

Evolution

Mitochondrial genome characterization and mitogenome phylogenetics in the central Mexican *Stenopelmatus talpa* complex (Orthoptera: Stenopelmatidae: Stenopelmatini)

Caracterización del genoma mitocondrial y filogenia del complejo Stenopelmatus talpa del centro de México (Orthoptera: Stenopelmatidae: Stenopelmatini)

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Abstract

The *Stenopelmatus talpa* species-group (Stenopelmatidae) comprises cricket-like orthopterans distributed across the Trans-Mexican Volcanic Belt (TMVB) morphotectonic province and adjacent areas in central Mexico. Despite recent efforts, the taxonomy and evolutionary relationships for members of this complex still are far from completely known. Here we generated and characterized the mitochondrial (mt) genome of 14 specimens of the *S. talpa* species-group and evaluated its species limits with the *cox1* barcoding locus. Moreover, based on the mt genome DNA sequence data, we also reconstructed its phylogenetic relationships and made inferences about its biogeographic history based on a relaxed molecular clock analysis. A total of 9 species were delimited using a 2% pairwise distance criterion, which were consistent with our best estimate of phylogeny. The relationships recovered for the *S. talpa* species-group were similar although with more recent divergence time estimates than those obtained in a previous phylogenetic study, suggesting that its origin and subsequent diversification in the TMVB followed an east-central pattern, with its earliest divergence occurring during the late Pliocene to early Pleistocene.

Keywords: Trans-Mexican Volcanic Belt; Ensifera; Mesoamerica; DNA barcoding

Resumen

El grupo de especies *Stenopelmatus talpa* (Stenopelmatidae) agrupa a ortópteros similares en apariencia a grillos que se encuentran distribuidos en la provincia morfotectónica del Eje Neovolcánico Transversal (ENT) y áreas adyacentes en el centro de México. A pesar de esfuerzos recientes, la taxonomía y las relaciones evolutivas de los miembros de este complejo aún están lejos de conocerse por completo. En este trabajo se caracteriza el genoma mitocondrial de 14 ejemplares del grupo de especies *S. talpa* y se evalúan sus límites de especie utilizando el marcador mitocondrial *cox1*. Con base en la secuencia completa del genoma mitocondrial se reconstruyeron las relaciones filogenéticas del grupo y se hicieron inferencias sobre su historia biogeográfica. Se delimitaron un total de 9 especies con base en un criterio de 2% de divergencia, las cuales fueron consistentes con nuestra reconstrucción filogenética. Las relaciones obtenidas para el grupo de especies *S. talpa* fueron similares, aunque con estimaciones de tiempos de divergencia más recientes a las obtenidas en un estudio filogenético anterior, lo que sugiere que su origen y subsecuente diversificación en el ENT siguió un patrón este-centro, ocurriendo el evento de divergencia más temprano entre el Plioceno tardío y el Pleistoceno temprano.

Palabras clave: Eje Neovolcánico Transversal; Ensifera; Mesoamérica; Código de barras de ADN

Introduction

The family Stenopelmatidae Burmeister, 1838 is a group of cricket-like orthopterans whose species occur in North and Central America, South and Central Africa, Southeast Asia, and the Indian subcontinent (Cigliano et al., 2022). This family was recently proposed by Gorochov (2021) to contain 3 subfamilies: Stenopelmatinae Burmeister, 1838, Schizodactylinae Blanchard, 1845, and Gryllacridinae Blanchard, 1845. However, only the former subfamily is currently recognized within Stenopelmatidae in the Orthoptera Species File (Cigliano et al., 2022). Following the latter classification, the Stenopelmatinae comprises 5 tribes: Maxentiini Gorochov, 2021, Oryctopini Kevan, 1986, Oryctopterini Gorochov, 1988, Siini Gorochov, 1988, and Stenopelmatini Burmeister, 1838 (Cigliano et al., 2022; Gorochov, 2021). Of these tribes, Stenopelmatini is the only one present in the American continent, with species distributed from British Columbia in Canada along the west coast of the USA to the Mesoamerican highlands from northern Mexico to Panama, and with one species from northern South America in Ecuador (Cigliano et al., 2022; Weissman et al., 2021).

Members of Stenopelmatini, commonly known as Jerusalem crickets in the U.S. and as ‘cara de niño’ or ‘niño de la tierra’ in Mexico, are amongst the most recognizable insects in North and Mesoamerica, (Weissman, 2005). They are mainly nocturnal, hiding under rocks or logs during the day (Weissman, 2001, 2005). Species of this tribe mainly feed on roots and are considered important organic matter decomposers (Weissman, 2001), although they also feed on small invertebrates (Weissman, 2001).

In a molecular phylogenetic study for the Stenopelmatoidae based on 2 nuclear and 1 mitochondrial (mt)

gene markers (Vandergast et al., 2017), the Stenopelmatini was recovered as monophyletic, although, only members of another tribe, Siini, were included. In a more recent integrative phylogenetic study that focused on the Stenopelmatini, Weissman et al. (2021) recovered 3 strongly supported main clades for the subfamily based on both anchor hybrid enrichment and mt data. One of these clades had the micropterous *S. piceiventris* Walker, 1869 from Oaxaca in southeast Mexico, whereas the remaining 2 clades included species from western North America, Canada to northern Mexico, and Mesoamerica, respectively. Based on these relationships and examination of morphological, behavioral, and chromosomal features, the tribe was restricted to contain only 2 genera: *Ammopelmatus* Tinkham, 1965 for the North American clade and *Stenopelmatus* Burmeister, 1838 for the 2 clades with Mesoamerican species.

The genus *Stenopelmatus* currently contains 19 described macropterous, micropterous, and apterous species that mainly inhabit highlands with pine-oak and cloud forests (Cigliano et al., 2022; Weissman et al., 2021). A recent phylogenomic study showed that the species richness in this genus is considerably higher, with several undescribed species with a marked geographic phylogenetic structure (Gutiérrez-Rodríguez et al., 2022). Based on these results, 4 species-groups were proposed: the *faulkneri*, *talpa*, Central American and *piceiventris* species-groups, whose geographic distribution and molecular clock estimates were concordant with some geological processes that took place in the Mexican Transition zone (Gutiérrez-Rodríguez et al., 2022).

The *S. talpa* species-group was proposed by Gutiérrez-Rodríguez et al. (2022) to contain 2 described (*S. talpa* Burmeister, 1838 and *S. typhlops* Rehn, 1903) and 5

undescribed species that occur across the Trans-Mexican Volcanic Belt (TMVB) and adjacent areas of the Sierra Madre Oriental (SMO) morphotectonic provinces in central Mexico. However, despite the effort of the above cited authors to assess the species boundaries in this group, additional information from different sources is still required to confirm its species limits, phylogenetic relationships, and biogeographic history.

Mt sequence data have been widely employed to elucidate evolutionary relationships, establish species boundaries, and carry out population genetics studies (Avice et al., 1987; Ballard & Pichaud, 2014). The metazoan mt genome typically consists of 15-18 kilobases, comprising 13 protein-coding genes, 22 transfer RNAs (tRNAs), and 2 ribosomal RNAs (rRNAs) (Boore, 1999), with this composition being generally conserved across bilaterian metazoans (Cameron, 2014; Shen et al., 2016). The use of this locus has given access to a set of orthologous genes with little or no recombination (Ballard & Rand, 2005) and has proved to provide valuable phylogenetic resolution at both shallow and deep evolutionary scales (Ballard & Rand, 2005; Cameron, 2014; DeSalle et al., 2017; Lumley & Sperling, 2010; Song et al., 2015).

With the advent of next-generation sequencing (NGS) techniques, phylogenetic studies of a number of biological groups, including insects, have enormously benefited due to the facilitated generation and use of genomic information, including the assembly of complete mt genomes (Maddock et al., 2016). In this respect, it has been shown that phylogenetic analyses of complete mt genome data increase resolution for resolving species diversification events (Bae et al., 2004; Song et al., 2015).

Here we generated and characterized 16 complete and partial mt genomes of *Stenopelmatus* species, 14 of which belong to species of the *S. talpa* species-group that were delimited in Gutiérrez-Rodríguez et al.'s (2022) study, and whose validity still needs to be further corroborated. We employed this mt genome data to assess the species limits among the examined taxa, estimate their phylogenetic relationships, and investigate their biogeographical history in central Mexico. Our results support the existence of at least 9 species within the *S. talpa* species-group, 2 of which have already been described, whose geographic structure is congruent with the geological history of the TMVB and adjacent morphotectonic provinces.

Materials and methods

A total of 16 *Stenopelmatus* specimens were included in this study, 14 of which belong to the *S. talpa* species-group. These specimens were collected in localities situated

in 7 Mexican states across the TMVB and SMO provinces (Hidalgo, Mexico City, Michoacán, Puebla, Querétaro, State of Mexico, and Veracruz; Fig. 1). Our sampling included specimens assigned to the only 2 described species of the *S. talpa* species-group, *S. talpa* and *S. typhlops*, which were collected from their type locality. We also included 2 undescribed species from 2 other species-groups: one belonging to the *faulkneri* species-group, *S. sp. aff. mineraldelmonte* (El Chico, Hidalgo, CNIN3922), and a second to the Central America species-group, rooting the trees with the latter species. A list with the examined species, their locality, DNA voucher, and GenBank accession numbers is provided in Table 1. All samples were collected between the years 2012 to 2018 and preserved in 96% ethanol at -20°C until they were processed for DNA sequencing.

Genomic DNA was extracted from smooth muscle of hind femur with the EZ-10 Spin Kit minipreps DNA Genomic Column Kit (BIOBasic, Toronto, Ontario, Canada) following the manufacturer's protocol. DNA fragmentation was performed with the Bioruptor Pico equipment (Diagenode Inc.) employing 3 cycles of sonication (15-90 sec. on-off pulse). We used 100 ng of input DNA for library preparation with the Kapa Hyper Prep kit (Kapa Biosystems Inc., Wilmington, MA, U.S.A.), and the TruSeq-style dual-indexing adapters (Glenn et al., 2016). Genomic DNA quantitations, pre- and post-PCR libraries, were performed using a Qubit 2.0 fluorometer (Invitrogen, Life Technologies, CA, USA). Whole-genome shotgun sequencing was conducted with an Illumina HiSeq X instrument at the Department of Environmental Health Science, University of Georgia, Athens, GA, USA.

Raw reads were trimmed and filtered for all samples using the program Geneious version 10.2.6 (Kearse et al., 2012). We carried out mt genomes *de novo* assemblies in the program GetOrganelle (Jin et al., 2018) using the mtDNA database. For 3 samples (CNIN 3669, 3741, 3922) we could not recover complete mt genomes in the *de novo* assembly. In these cases, we performed by-reference assemblies with Geneious to obtain longer contigs, using as reference either mt sequence genomes of geographically close samples or their cytochrome oxidase I (cox1) sequence previously obtained by Sanger sequencing. Mt genome annotations were performed with the MITOS 2 web server (Bernt et al., 2013) and verified using the protein-coding genes signal from the "protein plots" generated by MITOS. We used Geneious to confirm the accuracy of our assemblies and annotations. We also registered the order of the mt genome genes to identify possible gene arrangements, and then compared them to the Pancrustacea ground pattern, which is the proposed

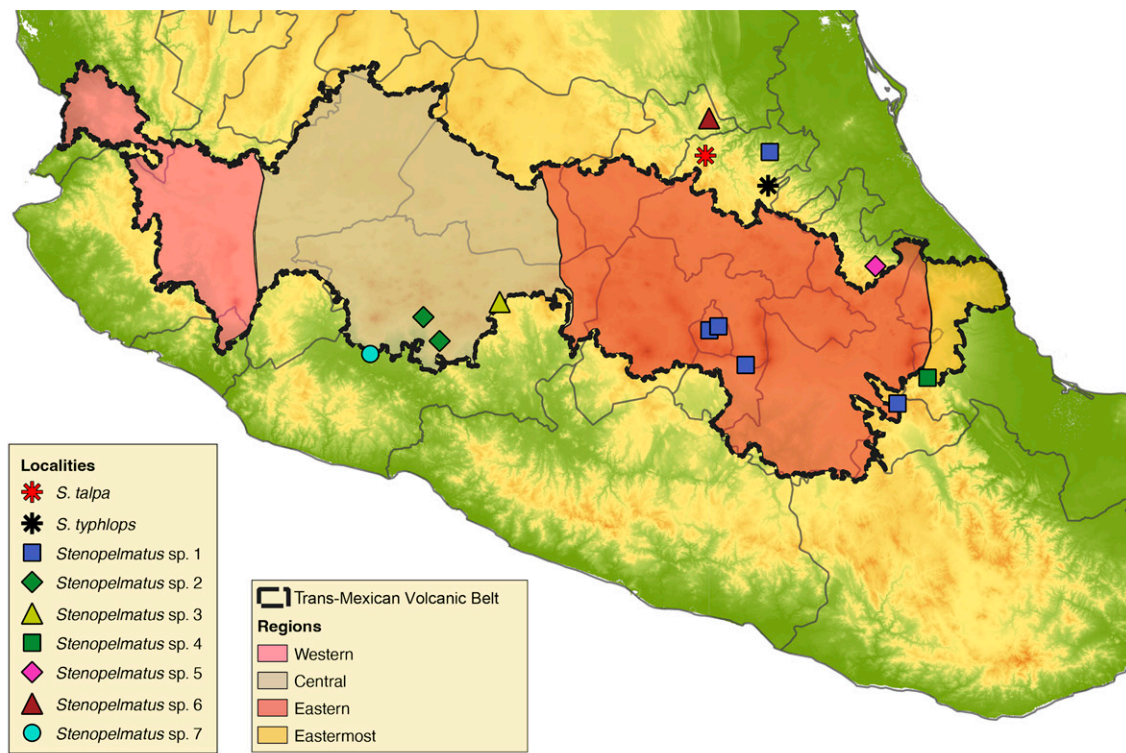


Figure 1. Map with the localities of the specimens examined in this study. The map shows the Trans-Mexican Volcanic Belt morphotectonic province.

Crustacea/Hexapoda common ancestor (Boore et al., 1995, 1998).

We extracted the sequences of the 13 protein-coding genes from all samples and independently aligned them using the program MAFFT version 7 (Katoh & Standley, 2013). We then verified each protein-coding gene alignment with respect to the reading frame (invertebrate mt genetic code) in Geneious. The 2 ribosomal genes (rRNA) and the 22 transfer genes (tRNA) from each sample were also extracted and manually aligned. The following 3 matrices were obtained: 1) a *cox1* matrix comprising the DNA barcoding locus (658 bp), 2) a protein-coding gene matrix (10,953 bp), and 3) a complete mt genome matrix (14,599 bp). The 3 matrices are available in figshare (<https://doi.org/10.6084/m9.figshare.21506583>).

Uncorrected genetic distances were calculated both for the complete mitogenome and *cox1* matrices with the program PAUP version 4.0a (Swofford, 2002). Mt species delimitation among the samples was assessed for the *cox1* data set following a 2% pairwise distance criterion. The use of this marker has been proven to be an efficient tool for species identification of most animal taxa

(Hajibabaei et al., 2007; Hebert et al., 2003). We included in this analysis 4 previously published *cox1* sequences belonging to specimens of the *talpa* species-group: 3 from the state of Morelos and one from Mexico City (Gutiérrez-Rodríguez et al., 2022). The uncorrected *cox1* distances were visualized building a neighbor-joining (NJ; Saitou & Nei, 1987) tree with PAUP.

The best-fit partitioning scheme for the protein-coding gene and complete mt genome matrices was obtained with ModelFinder (Kalyaanamoorthy et al., 2017) in the program IQTree version 2 (Minh et al., 2020). For both matrices we conducted maximum likelihood (ML) phylogenetic analyses using the same version of the program IQTree with 1,000 ultra-fast bootstrap (BTP) replicates.

We carried out the divergence times estimates for the complete mitogenome matrix with the program BEAST2 version 2.4.7 (Bouckaert et al., 2014) using the Birth-Death model (Nee, 1994) and an uncorrelated lognormal relaxed clock (Drummond et al., 2006). A single partition was considered, using the GTR + I + G model of evolution. To calibrate the tree, we used the mutation rate reported by Papadopoulou et al. (2010) for the mt genome (2.4%

Table 1

Specimens examined in this study, their locality, geographic coordinates, DNA voucher, Sequence Read Archive (SRA) and Mt GenBank accession numbers.

Species	Locality	Latitude	Longitude	DNA voucher No.	Raw data SRA accession No.	Mt genome Genbank accession No.
<i>S. talpa</i>	Mexico: Hidalgo, Jacala de Ledezma	20.948	-99.211	CNIN4350	SRR18074866	OP778777
<i>S. typhlops</i>	Mexico: Hidalgo, Zacualtipán	20.636	-98.628	CNIN3983	SRR18074868	OP699672
<i>Stenopelmatus</i> sp. 1	Mexico: Estado de México, Nepantla	18.980	-98.833	CNIN3651	SRR18074864	OP778769
<i>Stenopelmatus</i> sp. 1	Mexico: CDMX, Coyoacán	19.306	-99.174	CNIN3667	SRR18074875	OP778770
<i>Stenopelmatus</i> sp. 1	Mexico: CDMX, Iztapalapa	19.343	-99.089	CNIN3669	SRR18074869	OP778771
<i>Stenopelmatus</i> sp. 1	Mexico: Puebla, Chapulco	18.619	-97.410	CNIN3989	SRR18074862	OP778775
<i>Stenopelmatus</i> sp. 1	Mexico: Hidalgo, Tlanchinol	-	-	CNIN4152	SRR18074861	OP699671
<i>Stenopelmatus</i> sp. 2	Mexico: Michoacán, Ario de Rosales	19.205	-101.704	CNIN3934	SRR18074872	OP778774
<i>Stenopelmatus</i> sp. 2	Mexico: Michoacán, Tingambato	19.430	-101.856	CNIN3736	SRR18074874	OP778772
<i>Stenopelmatus</i> sp. 3	Mexico: Michoacán, Morelia	19.572	-101.140	CNIN3737	SRR18074863	OP699673
<i>Stenopelmatus</i> sp. 4	Mexico: Veracruz, Orizaba	18.860	-97.128	CNIN3639	SRR18074873	OP778767
<i>Stenopelmatus</i> sp. 5	Mexico: Puebla, Zacapoaxtla	19.908	-97.617	CNIN3662	SRR18074876	OP699674
<i>Stenopelmatus</i> sp. 6	Mexico: Querétaro, Valle de Guadalupe	21.299	-99.177	CNIN3646	SRR18074867	OP778768
<i>Stenopelmatus</i> sp. 7	Mexico: Michoacán, Apatzingán	19.083	-102.353	CNIN3741	SRR18074870	OP429107
<i>Stenopelmatus</i> sp. aff. <i>mineraldelmonte</i> (<i>faulkneri</i> species-group)	Mexico: Hidalgo, El Chico	20.184	-98.716	CNIN3922	SRR18074871	OP778773
<i>Stenopelmatus</i> sp. (Central America species-group)	Costa Rica: San José, Rivas Chirripó	9.464	-83.577	CNIN4267	SRR18074865	OP778776

per My; 0.012 ± 0.0012). The analysis ran for 100 million generations, sampling trees every 10,000 generations with a burn-in of 25%. The Maximum Clade Credibility Tree (MCCT) was obtained with TreeAnnotator version 1.7.4 (Drummond et al., 2012).

Results

We obtained 7 complete and 9 partial mt genomes with lengths between 13,056 and 15,785 base pairs, and a GC proportion between 25.0 and 26.5% (Table 2). Of the 16 mitogenome sequences, 12 were recovered as a single contig, whereas the remaining 4 were recovered in 2 or more fragments. We did not find arrangement variation in any of the assembled mitogenomes, with all of them being similar to the Pancrustacea ground pattern (Boore et al., 1998) (Fig. 2, Appendix 1).

Uncorrected genetic distances for the *cox1* and complete mitogenome matrices are shown in Table 3. The pairwise distances observed for the complete mt genome data set generally were slightly lower compared to the ones obtained with *cox1*. The NJ tree built with the *cox1* data set (Fig. 3a) grouped the ingroup taxa in 9 separate clusters that were mostly congruent with the sequence divergence gap of 2%. One of the cases of incongruence was found within the putative *S. sp. 1* from the Valley of Mexico, where 6 of the 8 pairwise comparisons that involved the specimen from Nepantla, Estado de Mexico (CNIN3669) were marginally higher than 2% (2.1-2.59%). Furthermore, the only sample from Apatzingán, in the state of Michoacán (*S. sp. 7*; CNIN3741), had a distance slightly lower than 2% with a sample of *S. sp. 1* from Mexico City (CNIN3669; 1.87%) and the sample belonging to *S. sp. 3* from Morelia, Michoacán (CNIN3737; 1.9%).

Table 2

Main features of the assembled mt genomes in this study: assembly size (bp), mean coverage, and genes not found.

DNA voucher	Assembly size	GC (%)	Mean coverage	Mt genes not recovered
CNIN3662	15,270	25.6	18.3	-
CNIN3667	14,249	25.4	19.7	-
CNIN3983	15,806	25.3	11.4	-
CNIN3646	15,440	25.0	7.9	-
CNIN4350	13,558	25.2	24.7	trnN, trnS1, trnE, trnF, nad4L, trnT, trnP, trnQ
CNIN4267	15,485	26.1	15.6	-
CNIN3651	13,485	25.9	14.5	trnI, trnA, trnR, trnN, trnS1, trnE, rrnS, trnV, trnF, trnQ
CNIN3737	15,312	25.2	15.0	-
CNIN3989	14,146	25.3	13.2	trnV, rrnS
CNIN4152	15,483	32.3	20.5	-
CNIN3736	15,356	25.1	134.1	trnN, trnR, trnS1
CNIN3639	13,056	26.5	15.3	trnI, trnQ, trnM, trnV, rrnS
CNIN3934	13,315	25.6	5.2	trnE, rrnL
CNIN3922	15,785	27.5	12.9	trnE
CNIN3741	14,734	24.5	16.4	trnA, trnD, trnF, trnL1, trnR, trnY, rrnL
CNIN3669	14,860	25.2	14.8	trnN, trnS1

The ML phylograms derived from the complete mt genome and protein-coding gene matrices showed identical relationships, only varying in the BTP support values of some of their clades (Fig. 3b and Appendix 2, respectively). The putative species *S. sp. 7* from Apatzingán, Michoacán, in the northernmost part of the Sierra Madre del Sur (SMS) province was sister to the remaining ingroup taxa (BTP = 100), which appeared divided into 2 main clades. One of these clades (BTP = 79) had *S. talpa*, which is distributed in the limits between the SMO and the TMVB provinces, as sister to the taxa from the eastern part of the TMVB. The second main clade (BTP = 100) contained the putative species from the central portion of the TMVB in the Valley of Mexico and central Michoacán.

The reconstructed chronogram (Fig. 4) shows that the origin of the *S. talpa* species-group took place during the late Miocene to late Pliocene, 5.81-2.38 Mya, whereas the divergence between the putative undescribed species from the northernmost portion of the SMS in Apatzingán, Michoacán, and those from the TMVB and SMO provinces probably occurred during the late Pliocene 3.55-2.5 Mya. The split of the members from the central and eastern portions of the TMVB on the other hand probably occurred during the late Pliocene to early Pleistocene, between 2.94 and 1.32 Mya.

Discussion

Mitogenome features in the talpa species-group

Although the Orthoptera is one of the orders whose species are known to have among the largest nuclear insect genomes and thus are more prone to the insertion of nuclear mitochondrial paralogs (Song et al., 2014), its mt gene arrangement has remained generally stable through time (Cameron, 2014; Song et al., 2015). Currently, the only mt gene rearrangement that has been reported as a probable synapomorphy at the supraspecific level within the order is the *trnD-trnK* tRNA gene translocation within Caelifera (Song et al., 2015).

Here, we have generated and assembled the complete and nearly complete mt genome of various *Stenopelmatus* specimens, a poorly-known group of ensiferan orthopterans mainly distributed across the Mesoamerican highlands. Previous to this study, only one mitogenome had been published for the genus and the whole family Stenopelmatidae, i.e., *Ammopelmatus fuscus* Haldeman, 1852 (Song et al., 2015). Thus, our results provide valuable information for a better understanding of the molecular evolution of this orthopteran family and Ensifera in general.

Table 3

Uncorrected distances for the complete mt genome (above) and *coxI* (below) data sets that were obtained for the putative species belonging to the *S. talpa* species-group. Bold numbers are cases of incongruence based on the 2% pairwise distance criterion employed for the *coxI* barcoding locus (see results).

Species	1	2	3	4	5	6	7	8	9	10	11
1. <i>S. sp. 1</i> (Valley of Mexico)	0-2.59	3.85-4.43	3.73-4.29	5.47-6.08	5.3-5.77	4-57-4.99	5.29-5.82	2.47-3.18	2.76-3.5	9.61-10.24	11.88-12.81
2. <i>S. sp. 2</i> (Tingambato, Ario de Rosales, Michoacán)	3.8-5.01	0.45	3.98-4.08	5.82-6.45	5.62-5.64	5.13-5.32	5.97-6.05	2.97-3.03	3.57-3.61	10.31-10.47	12.38-12.88
3. <i>S. sp. 3</i> (Morelia, Michoacán)	2.6-3.95	3.95-4.4	-	6.36	5.83	4.91	6.18	2.75	3.52	10.26	12.6
4. <i>S. sp. 4</i> (Orizaba, Veracruz)	5.17-6.23	5.77	5.77	-	2.28	4.91	5.62	4.51	4.23	10.53	12.71
5. <i>S. sp. 5</i> (Zacapoaxtla, Puebla)	5.21-6.53	5.62-6.07	6.07	2.12	-	4.16	4.91	3.77	3.74	10.18	12.55
6. <i>S. sp. 6</i> (Valle de Guadalupe, Querétaro)	5.24-6.29	6.43-6.59	6.14	5.36	5.36	-	4.59	3	3.29	8.92	11.32
7. <i>S. typhlops</i> (Zacualtipán, Hidalgo)	5.68-6.83	6.68-6.83	6.99	5.77	5.77	5.84	-	4.15	4.16	10.16	12.62
8. <i>S. sp. 7</i> (Apatzingán, Michoacán)	1.9-3.13	2.51-2.83	1.87	4.72	4.85	5.54	5.65	-	19.6	7.13	10
9. <i>S. talpa</i> (Jacala, Hidalgo)	2.33-3.39	3.1-3.25	2.78	4.31	4.16	4.34	4.15	2.04	-	8.52	10.98
10. <i>S. sp. aff. mineraldelmonte</i> (<i>faulkneri</i> species-group)	10.05-11.56	10.65	9.87	10.94	11.26	11.53	10.96	7.67	9.2	-	9.97
11. <i>S. sp.</i> (Central American species-group)	11.27-12.61	11.44-12.31	12.15	11.85	12.31	11.79	11.85	9.54	10.6	11.09	-

Our results indicate that all the assembled mt genomes follow the ancestral insect mt genome structure (Boore et al., 1998), thus supporting the high conservativeness of the mt genome structural organization within Orthoptera. Also, other mt genome features among the examined species, such as the conservation of the trnK-trnD pattern of the tRNAs between the protein-coding genes *cox2* and *atp8*, appear to be similar to those described for other ensiferans (Fenn et al., 2008; Song et al., 2015) as well as a high AT content reported for orthopterans in general (Fen et al., 2008; Sheffield et al., 2010; Song et al., 2015). Further assembling of additional mt genomes of other stenopelmatid taxa will reveal whether the ancestral mt gene organization and other relevant mt features persist in all members of the family.

Barcoding-based species delimitation

A recent molecular phylogenetic study based on mt and 3RAD nuclear data showed the existence of an extensive,

highly neglected species diversity in *Stenopelmatus* (Gutiérrez-Rodríguez et al., 2022). Of the 34 species that were delimited in the latter study, 8 belonged to a clade named the *S. talpa* species-group, whose species are characterized by having a large size and a generally reddish-orange and black color pattern (although it appears to be exclusively black in *S. typhlops*; Gutiérrez-Rodríguez et al., 2022).

Our inclusion of additional specimens from the same and newly sampled localities with respect to the above study resulted in 9 barcoding-based species, although the delimitation of samples from Mexico City and surrounding localities decreased to only one putative species in comparison with Gutiérrez-Rodríguez et al.'s (2022) study, which discriminated 3 potential evolutionary lineages. The species delineation obtained here, however, must be taken with caution, since some of the *coxI* distances that involved specimens from the populations that had been regarded as a different lineage

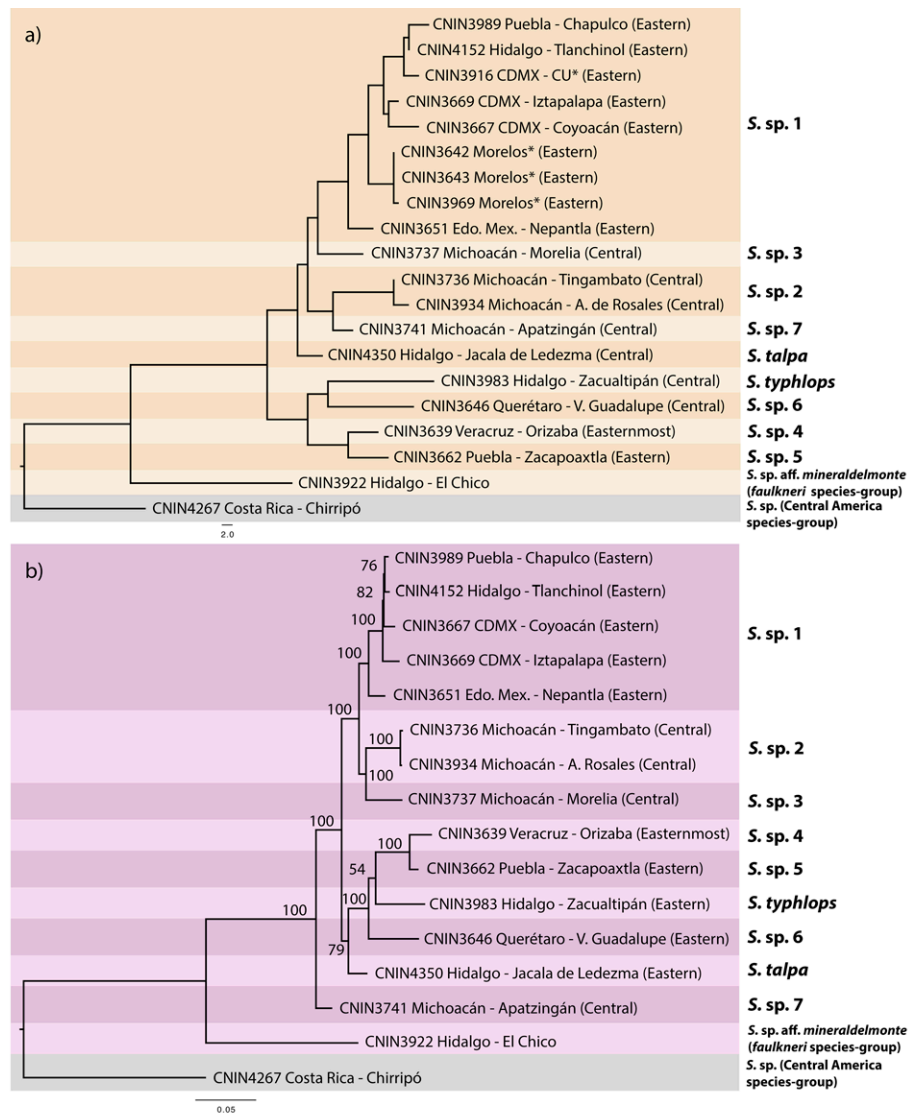


Figure 3. a) Neighbor-joining tree reconstructed using the *cox1* barcoding locus (658 bp) showing the 9 species delimited for the *S. talpa* species-group based on the 2% sequence divergence criterion. b) Phylogram for the *S. talpa* species-group reconstructed with the maximum likelihood method using the complete mitogenome sequence. Numbers near nodes represent ultra-fast bootstrap values.

western Mexico (Ferrari et al., 2012). The TMVB is characterized by having an east-west (E-W) orientation, being divided into 4 distinct episodes: 1) early- to mid-Miocene, 2) late Miocene, 3) late Miocene-early Pliocene, and 4) late Pliocene and Pleistocene (Ferrari et al., 2012; Gómez-Tuena et al., 2007). In addition, this province is divided into 4 sectors according to their age of inception: Easternmost (~16 Mya), Eastern (~19 Ma), Central (~11 Ma) and Western (~11 Ma) (Ferrari et al., 2012).

According to our results, the *S. talpa* species-group apparently followed an east-central diversification pattern

along the TMVB province. The earliest divergence within the *S. talpa* species-group was estimated to occur ca. 3.55-1.58 Mya and involved *S. sp. 7*, a species distributed between the SMS and TMVB border, followed by the clade with the species from the TMVB and adjacent areas of the SMO provinces, which started to diverge ca. 2.94-1.32 Mya. Moreover, most of the earlier divergences within the group occurred along the northern borders of the Eastern sector, while the most recent ones can be found across the Eastern and Central sectors. This is congruent with the aforementioned topographic evolution of the TMVB,

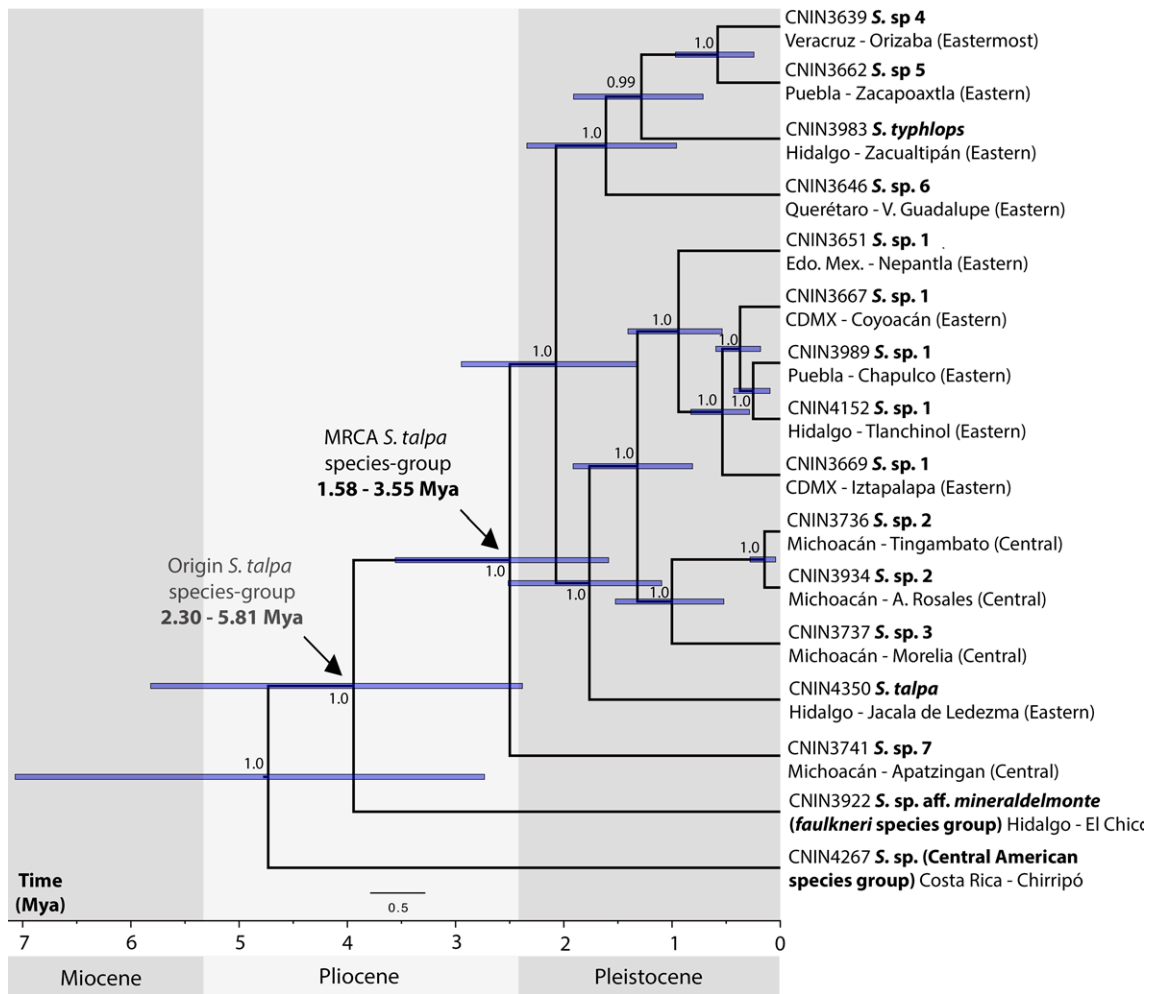


Figure 4. Bayesian ultrametric tree obtained with BEAST2 using the complete mitochondrial genome data set for the *S. talpa* species-group. Gray bars represent geological time periods.

which began in the early to mid-Miocene and finished in the late Pliocene to Pleistocene.

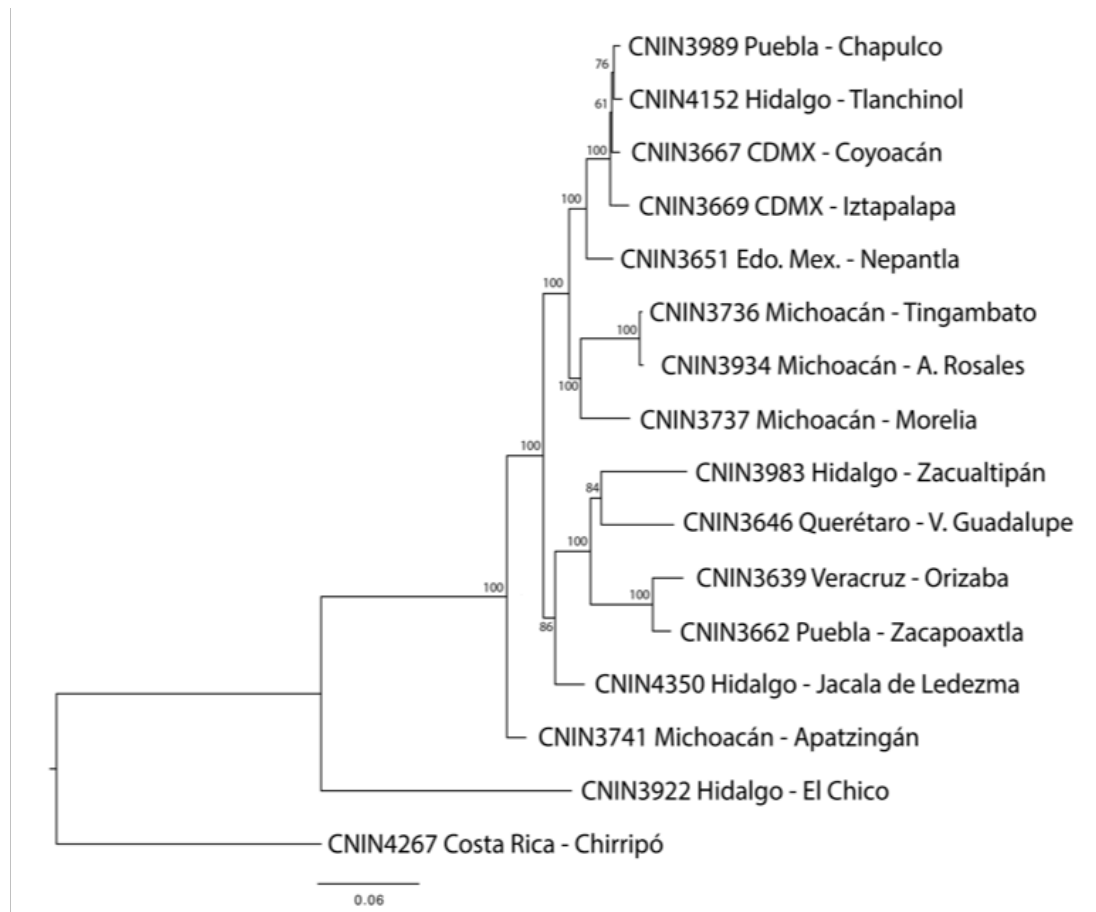
The biogeographic inferences drawn here for this apparently recently diverged group of orthopterans endemic to central Mexico will serve as a basis for future evolutionary studies of other groups of organisms with a similar geographic distribution. This work also highlights the utility that the mt genome sequence data have for phylogenetic reconstruction studies of animal taxa, and advocates for more studies that make use of this valuable, highly informative locus.

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Appendix 2. Phylogram for the *S. talpa* species-group reconstructed with the maximum likelihood method using a protein-coding genes matrix. Numbers near nodes represent ultra-fast bootstrap values.



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