	6139	6205	67			GCU	F
tnrE	6206	6274	69			UUC	F
trnF	6273	6338	66			GAA	R
nad5	6349	8088	1740	ATT	TAA		R
trnH	8089	8154	66			GUG	R
nad4	8155	9493	1336	ATG	T--		R
nad4l	9487	9783	297	ATG	TAA		R
trnT	9786	9849	64			UGU	F
trnP	9849	9913	65			UGG	R
nad6	9915	10442	528	ATT	TAA		F
cob	10442	11578	1137	ATG	TAA		F
trnS2	11582	11649	68			UGA	F
nad1	11666	12616	951	TTG	TAA		R
trnL1	12617	12681	65			UAG	R
rrnL	12659	13986	1328				R
trnV	13986	14056	71			UAC	R
rrnS	14058	14842	785				R
A-T region	14843	15755	913				

Table 3. Nucleotide composition (%) of protein-coding genes (PCGs) by codon position in *Saga hakkarica*.

	Overall Base Composition (%)				1st Codon Position (%)				2nd Codon Position (%)				3rd Codon Position (%)			
PCG's	%A	%C	%T	%G	%A1	%C1	%T1	%G1	%A2	%C2	%T2	%G2	%A3	%C3	%T3	%G3
nad1	25.03	9.67	45.22	20.08	23.66	8.20	42.90	25.24	17.98	17.98	46.37	17.67	33.44	2.84	46.37	17.35
nad2	29.35	23.32	36.83	10.50	33.82	19.83	31.78	14.58	18.66	23.32	45.77	12.24	35.57	26.82	32.94	4.66
nad3	29.66	23.16	35.59	11.58	33.05	18.64	29.66	18.64	14.41	23.73	48.31	13.56	41.53	27.12	28.81	2.54
nad4	26.66	8.14	43.91	21.28	27.07	7.16	43.18	22.60	16.59	15.02	47.98	20.40	36.32	2.24	40.58	20.85
nad4L	24.92	7.74	45.45	21.89	21.21	11.11	40.40	27.27	18.18	12.12	54.55	15.15	35.35	0.00	41.41	23.23
nad5	29.64	9.59	40.83	19.94	31.90	7.77	35.87	24.46	19.01	17.19	45.45	18.35	38.02	3.80	41.16	17.02
nad6	30.49	26.89	36.17	6.44	42.05	21.02	26.14	10.80	21.02	25.00	47.16	6.82	28.41	34.66	35.23	1.70
cox1	27.01	22.34	34.94	15.71	27.63	22.37	22.37	27.63	17.74	24.95	41.13	16.18	35.67	19.69	41.33	3.31
cox2	32.40	23.61	31.52	12.46	32.46	21.93	23.25	22.37	26.87	22.47	38.33	12.33	37.89	26.43	33.04	2.64
cox3	27.95	23.25	34.43	14.36	26.62	23.19	27.38	22.81	20.61	23.28	37.79	18.32	36.64	23.28	38.17	1.91
atp6	28.68	22.59	37.89	10.85	34.67	21.33	26.22	17.78	15.18	25.89	46.43	12.50	36.16	20.54	41.07	2.23
atp8	32.72	25.31	36.42	5.56	29.63	31.48	31.48	7.41	27.78	24.07	40.74	7.41	40.74	20.37	37.04	1.85
cytb	27.35	24.54	36.15	11.96	29.02	23.75	25.86	21.37	21.90	22.16	43.01	12.93	31.13	27.70	39.58	1.58
total	28.61	19.24	38.10	14.05	30.21	18.29	31.27	20.23	19.69	21.32	44.85	14.14	35.91	18.12	38.21	7.76

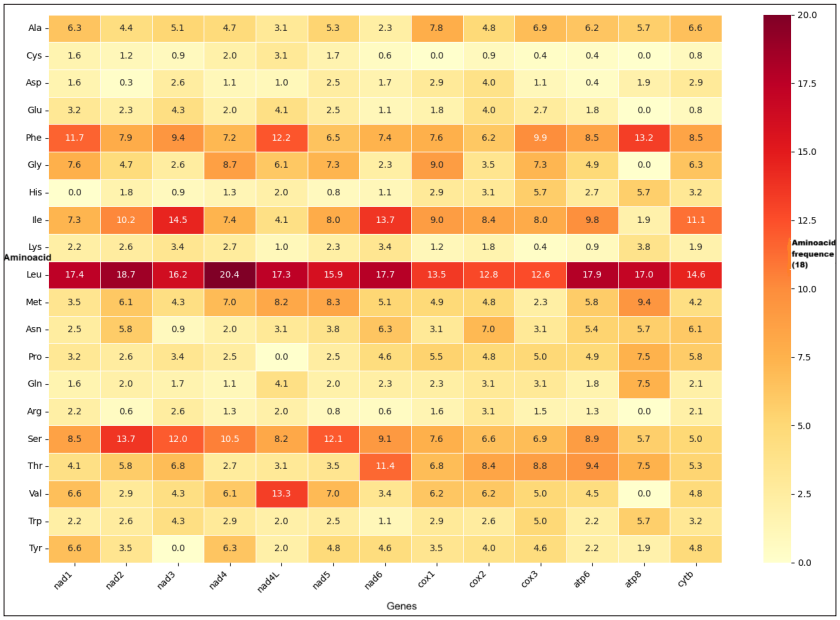

Figure 3. Heatmap illustrating the codon–amino acid usage frequencies across the protein-coding genes (PCGs) of *Saga hakkarica*.

Characterization of tRNA and rRNA genes

The complete mitogenome analysis of *S. hakkarica* revealed the presence of 22 tRNA genes, with a total length of 1-,458 bp. Among these, 14 tRNA genes are located on the F strand of the mtDNA, while the remaining 8 are positioned on the R strand. The total length of tRNA genes on the F strand is 927 bp, whereas those on the R strand collectively span 531 bp. Based on the analysis, the longest tRNA gene in *S.*

hakkarica is *trnV*, which is 71 bp in length and located on the R strand. Conversely, the shortest tRNA gene is *trnC*, measuring 63 bp, also situated on the R strand. In general, the lengths of tRNA genes in the mitogenome range from 63 to 71 bp (Table 1-2). Analysis of the rRNA genes showed that they are located between tRNA genes within the mitogenome. The precise positions and arrangements of the tRNA genes are crucial for reconstructing the mitochondrial genome and understanding the functional organization of these regions. The mitogenome of *S. hakkarica* includes two rRNA genes, both situated on the R strand. The loci of these rRNA genes are consistent with those found in ancestral insect mitogenomes (Wolstenholme, 1992; Boore, 1999; Cameron et al., 2014). The first rRNA gene, *rrnS* (small ribosomal subunit), is located between the *trnV* gene and the origin of heavy-strand replication (OH), with a length of 785 bp. The second rRNA gene, *rrnL* (large ribosomal subunit), is positioned between *trnL1* and *trnV*, and spans 1,328 bp (Table 1-2).

Characterization of intergenic and overlapping regions

A total of 12 intergenic regions were identified in the mitogenome of *S. hakkarica*. The combined length of these non-coding regions is 110 bp, with individual region lengths ranging from 1 to 21 bp. The locations of these intergenic spacers are listed in Table 3-5. The complete mitogenome analysis of *S. hakkarica* revealed 11 overlapping regions. The total length of these overlaps was determined to be 58 bp. The overlapping regions are presented in Table 3-5. Among these, the shortest overlaps are 1 bp in length, occurring between the *nad6-cob*, *rrnL-trnV*, and *trnT-trnP* gene pairs. The longest overlap spans 23 bp and is located between the *trnL1* and *rrnL* genes.

Table 4. Non-coding regions of the mitochondrial genome in *Saga hakkarica*.

1) trnQ - trnM (21 bp)	2) trnL2 - cox2 (5 bp)	3) cox2 - trnK (9 bp)
4) nad3-trnA (13 bp)	5) trnR - trnN (21 bp)	6) trnN - trnS1 (8 bp)
7) trnF - nad5 (10 bp)	8) nad41-trnT (2 bp)	9) trnP - nad6 (1 bp)
10) cob - trnS2 (3 bp)	11) trnS2 - nad1 (16 bp)	12) trnV - rrnS (1 bp)

Table 5. Overlapping coding regions identified in the mitochondrial genome of *Saga hakkarica*.

1) nad2-trnW (2 bp)	2) trnW-trnC (8 bp)
3) trnY-cox1 (8 bp)	4) trnK-trnD (1 bp)
5) atp8-atp6 (4 bp)	6) trnE-trnF (2 bp)
7) nad4-nad4l (7 bp)	8) trnT-trnP (1 bp)
9) nad6-cob (1 bp)	10) trnL1-rrnL (23 bp)
11) rrnL-trnV (1 bp)	

DISCUSSION

The mitogenome of *S. hakkarica* shows gene content consistent with *Saga natoliae* and other Tettigoniidae representatives (Cameron, 2014; Zhang et al., 2017; Yuan et al., 2021; Karakaş et al., 2025). Insect mitochondrial genomes are small, circular DNA molecules (15-18 kb), typically with 37 genes: 13 protein-coding genes (PCGs), 2 rRNA genes, and 22 tRNA genes (Cameron, 2014). This 37-gene structure is highly conserved (Boore, 1999). Besides genes, the largest non-coding region (the control

(AT-rich) region) contains replication and transcription origins (Cameron, 2014). The *S. hakkarica* mitogenome is 15,755 bp, with 22 tRNAs, 2 rRNAs, 13 PCGs, and one replication origin in the AT-rich region. PCGs include seven NADH dehydrogenase subunits, three cytochrome c oxidases, one cytochrome b, and two ATP synthase subunits. Genes are distributed on forward and reverse strands, with lengths largely similar to those obtained in prior studies (Zhang et al., 2016; Pang et al., 2024).

Pang et al. (2024) found mitogenome sizes in Tettigoniidae ranging 15,627-17,461 bp, all with 37 genes; *nad5* was longest (~1,732 bp), close to *S. hakkarica* (1,740 bp). Several genes showed conserved lengths across species, with minor differences, confirming variable evolutionary rates (Pang et al., 2024). Mitochondrial genomes are more conserved within genera, as seen in the comparison of *S. hakkarica* (15,755 bp) and *S. natoliae* (15,806 bp), both with typical Ensifera mitogenomes. Their 52 bp size difference mainly reflects control region variation (Pang et al., 2024), though gene length differences exist, especially in *nad2*, *cox1*, and *atp6*. For instance, *nad2* is 1,029 bp in *S. hakkarica* and 987 bp in *S. natoliae*; *cox1* is 1,540 bp vs. 1,531 bp; and *atp6* is 673 bp vs. 658 bp, respectively. Among the 13 PCGs, seven showed length variation, particularly *cox1, cox2, atp6, nad2*, and *nad5*. In addition to gene lengths, differences in start and stop codons were also observed among families and species. Variation in genetic codes particularly affects translation initiation and termination codons (Watanabe, & Yokobori, 2011). Many mitochondrial PCGs use incomplete stop codons completed post-transcriptionally (Ojala et al., 1988; Taanman, 1999). The genomic divergence observed between *S. hakkarica* and *S. natoliae* may have been influenced by the complex tectonic uplift and climatic fluctuations that shaped Anatolia's heterogeneous topography during the Miocene and Pleistocene (Sevgili, 2023). These geological processes likely created multiple montane refugia and biogeographic barriers, which are recognized as major drivers of speciation and endemism in Anatolian Orthoptera.

Furthermore, in several invertebrate groups, non-canonical start codons have been frequently reported. In taxa using translation Table 1 (invertebrate mitochondrial code), non-canonical start codons such as TTA (a member of the TTR box), ACG, CAA, CCG, CGA, and TCG have been annotated in RefSeq entries (Donath et al., 2019). In *S. hakkarica*, identified start codons include: ATT (*atp8, nad3, nad5,* and *nad6*), ATA (*atp6*), ATG (*cob*, *cox2*, *cox3*, *nad4*, and *nad4L*), GTG (*nad2*), TTG (*nad1*), and ATC (*cox1*). Pang et al. (2024) reported that most PCGs in Tettigoniidae begin with ATN codons, but the *nad1* gene starts with TTG in seven out of nine species consistent with the pattern seen in both *S. hakkarica* and *S. natoliae* (Karakaş et al., 2025). According to stop codons, notable differences were found between species. Pang et al. (2024) reported that incomplete stop codons (T or TA) are common, and TAG was observed only in *cob* and *nad1*. In *S. hakkarica*, only TAA (*atp8*, *nad1*, *nad2*, *nad3*, *nad4L*, *nad5*, *nad6*, and *cob*) and incomplete stop codons (T) (*cox1*, *cox2*, *cox3*, *nad4, and atp6*) were identified. In *S. natoliae*, T-incomplete stop codons were reported only in *cox2* and *nad4* (Karakaş et al., 2025). Similarly, *Tonkinacris sinensis* showed incomplete stops in *cox2* and *nad2*, while TAG was found in *nad1* (Zhang et

al., 2016). From an intrageneric perspective, the mitogenomes of *S. hakkarica* and *S. natoliae* exhibit 83.40% sequence similarity. This divergence is likely influenced by evolutionary background (e.g., divergence time), variation within control regions, and third codon position variability. However, differences in amino acid lengths and compositions in 7 of the 13 PCGs may also reflect annotation errors rather than true biological variation. These findings emphasize the importance of carefully considering non-canonical start codons and incomplete stop codons in Tettigoniidae (Ensifera) mitogenomes. Sole reliance on automated annotation tools is not recommended. Instead, manual correction based on comparisons with reference species is essential (Pang et al., 2024).

Indeed, annotation errors have been frequently reported by researchers using MITOS (Cameron, 2014; Hall, Hanzak, Allcock, Cooke, Ogura, & Strugnell, 2016). Although probabilistic improvements in MITOS2 have been introduced (Donath et al., 2019), this study found that both MITOS (via Proksee.ca) and MITOS2 (via Galaxy tools) produced similar annotation discrepancies for the *S. hakkarica* mitogenome FASTA. As a result, manual refinement through comparison with closely related species was necessary.

The nucleotide distribution across codon positions showed marked differences, with the third codon position having a notably higher A+T content (~74%) than the first and second positions (Table 3). This confirms that third codon positions in mitochondrial genes are particularly susceptible to compositional bias and skew. As noted in previous studies, the inclusion or exclusion of third codon positions in phylogenetic analyses should be carefully evaluated, as they may either obscure phylogenetic signal (e.g., in Dictyoptera) or serve as the primary source of signal (e.g., in Calliphoridae) (Cameron, 2014). These results demonstrate that codon positions not only influence the overall nucleotide composition of the mitogenome but also significantly shape amino acid composition in protein sequences. In conclusion, the analysis of the 13 PCGs in *S. hakkarica* revealed features highly consistent with mitogenomes of other Orthoptera species available in GenBank. The nucleotide sequences and codon usage of these PCGs are crucial determinants of mitochondrial genome functionality. The findings contribute to our understanding of the genetic similarities between *S. hakkarica* and other orthopteran taxa and offer a valuable foundation for future phylogenetic and evolutionary studies.

A total of 12 intergenic spacers were identified in the mitogenome of *S. hakkarica*, with a cumulative length of 110 base pairs (bp), and individual lengths ranging from 1 to 21 bp (Table 4). Likewise, in species of the subfamily Meconematinae, 8 to 11 intergenic spacers have been reported, with lengths similarly varying between 1 and 21 bp (Pang et al., 2024). For instance, in *Grigoriora cheni*, the spacer between *trnS2* and *nad1* is 21 bp long, while in eight other species it is 16 bp; and between *trnP* and *nad6*, a 1 bp intergenic spacer is present in nine species. In *S. hakkarica*, these spacers are also 16 bp and 1 bp, respectively. A total of 11 overlapping regions were identified in the *S. hakkarica* mitogenome, with a cumulative overlap length of 58 bp (Table 3-5). In Orthoptera, the number of overlapping regions typically ranges between 12 and 17 in various species (Zhang et al., 2016; Pang et al., 2024; Karakaş et al., 2025). *S. hakkarica* shows complete congruence with nine Meconematinae species (Pang

et al., 2024) at six overlapping loci: 1 bp overlaps between *trnK-trnD*, *trnT-trnP,* and *nad6-cob*; a 2 bp overlap between *nad2-trnW*; and 8 bp overlaps between *trnW-trnC* and *trnY-cox1*. Among these, only the *trnY-cox1* 8 bp overlap differs in *S. natoliae* (2 bp) (Karakaş et al, 2025). In the mitochondrial genome of *S. hakkarica*, a total of 22 tRNA genes were identified; their lengths range from 63 to 71 bp. These values align with tRNA lengths reported in other Orthoptera species. For example, Pang et al, (2024) observed a 63-71 bp range in nine Meconematinae species, while *S. natoliae* exhibits a 62-71 bp range (Karakaş et al., 2025), and *Tonkinacris sinensis* (Acrididae) shows a broader range of 63-76 bp (Zhang et al., 2016). Pang et al. (2024) noted conserved lengths for *trnW* (66 bp), *trnK* (70 bp), *trnS1* (61 bp), *trnS2* (69 bp), and *trnV* (71 bp) among the nine Meconematinae species. In *S. hakkarica*, *trnW*, *trnK*, and *trnV* lengths are consistent with those. In comparison with *S. natoliae*, only seven tRNA genes (*trnM*, *trnW*, *trnC*, *trnY*, *trnL2*, *trnE*, and *trnF*) differ in length, and only by 1 bp, indicating high conservation of tRNA gene lengths between closely related species. Relative to rRNA genes, *rrnL* is situated between *trnL1* and *trnV*, while *rrnS* is located between *trnV* and the control region consistent with the overall mitogenome organization of Orthoptera (Zhang et al., 2016; Pang et al., 2024; Karakaş et al., 2025). In *S. hakkarica*, *rrnL* is 1,328 bp and *rrnS* is 785 bp. Meconematinae species typically show ranges of 1,308-1,316 bp for *rrnL* and 787-792 bp for *rrnS* (Pang et al., 2024). In *S. natoliae*, these values are 1,304 bp (*rrnL*) and 780 bp (*rrnS*) (Karakaş et al., 2025), whereas *Tonkinacris sinensis* has slightly different values of 1,314 bp and 807 bp for *rrnL* and *rrnS*, respectively (Zhang et al., 2016).

In addition to protein-coding genes, rRNA and tRNA genes, mitochondrial genomes typically contain a non-coding region known as the control region (CR). This region plays a critical role in regulating mitochondrial function, as it includes the initiation sites for both DNA replication and transcription (Boore, 1999; Zhang et al., 2016). The control region is often referred to in the literature as the “AT-rich region” due to its high adenine and thymine content, or as the “D-loop” in vertebrates, owing to its triplex DNA structure (Clayton, 1991). It is generally located between the *rrnS* gene and a neighbouring tRNA gene (e.g*., trnI, trnQ,* or *trnV*), and exhibits substantial variation in length and structure across different species. This variation not only provides insight into the evolutionary dynamics of mitogenomes but also serves as a valuable phylogenetic and taxonomic marker in comparative studies (Cameron, 2014). In *S. hakkarica*, the control region is 913 bp in length and is situated between *rrnS* and *trnI*, encompassing the origin of heavy-strand replication (OH). It exhibits a high A+T content of 77.2%. The presence of tandem repeats and their length polymorphisms within this region are frequently used in interspecific comparisons (Pang et al., 2024). Notably, considerable variability is observed in both the length of the control region and the structure of tandem repeats among species. For instance, the control region in *S. natoliae* has been reported to be approximately 910 bp (Karakaş et al., 2025), while in the more distantly related species *Tonkinacris sinensis*, it is 795 bp in length (Zhang et al., 2016). Pang et al. (2024) highlighted that the tandem repeat sequences in the control region are entirely distinct among the nine species examined in their study.

CONCLUSIONS

In this research, the mitogenome of *S. hakkarica*, a species within the subfamily Saginae of the family Tettigoniidae (Orthoptera), was characterized for the first time. The obtained mitogenomic data revealed that the species possesses a typical animal mitogenome structure, comprising a total length of 15,755 base pairs, 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, 2 ribosomal RNA (rRNA) genes, and one control region. This genomic structure shows a high degree of similarity to that of *S. natoliae* and other members of the Tettigoniidae family. As a result of the study, the mitogenome of *S. hakkarica* was analysed in detail in terms of both structure and function. The positions and lengths of the genes, their start and stop codons, intergenic spacers, and overlapping regions were identified, while the tRNA and rRNA genes were thoroughly characterized. Notably, the presence of non-canonical start and stop codons, as well as incomplete stop codons, underscores the importance of careful manual correction during the annotation process. Intrageneric comparisons revealed a mitogenomic similarity rate of 83.40% between *S. hakkarica* and *S. natoliae*. Nevertheless, differences in gene lengths, codon usage, and control region structure indicate evolutionary divergence between the two species. Additionally, the results highlighted a high A+T content in third codon positions, which may provide both phylogenetic signal and methodological challenges in evolutionary analyses. With this study, the mitogenome of a second species within the genus *Saga* has been introduced to the literature, enabling intrageneric comparative analyses for the first time. The availability of the *S. hakkarica* mitogenome in public databases will serve as a valuable reference for future studies in evolutionary biology, molecular systematics, and biodiversity research.

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