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Mitogenomics of *Psorodonotus ebneri* Brunner von Wattenwyl, 1861 (Orthoptera: Tettigoniidae): Selection Profile and Patterns of Intraspecific and Interspecific Divergence

Psorodonotus ebneri Brunner von Wattenwyl, 1861 (Orthoptera, Tettigoniidae) mitogenomiği: Seçilim Profili ve Türiçi ve Türler Arası Farklılaşma Örüntüleri

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ABSTRACT

Advances in DNA sequencing technologies have made comparative mitogenome studies available. However, studies on intraspecific mitogenome variation are still rare. This study reports total mitogenome of *Psorodonotus ebneri* Brunner von Wattenwyl, 1861 (Orthoptera: Tettigoniidae), highlighting selection profile and intraspecific and interspecific divergence in protein-coding genes (PCGs). Here, the analyses were performed with two datasets, one including five *P. ebneri* mitogenomes and the another including a mitogenome of *P. venosus* (Fischer von Waldheim, 1839). Results showed that the mitogenome of *P. ebneri* consists of 37 genes and an AT-rich region, ordering as in Pancrustacean, constituting 15645-15668 base pairs. Among 38 gene borders, eight had intergenic sequences (IGS) and seven overlapping (OR). All tRNAs exhibited the typical clover-leaf structure except for trnS1. Pairwise distance ranges between 0.001-0.003 among sequences of *P. ebneri* and ~0.12 between *P. ebneri* and *P. venosus*. The dN/dS and neutrality index indicated purifying selection in PCGs. We reached the following conclusions: (i) *P. ebneri* mitogenomes show characteristics of Orthoptera and Pancrustacea, (ii) IGS and OR appear conserved in Tettigoniidae, (iii) low intraspecific variation is likely due to small population size, and (iv) most of the PCGs evolved under purifying selection suggesting no specific adaptation to montane habitats.

Key Words

Mitogenomics, Orthoptera, selection, Tettigoniidae.

ÖZ

DNA dizileme teknolojilerindeki gelişmeler, karşılaştırmalı mitogenom çalışmalarını olası kılmıştır. Ancak, türiçi mitogenom varyasyonu üzerine yapılan çalışmalar hâlâ nadirdir. Bu çalışma, Psorodonotus ebneri Brunner von Wattenwyl, 1861 (Orthoptera: Tettigoniidae)'in total mitogenomunu rapor etmesinin yanısıra, türiçi ve türler arası farklılaşma oranı ve protein kodlayan genlerde (PKG) görülen seçilim profillerine odaklanmaktadır. Bu çalışmada, iki veri seti analiz edilmiştir: biri beş *P. ebneri* mitogenomunu, diğeri ise ek olarak *P. venosus* (Fischer von Waldheim, 1839)'u içermektedir. Sonuçlar, *P. ebneri* mitogenomunun 37 gen ve AT zengin bir bölgeden oluştuğunu, bunların Pancrustacea gen sırasına sahip olduğu ve toplamda 15645–15668 baz çifti uzunluğunda olduğunu göstermektedir. Otuz sekiz gen sınırının sekizinde genler arası bölgeler bulunurken, yedisinde genler örtüşmektedir. trnS1 dışındaki tüm tRNA'lar tipik yonca yaprağı yapısını göstermektedir. Beş *P. ebneri* dizisi arasındaki ikili uzaklıklar 0.001–0.003, *P. ebneri* ile *P. venosus* arasındaki ise ~0.12 olarak hesaplanmıştır. dN/dS oranı ve nötralite indeksi PKG'lerde saflaştırıcı seçilim profiline işaret etmektedir. Verilerden şu çıkarımlara varılmıştır: (i) *P. ebneri* mitogenomları Orthoptera ve Pancrustacea genel özelliklerini taşımaktadır, (ii) IGS ve OR örüntüleri Tettigoniidae'de korunmuş görünmektedir, (iii) düşük türiçi varyasyon olasılıkla populasyonun küçük olmasından kaynaklanmaktadır ve (iv) çoğu PKG, saflaştırıcı seçilim altında evrimleşmiştir ve dağlık habitatlara adaptasyonu gösteren bir işaret bulunmamıştır.

Anahtar Kelimeler

Mitogenomik, Orthoptera, seçilim, Tettigoniidae.

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INTRODUCTION

Mitochondrion, a common organelle to eukaryotic cell, is arguably the most extensively studied. One reason for this intense focus is its possession of an independent genome that is notably different than the nuclear genome. The matrilineal inheritance of the mitogenome, the low recombination potential, and the faster evolutionary rates compared to the nuclear genome have made the mitochondrion a highly useful tool for evolutionary studies [1,2]. For this purpose, specifically, the mitochondrial cytochrome C oxidase subunit I gene has been proposed and widely used as a barcode gene for animals [3]. Early mitochondrial studies primarily relied on sequencing single genes via Sanger sequencing techniques. Over the past two decades, the development of next-generation sequencing technologies has led to a rapid increase in the number of complete mitogenome sequences available in databases [4]. For instance, currently, over 134.000 complete mitogenome sequences from Animalia and more than 19.000 from Insecta are available in GenBank database (NCBI, https://www.ncbi.nlm.nih.gov/genbank/, keyword: mitochondrion complete genome; accessed on 09.01.2025).

Following the first complete mitogenome description, subsequent studies primarily focused on characterizing mitogenome features based on single sequences [5]. Comparative evolutionary studies involving multi-mitogenome sequences per species have seen a recent surge. Topics such as intraspecific and interspecific mitogenome differentiation and selection patterns in protein-coding genes (PCGs) have received significant attention. These studies have revealed that patterns of divergence vary among lineages and genes, and purifying selection seems to be the common phenomenon in mitogenomes [2, 6-16]. However, these general findings are still limited to a relatively small number of lineages and await further validation. Approximately 900 complete mitogenome sequences representing around 500 orthopteran species are available in the GenBank database (NCBI, https://www.ncbi.nlm.nih.gov/genbank/, keywords: mitochondrion complete genome; Orthoptera; accessed on 09.01.2025). This number represents approximately 1.7% of the total 30.180 species in the order [17]. However, a significant number of these studies primarily focus on the mitogenome description of single species. Only a few have addressed Orthoptera mitogenomics from a comparative evolutionary perspective [18-21]. Although there are some comparative studies [22,23] intraspecific and interspecific mitogenome divergence is rarely explored. Taken together, these observations suggest that Orthoptera mitogenomics is still in its early stages, with many unanswered questions remaining. Addressing these gaps is one of the primary motivations of this study.

The genus *Psorodonotus* Brunner von Wattenwyl, 1981 (Tettigoniidae, Ensifera, Orthoptera), belonging to the tribe Pholidopterini, includes 17 species distributed across the Caucasus, Anatolia, and the Balkans [24-28]. Among the three species found in the southern part of the generic range, two (*P. salmani* Ünal and *P. anatolicus* Karabağ) are reported as extinct, while *P. ebneri* Karabağ, endemic to the Beydağları Mts. in Antalya, Turkey remains extant [26,29]. Although numerous studies have reported partial mitochondrial gene sequences of *Psorodonotus* [24-26], only one study has characterized a complete mitogenome of *P. venosus* (Fischer de Waldheim) [30]. Therefore, this study aims to focus the mitogenomics of a second member of the genus, *P. ebneri*, one of the world's most threatened 100 species [31].

There are several reasons for selecting *P. ebneri* as a model organism for this study. First, it is critically endangered under IUCN criteria [29,31], and obtaining genetic diversity data for this species could provide valuable insights into its conservation genetics. Second, members of the genus *Psorodonotus* are cold-adapted montane species, whose speciation and distribution were shaped by Pleistocene glacial cycles. These species likely experienced range contractions and population bottlenecks during Holocene due to erosion from their southern margins [25,26,29]. This study represents a pioneering effort to assess intraspecific mitogenome diversity within a likely bottlenecked population, offering insights into Orthoptera demography, and conservation. Third, mitochondria are important functional organelles that provide most of the adenosine triphosphate (ATP) required by cells through oxidative phosphorylation (OXPHOS) and the gene complex encoding these enzymes is in the mitogenome making it significant in the context of temperature adaptation [9,11] and thermoregulation [32]. As a cold adapted species, *P. ebneri* provides an opportunity to evaluate whether mitochondrial PCGs evolve neutrally or under purifying or positive selective pressures, which may allow to assess the species' resilience to montane habitats. Fourth and less critically, the published mitogenome of *P. venosus* [30] provide an opportunity to comparative assessments. The conclusions

derived from analyses of intraspecific and interspecific mitogenome differentiation may serve as a rare example for successive higher taxonomical levels, particularly within Orthoptera. To this end, complete mitogenome sequences from five different *P. ebneri* samples were analyzed to; (i) describe the mitogenome characteristics, including gene content and strand location, gene lengths, intergenic spacers (IGSs), overlapping regions (ORs), base composition per genes and gene cluster, start/stop codons of PCGs, tRNA secondary structures, anticodons, and relative synonymous codon usage (RSCU); (ii) determine diversity indices such as haplotype and nucleotide at the mitogenome level, (iii) evaluate intraspecific and interspecific divergence per gene, per gene cluster and for the total mitogenome, and (iv) assess selection profiles for each PCG.

MATERIALS and METHODS

Mitogenome data

We studied complete mitochondrial genomes of five *Psorodonotus ebneri* specimens collected from Antalya, Turkey between 2007 and 2019 (Table 1.). Mitochondria-rich tissue samples from the third leg (hind femur) were used to extract genomic DNA using the PureLink Genomic DNA Isolation Kit (ThermoFisher Scientific) following the manufacturer's protocol. The extracted amount of DNA was measured using a Qubit Fluorometer 4.0. Next generation sequencing (NGS) was performed at Novogene Inc. (China) using the Illumina HiSeq 2500 platform, for 150 bp paired-end reads with a total data output of 5 GB. Raw sequencing data were processed to remove adaptors and contaminant sequences, and low-quality reads by the sequencing company and the resulting clean reads were used for dataset establishment and analyses.

Clean NGS reads from five *Psorodonotus ebneri* samples were processed on Galaxy EU platform (https://usegalaxy.eu) [33] for mitogenome assembly. Reads were mapped to the only currently available complete mitogenome within the genus, *Psorodonotus venosus* (GenBank accession: MK951778, [30]), using the Bowtie2 [34]. This reference genome was also used for subsequent comparative analysis.

Mapped genomes were transferred to GENEIOUS v9.0.5 (https://www.geneious.com, [35]) to generate consensus sequences. These sequences were subsequently annotated using MITOS2 annotation tool [1] on the Galaxy portal. To ensure accuracy, tRNA genes were further analyzed, verified and the secondary structures were visualized using ARWEN online tool [36]. Key characteristics of the annotated mitogenomes (*P. ebneri* and *P. venosus*) including strand location, start and end sites, lengths, start and stop codons (for PCGs), and anticodons (for tRNAs) were determined using GENEIOUS v9.0.5. Furthermore, features of overlapping regions and intergenic spacers were identified. Additionally, each mitogenome was visualized as a circular representation.

Comparative analysis

The mitochondrial gene matrices for each 13 protein coding genes (PCGs), 22 tRNAs, 2 rRNAs and AT-rich region from two *Psorodonotus* species were prepared separately for intraspecific (only *P. ebneri*) and interspecific (*P. ebneri and P. venosus*) comparative analyses. Each gene

Table 1. GenBank accession numbers, sample IDs, and collection localities for *Psorodonotus* samples used in this study.

GenBank Accession No.	Sample ID	Localities
PV836850	*P. ebneri_1*	Turkey, Antalya, between Antalya and Bakırlıdağ Pozan, N: 36.983333, E: 30.304722, 1887 m, 15.07.2007
PV836851	*P. ebneri_2*	Turkey, Antalya, İmecik, Güzle village, N: 36.84989, E:30.30722, 1755 m, 16.06.2019
PV836852	*P. ebneri_3*	Turkey, Antalya, İmecik, Güzle village, N:36.870614, E:30.301105, 1755 m, 19.05.2019
PV836853	*P. ebneri_4*	Turkey, Antalya, Imecik, Güzle village, N:36.9075, E:30.50111, 1852 m, 01.07.2012
PV836854	*P. ebneri_5*	
MK951778	*P. venosus*	-

matrix was aligned using the MAFFT v.7 (https://mafft.cbrc.jp/alignment/server/, [37]) with default settings and the alignments verified using MEGA v7.0 [38]. For each gene, conserved and variable sites were obtained for both matrices. The nucleotide and amino acid composition, and also RSCU values were calculated for the PCGs of all five samples. Codon mean data and RSCU values were formatted using *pandas* v2.2.3 [39] and visualized using *numphy* v2.1 [40], *pyCirclize* v 1.7.1. [41] and *plotly* v5.24.1 [42].

To assess the overall distances in the *P. ebneri* and *P. venosus + P.ebneri* datasets, all genes were first concatenated using the Concatenator [43]. Next, pairwise distance ratios were calculated within and between species using Maximum Composite Likelihood model [44] in MEGA v7.0. The signs of selection on the PCGs were assessed using the dN/dS ratio (nonsynonymous substitutions/synonymous substitutions ratio), which measures nonsynonymous and synonymous changes across sequences, and the McDonald-Kreitman test [45], which compares polymorphism and divergence to identify neutral, positive, or purifying selection patterns in the given datasets. Both tests were performed using DnaSP v5 [46].

RESULTS and DISCUSSION

Genome characteristics

A total of five complete mitochondrial genomes were successfully obtained for *Psorodonotus ebneri,* each comprising 13 PCGs, 22 tRNAs, 2 rRNAs and an AT-rich region. The gene content and order were fully consistent with the defined structure for Pancrustacea and Tettigoniidae [5,18,20,21,47,48]. Circular representation for one sample of the genome of *P. ebneri* is provided in Figure 1. Detailed characteristics of the PCGs are presented in Table 2, and those of the remaining tRNA and rRNA genes and the AT-rich region are shown in Table 3.

The lengths of the PCGs ranged from 171 bp (*atp8*) to 1732 bp (*nad5*) in *P. ebneri*. No intraspecific length variation was found among the five *P. ebneri* samples. Start codons were predominantly conserved, with most PCGs beginning with ATN codons, except for *cox1* (CAA) and *nad1* (TTG). Similarly, stop codons starting with T-- were identified in *nad2, cox1, cox3, nad3, nad5, nad4 nad6* and *cytb*; TAA in *atp8, atp6* and *nad4L*; and TAG in *nad1*. Strand orientation revealed four genes (*nad5,*

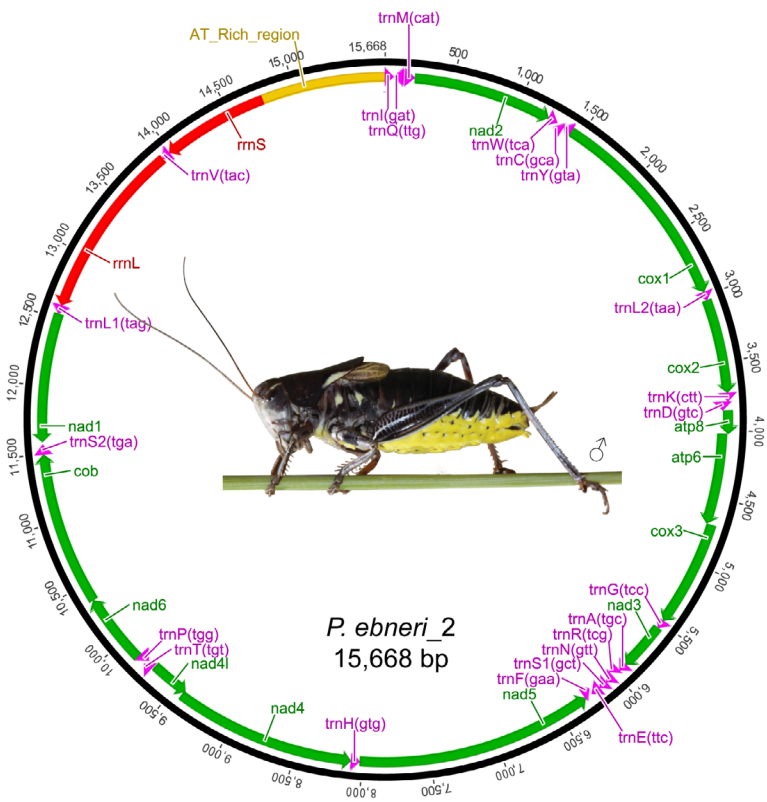

Figure 1. Representative circular mitogenome of *P. ebneri.*

Table 2. The characteristics of 13 PCGs.

PCGs	Samples	Strand	Start site	End site	Lenght	Start codon	Stop codon
nad2	*P. ebneri_1/2/4*	J	216	1242	1027	ATT	T..
	P. ebneri_3/5		218	1244	1027		
	P. venosus		221	1247	1027		
cox1	*P. ebneri_1/2/4*	J	1434	2964	1531	CAA	T..
	P. ebneri_3/5		1436	2966	1531		
	P. venosus		1435	2965	1531		
cox2	*P. ebneri_1/2/4*	J	3030	372	691	ATG	T..
	P. ebneri_3/5		3032	3722	691		
	P. venosus		3032	3722	691		
atp8	*P. ebneri_1/2/4*	J	3856	4026	171	ATC	TAA
	P. ebneri_3/5		3858	4028	171		
	P. venosus		3858	4028	171		
atp6	*P. ebneri_1/2/4*	J	4023	4697	675	ATA	TAA
	P. ebneri_3/5		4025	4699	675		
	P. venosus		4025	4699	675		
cox3	*P. ebneri_1/2/4*	J	4697	5483	787	ATG	T..
	P. ebneri_3/5		4699	5485	787		
	P. venosus		4699	5487	789		TAA
nad3	*P. ebneri_1/2/4*	J	5548	5899	352	ATC	T..
	P. ebneri_3/5		5550	5901	352		
	P. venosus		5551	5902	352		
nad5	*P. ebneri_1/2/4*	N	6287	8018	1732	ATT	T..
	P. ebneri_3/5		6289	802	1732		
	P. venosus		6293	8024	1732		
nad4	*P. ebneri_1/2/4*	N	8083	9421	1339	ATG	T..
	P. ebneri_3/5		8083	9421	1339		
	P. venosus		8089	9427	1339		
nad4L	*P. ebneri_1/2/4*	N	9415	9711	297	ATG	TAA
	P. ebneri_3/5		9417	9713	297		
	P. venosus		9421	9717	297		
nad6	*P. ebneri_1/2/4*	J	9842	1037	529	ATT	T..
	P. ebneri_3/5		9844	10372	529		
	P. venosus		9847	10377	531		TAA
cytb	*P. ebneri_1/2/4*	J	10371	11505	1135	ATG	T..
	P. ebneri_3/5		10373	11507	1135		
	P. venosus		10377	11511	1135		
nad1	*P. ebneri_1/2/4*	N	11590	12537	948	TTG	TAG
	P. ebneri_3/5		11592	12539	948		
	P. venosus		11596	12543	948		

nad4, nad4L, and *nad1*) located on the N-strand (minority strand), while the remaining nine PCGs on the J-strand (majority strand). The orientation and start/stop codon patterns were identical in both *P. ebneri* and *P. venosus*. Although the lengths of the PCGs were mostly identical, the *cox3* and *nad6* genes were 2 bp longer in *P. venosus*, due to TAA stop codon, which was observed as an incomplete T-- in *P. ebneri* (see [30]).

More varied patterns were observed in the lengths of tRNAs, rRNAs, and the AT-rich region among mitogenomes. tRNA lengths ranged from 63 bp (*trnR*) to 70 bp (*trnK* and *trnV*) in five *P. ebneri* samples. Significant length variation was observed in 13 tRNA genes of *P. ebneri* and *P. venosus* (Table 3; [30]).

In terms of strand location and anticodon patterns, no significant differences were detected between *P. ebne-*

Table 3. The characteristics of 22 tRNAs, 2 rRNAs and AT-rich region.

Gene	Samples	Strand	Start site	End site	Lenght	Anticodon
trnI	*P. ebneri_1/2/3/4/5*	J	1	66	66	GAT
	P. venosus		1	64	64	
trnQ	*P. ebneri_1/2/3/4/5*	N	67	135	69	TTG
	P. venosus		64	139	76	
trnM	*P. ebneri_1/2/3*	J	150	215	66	CAT
	P. ebneri_4/5		152	217	66	
	P. venosus		156	220	65	
trnW	*P. ebneri_1/2/3*	J	1243	1310	68	TCA
	P. ebneri_4/5		1245	1312	68	
	P. venosus		1248	1313	66	
trnC	*P. ebneri_1/2/3*	N	1303	1366	64	GCA
	P. ebneri_4/5		1305	1368	64	
	P. venosus		1306	1368	63	
trnY	*P. ebneri_1/2/4*	N	1367	1432	66	GTA
	P. ebneri_3/5		1369	1434	66	
	P. venosus		1369	1433	65	
trnL2	*P. ebneri_1/2*	J	2965	3028	64	TAA
	P. ebneri_3/5		2967	3030	64	
	P. ebneri_4		2966	3030	65	
	P. venosus		2966	3030	65	
trnK	*P. ebneri_1/2/4*	J	3721	379	70	CTT
	P. ebneri_3/5		3723	3792	70	
	P. venosus		3723	3792	70	
trnD	*P. ebneri_1/2/4*	J	3790	3855	66	GTC
	P. ebneri_3/5		3792	3857	66	
	P. venosus		3792	3857	66	
trnG	*P. ebneri_1/2/4*	J	5484	5547	64	TCC
	P. ebneri_3/5		5486	5549	64	
	P. venosus		5489	5550	62	
trnA	*P. ebneri_1/2/4*	J	5900	5963	64	TGC
	P. ebneri_3/5		5902	5965	64	
	P. venosus		5903	5966	64	

Table 3. The characteristics of 22 tRNAs, 2 rRNAs and AT-rich region. Continue

trnR	*P. ebneri_1/2/4*	J	5963	6025	63	TCG
	P. ebneri_3/5		5965	6027	63	
	P. venosus		5967	6029	63	
trnN	*P. ebneri_1/2/4*	J	6026	6090	65	GTT
	P. ebneri_3/5		6028	6092	65	
	P. venosus		6030	6095	66	
trnS1	*P. ebneri_1/2/4*	J	6091	6157	67	GCT
	P. ebneri_3/5		6093	6159	67	
	P. venosus		6096	6162	67	
trnE	*P. ebneri_1/2/4*	J	6158	6224	67	TTC
	P. ebneri_3/5		6160	6226	67	
	P. venosus		6164	6229	66	
trnF	*P. ebneri_1/2/4*	N	6223	6286	64	GAA
	P. ebneri_3/5		6225	6288	64	
	P. venosus		6228	6292	65	
trnH	*P. ebneri_1/2/4*	N	8019	8082	64	GTG
	P. ebneri_3/5		8021	8084	64	
	P. venosus		8025	8088	64	
trnT	*P. ebneri_1/2/4*	J	9713	9776	64	TGT
	P. ebneri_3/5		9715	9778	64	
	P. venosus		9719	9782	64	
trnP	*P. ebneri_1/2/4*	N	9776	9840	65	TGG
	P. ebneri_3/5		9778	9842	65	
	P. venosus		9782	9845	64	
trnS2	*P. ebneri_1/2/4*	J	11506	11573	68	TGA
	P. ebneri_3/5		11508	11575	68	
	P. venosus		11512	11579	68	
trnL1	*P. ebneri_1/2/4*	N	12538	12602	65	TAG
	P. ebneri_3/5		12540	12604	65	
	P. venosus		12544	12607	64	
trnV	*P. ebneri_1/2/4*	N	13912	13981	70	TAC
	P. ebneri_3		13917	13986	70	
	P. ebneri_5		13914	13983	70	
	P. venosus		13915	13984	70	
rrnaL	*P. ebneri_1/2/4*	N	12603	13911	1309	-
	P. ebneri_3		12605	13916	1312	
	P. ebneri_5		12605	13913	1309	
	P. venosus		12608	13914	1307	
rrnaS	*P. ebneri_1/2/4*	N	13982	14757	776	
	P. ebneri_3		13987	14762	776	
	P. ebneri_5		13984	14759	776	
	P. venosus		13985	14763	779	
AT-Rich region	*P. ebneri_1*	-	14758	15645	888	
	P. ebneri_2		14758	15668	911	
	P. ebneri_3		14763	15664	902	
	P. ebneri_4		14758	15668	911	
	P. ebneri_5		14760	15650	891	
				15844	1081	

ri and *P. venosus* (see [30]). A total of 14 tRNAs were situated on the J-strand, while 8 tRNAs, along with both rRNAs were located on the N-strand. Additionally, no major differences were detected in the secondary structures of tRNAs across *P. ebneri* samples and *P. venosus*. The secondary structures were consistent with previous reports for insects [20-23,49]. All tRNAs exhibited the typical clover-leaf structure except for *trnS1* (where the D-stem is missing), which seems to be a general pattern in Orthoptera [20-23,49,50]. In both *P. ebneri* and *P. venosus*, both rRNA genes were located on the N-strand, both differing in length. The *rrnL* is 1309 or 1312 bp in the five samples of *P. ebneri* while 1307 bp in *P. venosus*. Again, the *rrnS* is 776 bp in all *P. ebneri* while 779 bp in *P. venosus*. The most variable segment of mitogenomes is the AT-rich or control region, ranging from 888 bp to 911 bp in five samples of *P. ebneri*, while 1081 bp in *P. venosus*.

The nucleotide composition and AT ratio of PCGs for five *P. ebneri* samples are presented in Table 4. The AT ratio ranged from a minimum of 65.4% in the *cox3* gene to a maximum of 75.7% in the *nad4L* gene, with an overall mean of 69.8% across all genes. The mean base frequencies were approximately 40.4% for adenine (A), 29.4% for thymine (T), 15.9% for cytosine (C), and 14.3% for guanine (G). The 1st and 2nd codon positions exhibited similar AT ratios, whereas the 3rd codon position showed the highest AT ratio, ranging between 75.1% and 85.2%. The overall AT ratio for *P. ebneri* was identical for five mitogenomes at 71%, similar to that of *P. venosus* [30]. There is no significant difference between *P. ebneri* and *P. venosus*, as well as other orthopteran, regarding AT ratio per gene and per position [19-23, 30].

The mean amino acid compositions and RSCU for each PC of *P. ebneri* displayed patterns consistent with those previously reported for Orthoptera [19-23,48] (see Figure 2). Significant variation was observed in amino acid usage among PCGs. Notably, *leucine*, *isoleucine*, *phenylalanine*, and *serine* were the most frequent amino acids observed across the genes, consistent with findings for *P. venosus* [30]. The RSCU among PCGs is given in Figure 3. *Serine*, *leucine*, *valine*, *proline*, *threonine*, *alanine*, *arginine*, and *glycine* had the highest RSCU values, corresponding to the maximum number of codons and codon types, respectively, as also observed in *P. venosus* [30].

Pattern of intergenic spacers and overlapping regions

The data revealed a mostly conserved pattern in IGS and OR across the five *P. ebneri* specimens, as well as in *P. venosus* (Table 5). Of the 38 gene borders, 18 had neither OR nor IGS, eight had IGS, and seven had OR, all of which were consistent across the five *P. ebneri* samples in terms of location, length, and base composition—except for the IGS between *trnQ* and *trnM* (Table 5). The five *P. ebneri* samples exhibited two patterns of IGS at the border of these two genes: +14(AATATATCTTCTAG) in *P. ebneri*_1/2/5 and +16(AATATACNCTCCAAAG) in *P. ebneri*_3/4. The second pattern was also observed in *P. venosus*, indicating that the first type is derived, and the second plesiomorphic.

The presence/absence of IGS and OR, as well as their length and base composition, was largely conserved in *P. ebneri* and *P. venosus,* differing in only for 5/38 gene borders, while remaining 33 were identical in location pattern, base number and sequence. The five differences observed in *P. venosus* were as follows; presence of a +1(T) IGS between the *trnG-nad3* and *trnS1-trnE* pairs (absent in *P. ebneri*), -3(TTA) OR between *trnI-trnQ* (+2(TA) in *P. ebneri*), -1(A) OR between *nad6-cytb* (absent in *P. ebneri*), and no IGS/OR between *trnA-trnR* (+1(A) IGS in *P. ebneri*). These findings indicate that OR and IGS patterns (in terms of location, base number and sequence) are highly conserved within the genus, and even among other Tettigoniidae and some Orthoptera [20,21], while differing in some others [23].

Intra- and interspecific genetic diversity

The analysis of mitochondrial genes provided a detailed overview of genetic variability at both intraspecific and interspecific levels (Table 6.). Interspecifically, among the 13 PCGs, *atp8*, *cytb* and *nad1* are invariable, while the remaining 10 genes exhibit variation at one (*cox2* and *nad3*) to five (*atp6* and *nad4*) sites. The *nad6* gene shows the highest variation ratio, with 0.008 (variable sites/all sites; 4/529). Eighteen tRNA genes are completely identical across all *P. ebneri* samples, while the remaining four (*trnI, trnF, trnH* and *trnL1*) vary at a single position, with a variation ratio of 0.02 (1/65 for *trnL1* and 1/64 for the rest). Similarly, *rrnS* is invariable, whereas *rrnL* varies at only one position, with a variation ratio of 0.0008 (1/1312). The AT-rich region is the most variable segment of the mitogenome, with the 42 variable sites and a variation ratio of 0.05 (42/931).

Table 4. Nucleotide compositions of total codon, 1st, 2nd and 3rd position of the codon for PCGs.

PCGs	Total codon				
	T%	C%	A%	G%	AT%
nad6	36.9 (36.7/37.1)	22.3 (22.1/22.5)	33.5 (33.5/33.5)	7.4 (7.4/7.4)	70.4 (70.2/70.6)
nad5	44.4 (44.4/44.5)	9.88 (9.8/9.9)	27.9 (27.9/27.9)	17.8 (17.8/17.8)	72.3 (72.3/72.4)
nad4l	48.3 (48.1/48.5)	6.16 (6.1/6.4)	27.36 (27.3/27.6)	18.2 (18.2/18.2)	75.7 (75.4/75.8)
nad4	46.4 (46.4/46.4)	8.94 (8.9/9)	27 (27/27)	17.7 (17.6/17.8)	73.4 (73.4/73.4)
nad3	36.6 (36.4/36.6)	18.8 (18.8/19)	32.1 (32.1/32.1)	12.5 (12.5/12.5)	68.6 (68.5/68.7)
nad2	39.2 (39.1/39.3)	19.6 (19.5/19.6)	32 (32/32.1)	9.2 (9.2/9.2)	71.2 (71.2/71.3)
nad1	46.7 (46.7/46.7)	10.2 (10.2/10.2)	25.2 (25.2/25.2)	17.8 (17.8/17.8)	71.9 (71.9/71.9)
cytb	37 (37/37)	21.1 (21.1/21.1)	29.3 (29.3/29.3)	12.6 (12.6/12.6)	66.3 (66.3/66.3)
cox3	36.6 (36.5/36.6)	20.6 (20.6/20.7)	28.8 (28.8/29)	13 (13.9/14)	65.4 (65.4/65.5)
cox2	34.3 (34.3/34.4)	19.1 (19/19.1)	34.3 (34.3/34.3)	12.3 (12.3/12.3)	68.6 (68.6/68.7)
cox1	36.2 (36.1/36.2)	18.5 (18.5/18.5)	29.7 (29.7/29.7)	15.7 (15.6/15.7)	65.9 (65.8/65.9)
atp8	36.3 (36.3/36.5)	21.1 (21.1/21.2)	36.2 (35.9/36.3)	6.4 (6.4/6.5)	72.6 (72.4/72.6)
atp6	39.2 (39.1/39.4)	20.5 (20.3/20.6)	30.2 (30.1/30.4)	10.1 (9.9/10.2)	69.4 (69.2/69.5)
mean	40.4 (40.4/40.4)	15.9 (15.9/15.9)	29.4 (29.4/29.5)	14.3 (14.3/14.3)	69.8 (69.8/69.9)
	1st position of the codon				
nad6	23.2 (23/24)	20.2 (19.8/20.3)	42.9 (42.9/42.9)	13.6 (13.6/13.6)	66.1 (65.9/66.9)
nad5	38 (38/38)	8.8 (8.8/8.8)	30.22 (30.1/30.3)	22.7 (22.7/22.8)	68.2 (68.1/68.3)
nad4l	43.8 (43/44)	7.3 (7.1/8.1)	25.3 (25.3/25.3)	23.2 (23.2/23.2)	69.1 (68.3/69.3)
nad4	44 (44/44)	8.9 (8.9/8.9)	26.3 (26.2/26.4)	21.1 (21/21.3)	70.3 (70.2/70.4)
nad3	31 (31/31)	16.9 (16.9/16.9)	33.9 (33.9/33.9)	18.6 (18.6/18.6)	64.9 (64.9/64.9)
nad2	32 (32/32)	18.1 (18.1/18.1)	36.4 (36.4/36.4)	13.4 (13.4/13.4)	68.4 (68.4/68.4)
nad1	41 (41/41)	9.8 (9.8/9.8)	25.6 (25.6/25.6)	23.1 (23.1/23.1)	66.6 (66.6/66.6)
cytb	28 (28/28)	20.8 (20.8/20.8)	29.3 (29.3/29.3)	22.2 (22.2/22.2)	57.3 (57.3/57.3)
cox3	29 (29/29)	22.1 (22.1/22.1)	26.6 (26.6/26.6)	22.1 (22.1/22.1)	55.6 (55.6/55.6)
cox2	21 (21/21)	23.4 (23.4/23.4)	33.8 (33.8/33.8)	21.6 (21.6/21.6)	54.8 (54.8/54.8)
cox1	27 (27/27)	17.8 (17.8/17.8)	28 (28/28)	27.6 (27.6/27.6)	55 (55/55)
atp8	33 (33/33)	15.8 (15.8/15.8)	42.1 (42.1/42.1)	8.8 (8.8/8.8)	75.1 (75.1/75.1)
atp6	29 (29/29)	21.4 (21.3/21.8)	33.5 (33.3/33.8)	15.76 (15.6/16)	62.5 (62.3/62.8)
mean	33 (33/33)	15.7 (15.6/15.7)	30.5 (30.4/30.5)	21 (21/21)	63.5 (63.4/63.5)
	2nd position of the codon				
nad6	51 (51/51)	23.3 (23.3/23.3)	18.8 (18.8/18.8)	7.4 (7.4/7.4)	69.8 (69.8/69.8)
nad5	47 (47/47)	17.3 (17.3/17.3)	18.5 (18.5/18.5)	16.8 (16.8/16.8)	65.5 (65.5/65.5)
nad4l	55.8 (55/56)	10.1 (10.1/10.1)	18.4 (18.2/19.2)	16.2 (16.2/16.2)	74.2 (74.2/74.2)
nad4	49 (49/49)	14.6 (14.6/14.6)	16.8 (16.8/16.8)	19.4 (19.3/19.5)	65.8 (65.8/65.8)
nad3	50 (50/50)	18.8 (18.8/18.8)	17.1 (17.1/17.1)	14.5 (14.5/14.5)	67.1 (67.1/67.1)
nad2	46.2 (46/47)	25.64 (25.4/25.7)	17.3 (17.3/17.3)	10.5 (10.5/10.5)	63.5 (63.3/64.3)
nad1	49 (49/49)	18 (18/18)	16.8 (16.8/16.8)	15.8 (15.8/15.8)	65.8 (65.8/65.8)
cytb	43 (43/43)	22.5 (22.5/22.5)	20.9 (20.9/20.9)	13.5 (13.5/13.5)	63.9 (63.9/63.9)
cox3	39 (39/39)	22.1 (22.1/22.1)	21 (21/21)	18.3 (18.3/18.3)	60 (60/60)
cox2	39 (39/39)	21.3 (21.3/21.3)	27 (27/27)	13 (13/13)	66 (66/66)
cox1	41 (41/41)	24.9 (24.9/24.9)	18 (18/18)	16.3 (16.3/16.3)	59 (59/59)
atp8	39 (39/39)	26.3 (26.3/26.3)	24.6 (24.6/24.6)	10.5 (10.5/10.5)	63.6 (63.6/63.6)
atp6	45 (45/45)	26.2 (26.2/26.2)	15.6 (15.6/15.6)	12.9 (12.9/12.9)	60.6 (60.6/60.6)
mean	45 (45/45)	20.8 (20.7/20.8)	18.8 (18.8/18.8)	15.1 (15/15.1)	63.8 (63.8/63.8)

Table 4. Nucleotide compositions of total codon, 1st, 2nd and 3rd position of the codon for PCGs. Continue

			3rd position of the codon		
nad6	37 (36/38)	23.3 (22.7/23.9)	38.6 (38.6/38.6)	1.1 (1.1/1.1)	75.6 (74.6/76.6)
nad5	48 (48/48)	3.5 (3.3/3.5)	35 (34.8/35.2)	13.9 (13.7/14)	83 (82.8/83.2)
nad4l	45 (45/45)	1 (1/1)	38.4 (38.4/38.4)	15.2 (15.2/15.2)	83.4 (83.4/83.4)
nad4	46 (46/46)	3.2 (3.1/3.4)	37.9 (37.9/37.9)	12.6 (12.6/12.6)	83.9 (83.9/83.9)
nad3	29.8 (29/30)	20.7 (20.5/21.4)	45.3 (45.3/45.3)	4.3 (4.3/4.3)	75.1 (74.3/75.3)
nad2	39 (39/39)	14.8 (14.6/15.2)	42.5 (42.4/42.7)	3.5 (3.5/3.5)	81.5 (81.4/81.7)
nad1	49 (49/49)	2.8 (2.8/2.8)	33.2 (33.2/33.2)	14.6 (14.6/14.6)	82.2 (82.2/82.2)
cytb	40 (40/40)	20.1 (20.1/20.1)	37.6 (37.6/37.6)	2.1 (2.1/2.1)	77.6 (77.6/77.6)
cox3	42 (42/42)	17.7 (17.6/17.9)	38 (38.9/39.3)	1.4 (1.1/1.5)	80 (80.9/81.3)
cox2	43 (43/43)	12.5 (12.2/12.6)	42.2 (42.2/42.2)	2.2 (2.2/2.2)	85.2 (85.2/85.2)
cox1	41 (41/41)	12.7 (12.7/12.7)	43 (42.9/43.1)	3.1 (2.9/3.1)	84 (83.9/84.1)
atp8	37.2 (37/38)	21.2 (21.1/21.4)	41.9 (41.1/42.1)	0 (0/0)	79.1 (79.1/79.1)
atp6	43.2 (43/44)	13.9 (13.3/14.2)	41.5 (41.3/41.8)	1.6 (1.3/1.8)	84.7 (84.3/85.3)
mean	43 (43/43)	11.2 (11.2/11.3)	39.1 (39/39.1)	6.74 (6.7/6.8)	82.1 (82/82.1)

However, interspecifically, when *P. ebneri* and *P. venosus* are compared, the number and rate of variable positions per gene are much higher. Among the PCGs, the number of variable positions is particularly high in *nad5*, *nad2*, *cox1* and *nad4* (206, 166, 164 and 159 variable sites), while the remaining nine genes display variable sites ranging from 25 to 143. The lowest variation is found in *cox1, cox2 and nad4L* with the ratio of 0.1 (164/1532, 68/691 and 29/297) while the highest is observed in *nad6* with 0.2 (107/532). In tRNA genes, *trnS1*, *trnD*, *trnS2*, *trnP* and *trnT* are invariable, while the others vary at one to six sites, still demonstrating a considerable degree of conservation. In contrast, the rRNA genes show a notable level of variation, with *rrnS* differing at 43 sites with a variation ratio of 0.06 (43/780) and *rrnL* at 95 sites with a variation ratio of 0.07 (95/1313). As expected in insects [51], one of the highest numbers of variable positions and the highest variation ratio are observed in the largest non-coding AT-rich region, with 147 sites and a variation ratio of 0.13 (147/1096).

The five *P. ebneri* total mitogenomes showed very low intraspecific genetic variation, with pairwise distances ranging from 0.001 to 0.003 (Table 7). This pattern of intraspecies divergence, both at level of individual genes and gene clusters as well as for total mitogenome, is considerably low compared to other orthopterans [22,23], and some other animals [8-12,14]. Such low intraspecific divergence may result from a recent decline in population size, potentially leading to genetic loss and indicating its increased extinction vulnerability due to reduced genetic diversity [29].

The pairwise distance between total mitogenomes of *P. ebneri* and *P. venosus* was significantly large, with a value of 0.118. This represents considerable genetic diversity, and similar patterns have been observed in other orthopterans [22,23]. However, interspecific divergence levels are generally expected to align with phylogenetic relatedness. *Psorodonotus ebneri* and *P. venosus* are two distantly related species, as they belong to two different species groups [25-26]. This level of divergence would likely be much lower if *P. ebneri* were compared with species from the *Psorodonotus caucasicus* species group [27,29].

Signs of natural selection

The pattern of natural selection affecting PCGs was assessed by calculating dN/dS ratios (dN/dS=1, neutral selection; dN/dS>1, positive selection; and dN/dS<1, purifying selection), as shown in Table 8. Due to the absence and/or low variation in the six PCGs of *P. ebneri*, dN/dS and neutrality index (NI, NI>1, purifying selection; NI<1 for positive selection) calculations were not applicable. The calculations resulted significantly low dN/dS ratios for the remaining seven genes (*nad2*, *atp6*, *nad6*, *nad5*, *nad4*, and *nad4L*) indicating purifying selection. For the dataset including *P. ebneri* and *P. venosus*, dN/dS ratios for all PCGs were generally lower than those observed for *P. ebneri* alone, suggesting a stronger purifying selection for most of the PCGs. The NI values were similar to the pattern produced from the dN/dS ratios for both *P. ebneri* and the combined *P. ebneri* and *P. venosus* dataset.

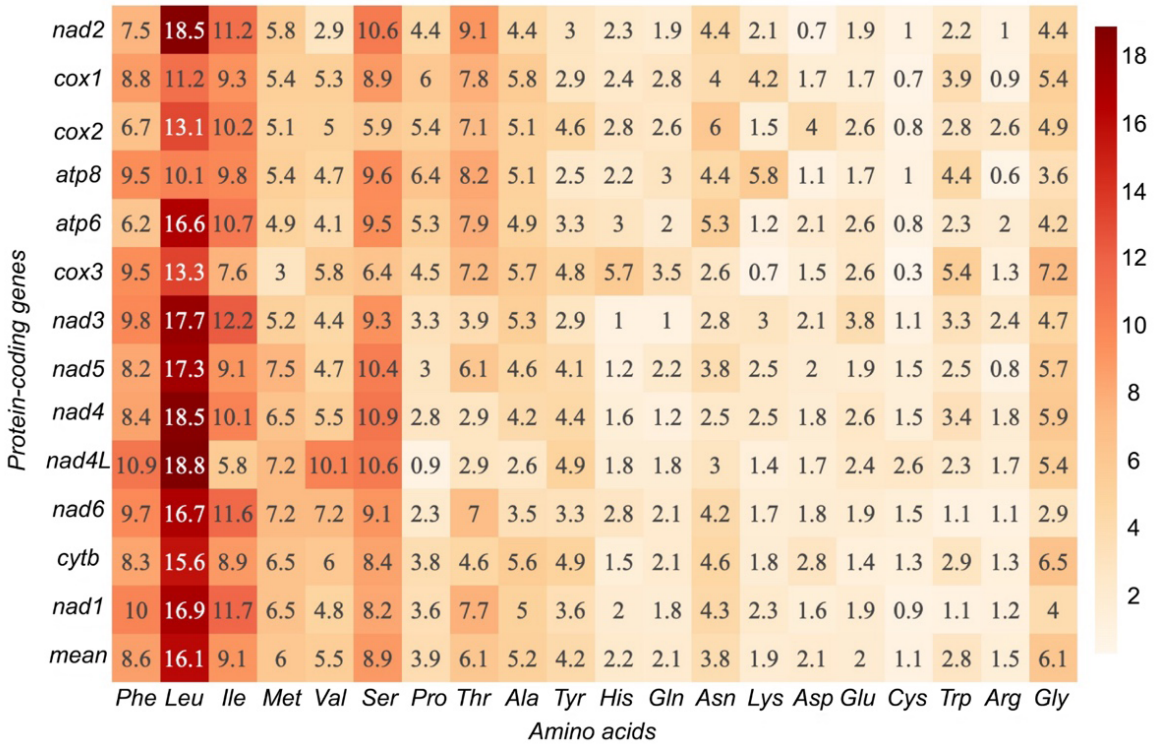

Figure 2. Mean amino acid values of PCGs in *P. ebneri* (amino acids as follows; *Phe* (*Phenylalanine*), *Leu* (*Leucine*), *Ile* (*Isoleucine*), *Met* (*Methionine*), *Val* (*Valine*), *Ser* (*Serine*), *Pro* (*Proline*), *Thr* (*Threonine*), *Ala* (*Alanine*), *Tyr* (*Tyrosine*), *His* (*Histidine*), *Gln* (*Glutamine*), *Asn* (*Asparagine*), *Lys* (*Lysine*), *Asp* (*Aspartic acid*), *Glu* (*Glutamic acid*), *Cys* (*Cysteine*), *Trp* (*Tryptophan*), *Arg* (*Arginine*), *Gly* (*Glycine*)).

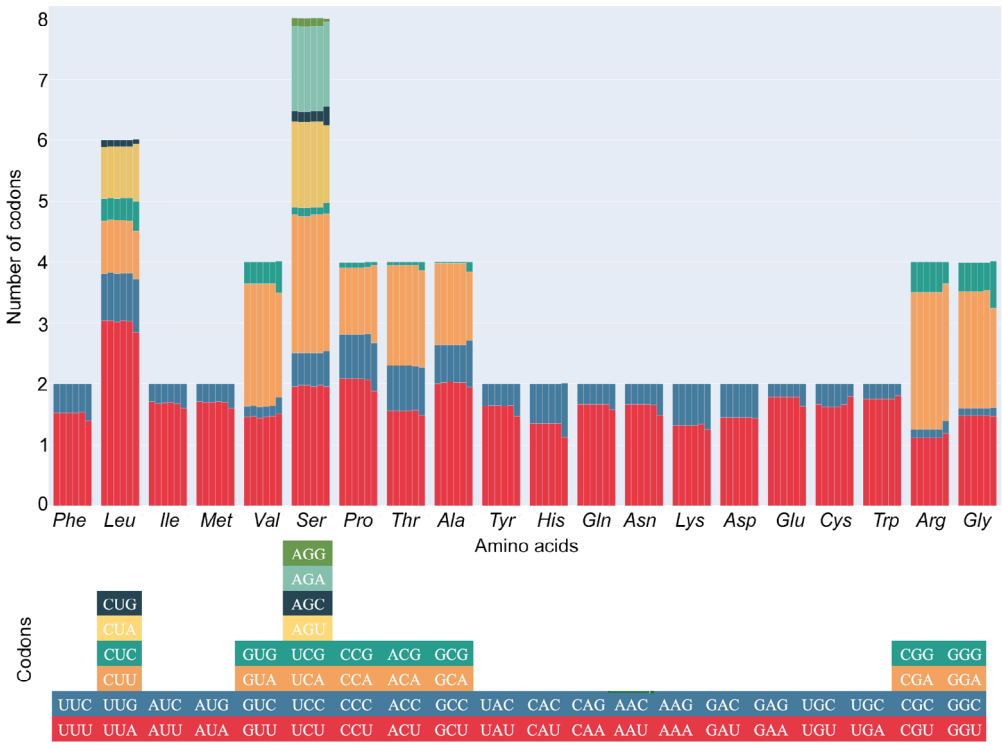

Figure 3. Relative synonymous codon usage (RSCU) in PCGs (amino acids as follows; *Phe* (*Phenylalanine*), *Leu* (*Leucine*), *Ile* (*Isoleucine*), *Met* (*Methionine*), *Val* (*Valine*), *Ser* (*Serine*), *Pro* (*Proline*), *Thr* (*Threonine*), *Ala* (*Alanine*), *Tyr* (*Tyrosine*), *His* (*Histidine*), *Gln* (*Glutamine*), *Asn* (*Asparagine*), *Lys* (*Lysine*), *Asp* (*Aspartic acid*), *Glu* (*Glutamic acid*), *Cys* (*Cysteine*), *Trp* (*Tryptophan*), *Arg* (*Arginine*), *Gly* (*Glycine*)).

Table 5. Overlapping regions (OR) and intergenic spacers (IGS) identified for 38 gene borders (- overlapping, + intergenic spacer, and β used for no overlapping and spacer).

Adjacent genes	*P. ebneri_1/2/5*	*P. ebneri_3/4*	*P. venosus*
trnI -trnQ	+2(TA)	+2(TA)	-3(TTA)
trnQ -trnM	+14 (AATATATCTTCTAG)	+16 (AATATACNCTCCAAAG)	+16 (AATATACACTCTAAAG)
trnM -nad2	β	β	β
nad2 -trnW	β	β	β
trnW -trnC	-8(AAACCTTA)	-8 (AAACCTTA)	-8 (AAACCTTA)
trnC -trnY	β	β	β
trnY -cox1	+1(G)	+1(G)	+1(G)
cox1 -trnL2	β	β	β
trnL2 -cox2	+1(T)	+1(T)	+1(T)
cox2 trnK	β	β	β
trnK -trnD	-1(A)	-1(A)	-1(A)
trnD -atp8	β	β	β
atp8 -atp6	-4(ATAA)	-4(ATAA)	-4(ATAA)
atp6 -cox3	-1(A)	-1(A)	-1(A)
cox3 -trnG	β	β	β
trnG -nad3	β	β	+1(T)
nad3 -trnA	β	β	β
trnA -trnR	+1(A)	+1(A)	β
trnR -trnN	β	β	β
trnN -trnS1	β	β	β
trnS1 -trnE	β	β	+1(T)
trnE -trnF	-2(TA)	-2(TA)	-2(TA)
trnF -nad5	β	β	β
nad5 -trnH	β	β	β
trnH -nad4	β	β	β
nad4 -nad4l	-7 (TTAACAT)	-7 (TTAACAT)	-7 (TTAACAT)
nad4l -trnT	+1(T)	+1(T)	+1(T)
trnT -trnP	-1(T)	-1(T)	-1(T)
trnP -nad6	+1 (C)	+1 (C)	+1(T)
nad6 -cytb	β	β	-1(A)
cytb -trnS2	β	β	β
trnS2 -nad1	+16 (TACTAAATAAAATACA)	+16 (TACTAAATAAAATACA)	+16 (TACTAAACAAAATACA)
nad1 -trnL1	β	β	β
trnL1 -rrnL	β	β	β
rrnL -trnV	β	β	β
trnV -rrnS	β	β	β
rrnS -A+T	β	β	β
A+T-trnI	β	β	β
IGS (bp)	37	39	38
OR (bp)	24	24	28

Table 6. The number of conserved sites (Ncs), number of variable sites (Nvs) and the variation ratio (VR; number of variable sites/all sites) for each gene and each matrix (Intraspecific/Interspecific matrix (*P. ebneri / P. ebneri+ P. venosus*)).

Gene	Ncs	Nvs	VR	Gene	Ncs	Nvs	VR
nad2	1023/861	4/166	0.004/0.16	*trnL2*	64/62	0/2	0/0.03
cox1	1527/1367	4/164	0.003/0.1	*trnK*	70/69	0/1	0/0.014
cox2	690/623	1/68	0.001/0.1	*trnD*	66/66	0/0	0/0
atp8	171/146	0/25	0/0.15	*trnG*	64/61	0/1	0/0.016
atp6	670/593	5/82	0.007/0.12	*trnA*	64/62	0/2	0/0.031
cox3	785/701	2/86	0.003/0.11	*trnR*	63/59	0/4	0/0.06
nad3	351/304	1/48	0.003/0.14	*trnN*	65/63	0/2	0/0.03
nad5	1728/1526	4/206	0.002/0.12	*trnS1*	67/67	0/0	0/0
nad4	1334/1180	5/159	0.004/0.12	*trnE*	67/64	0/3	0/0.04
nad4L	295/268	2/29	0.007/0.1	*trnF*	63/62	1/2	0.02/0.03
nad6	525/422	4/107	0.008/0.2	*trnH*	63/58	1/6	0.02/0.09
cytb	1135/992	0/143	0/0.13	*trnT*	64/64	0/0	0/0
nad1	945/834	0/111	0/0.12	*trnP*	65/65	0/0	0/0
trnI	63/61	1/3	0.02/0.05	*trnS2*	68/68	0/0	0/0
trnQ	69/68	0/1	0/0.013	*trnL1*	64/60	1/5	0.02/0.08
trnM	65/63	0/2	0/0.03	*trnV*	70/67	0/3	0/0.04
trnW	68/65	0/3	0/0.04	*rrnaL*	1308/1214	1/95	0.0008/0.07
trnC	64/60	0/4	0/0.06	*rrnaS*	776/733	0/43	0/0.06
trnY	66/61	0/5	0/0.08	*AT-rich r.*	878/783	42/147	0.05/0.13

Table 7. Intraspecific pairwise differences between five *P. ebneri* mitogenomes.

	P. ebneri_1	*P. ebneri_2*	*P. ebneri_3*	*P. ebneri_4*	*P. ebneri_5*
P. ebneri_1	****				
P. ebneri_2	0.002	****			
P. ebneri_3	0.003	0.001	****		
P. ebneri_4	0.001	0.001	0.001	****	
P. ebneri_5	0.002	0.002	0.003	*0.003*	****

Specifically, NI values were greater than 1 for *nad2*, *atp6*, *nad5*, *nad4*, and *nad4L* genes, whereas it was lower than 1 for *nad6*, *cox1*, *cox2* and *cox3*. However, due to low p-values, for all PCGs except *nad4*, these results should be interpreted with caution and considered neutral. The NI value for *nad4* indicated strong purifying selection, with a value of 8.543. The observed intraspecific and interspecific selection patterns mostly align with previous studies, which report purifying selection as the general pattern for mitochondrial PCGs [6,7,9,11,12,14,16].

Taken together, the findings provide some insights allowing to make some generalizations. First, the mitogenome characteristics, including gene content, gene order and strand location, gene lengths, IGSs, ORs, base composition per gene and gene cluster and per sites in the codons of PCGs, start/stop codons of PCGs, tRNA secondary structures, anticodons, and RSCU exhibit the general characteristics of Orthoptera, and more broadly Pancrustacea [5,19-23,52-54]. However, the conservation of IGS and OR patterns is particularly noteworthy, as they exhibit considerable stability across long-horned orthopterans [20,21,55,56]. Specifically, the IGS +16 (AATATATCTTCTAAG) between *trnQ–trnM*, the IGS +16 (TACTAAATAAAATACA) between *trnS2-nad1*, the OR -8 (AAACCTTA) between *trnW–trnC*, the OR -4(ATAA) between *atp8-atp6* and the OR -7 (TTAACAT) between *nad4-nad4L* seems to be functionally conserved sites across the lineage. Another important aspect

Table 8. Average dN/dS ratios for each matrix (Intraspecific/Interspecific matrix (*P. ebneri / P. ebneri + P. venosus*)) and Neutrality Index (NI) values with p-values (* indicates the values < 0.05) for interspecific matrix (NA: Not applicable).

Genes	dN/dS ratio	NI	p-value
nad2	0.153/0.0706	2.951	0.28
atp6	0.18/0.047	3.35	0.22
nad6	0.238/0.1044	0.595	1
nad5	0.135/0.0496	1.393	1
nad4	0.52/0.0396	8.543	0.03*
nad4L	0.291/0.0594	4.4	0.38
cytb	NA/0.026	NA	NA
nad1	NA/0.037	NA	NA
cox1	NA/0.008	0	1
cox2	NA/0.0302	0	1
atp8	NA/0.195	NA	NA
cox3	NA/0.0148	0	1

is the AT composition per gene, gene cluster and codon position in PCGs which is similar to hemimetabolous insects (see preceding references) but is lower than in holometabolous insects [18,57-63].

Second, this study is among one of the few studies reporting the amount of intraspecific variation per gene and across total mitogenome. The results indicate a low level of variation compared to previous studies [22,23], indicating that intraspecific variation rates are not a universal rule. This is not unexpected since intraspecific genetic divergence is affected by multiple factors related to evolutionary history and population dynamics. In the case of *P. ebneri*, this is particularly relevant, as the species has experienced a significant range contraction and is now restricted to a very small area, with an estimated annual population size of around 1,000 individuals [29]. Although intraspecific variation is low, interspecific divergence between *P. ebneri* and *P. venosus* is considerably high, reaching 12%. This level of divergence aligns with their phylogenetic distance, as both species belong to different species groups within the genus [17].

Third and last, the selection patterns estimated for PCGs should be emphasized. No results were available for six genes due to low genetic variation. However, for the remaining genes, results indicate purifying selection, which seems to be the general pattern for PCGs [6,7,9,11,12,14,16,64,65]. Importantly, no gene showed evidence of positive selection that could suggest adaptation to montane habitats.

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