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Article

The Complete Mitochondrial Genome of *Spirobolus bungii* (Diplopoda, Spirobolidae): The First Sequence for the Genus *Spirobolus*

Hanmei Xu [1,†], Yu Fang [1,†], Guohua Cao [2], Caiqin Shen [2], Hongyi Liu [1] and Honghua Ruan [1,*]

[1] Co-Innovation Center for Sustainable Forestry in Southern China, College of Biology and the Environment, Nanjing Forestry University, Nanjing 210037, China
[2] Dongtai National Forest Farm of Jiangsu Province, Dongtai 224200, China
* Correspondence: hhruan@njfu.edu.cn
† These authors contributed equally to this work.

Abstract: Millipedes (Diplopoda) comprise one of the most important groups of large soil arthropods in terrestrial ecosystems; however, their phylogenetic relationships are poorly understood. Herein, the mitochondrial genome (mitogenome) of *Spirobolus bungii* was sequenced and annotated, which was 14,879 bp in size and included 37 typical mitochondrial genes (13 protein-coding genes (PCGs), two ribosomal RNA genes (rRNAs), and 22 transfer RNA genes (tRNAs)). Most of the 13 PCGs had ATN (AT/A/T/G) as the start codon except for COX1, which used CGA, and most PCGs ended with the T end codon. By comparing the gene arrangements of the mitogenomes among Diplopoda species, rearrangement occurred between and within orders. In contrast to *Narceus annularus*, the mitogenome genes of *S. bungii* had consistent orders but were transcribed in completely opposite directions, which was a novel finding in Spirobolidae. Moreover, the phylogenetic relationships within Diplopoda, which were based on the sequences of 13 PCGs, showed that *S. bungii* was clustered with *N. annularus*, followed by *Abacion magmun*. This indicated that there might be a close relationship between Callipodida and Spirobolida. These results could contribute to further studies on the genetics and evolutionary processes of *S. bungii* and other Diplopoda species.

Keywords: Diplopoda; mitochondrial DNA; rearrangement; transcription direction; phylogenetic tree

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1. Introduction

Millipedes *Spirobolus bungii* (*S. bungii*) belongs to the Spirobolidae family of the Diplopoda class [1]. Diplopoda comprise one of the most important groups of large soil arthropods in the terrestrial ecosystems [2], with key decomposition and nutrient cycling functions in forests [3]. They also serve as model organisms for addressing myriad evolutionary, ecological, and biological concepts and questions [4]. Diplopoda are found worldwide and reside within forests, meadows, mountains, caves, farmlands, urban green spaces, and residential areas [1]. While there have been interesting studies on millipedes in recent years, they remain a largely unexplored group, with only 12,000 of the predicted 60,000 [5] to 80,000 [6] species that are currently described. To date, there are very few studies on Diplopoda and even fewer for species in China [7,8]. Furthermore, phylogenetic studies based on morphological characteristics between diplopod taxa are rare [9,10].

Molecular data have become increasingly important in recent years. In animals, the typical mitochondrial genome (mitogenome) is a circular double-stranded DNA molecule, which encodes 13 protein-coding genes (PCGs) for the enzymes required for oxidative phosphorylation, two ribosomal RNA genes (rRNAs), and 22 transfer RNA genes (tRNAs) necessary for the translation of the proteins encoded by the mitogenome [11,12]. Compared with individual genes, the mitogenome remains a promising tool for inferring phylogenetic relationships due to its high information content. Recently, some mitogenomes

in Diplopoda were published and applied to explore phylogenetic relationships [13–16]. However, only a few mitogenomes have been published for Spirobolida [17]. Further, the arrangement of genes in mitogenomes is remarkably variable across Diplopoda [13,17,18].

In this study, for the first time, the *S. bungii* mitogenome was assembled and characterized. The structural organization, nucleotide composition, codon usage, and AT/GC-skew were analyzed. Additionally, we conducted phylogenetic analyses based on 13 PCGs available elsewhere for the purpose of investigating the phylogenetic position of *S. bungii* within Diplopoda, which might further elucidate the genetics and evolutionary processes of *S. bungii* and other Diplopoda species.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

The specimens used in this study were collected from the Purple Mountain (30°01′ N, 118°48′ E) in 2019, where an existing deciduous broadleaved mixed forest is dominated by oaks (e.g., *Quercus varialis* BL, *Q. accutissima* Carruth), in Nanjing, Jiangsu Province, China. Following morphological identification, the samples were stored at −20 °C in the Ecology Laboratory of Nanjing Forestry University (Accession No: NFU20191103). The total genomic DNA was prepared from a small portion of body segments of a single individual using the SDS-protease K-alcohol phenyl-trichlormethane method. The remaining tissue was stored at −20 °C in 90% ethanol to preserve the specimens.

2.2. Mitogenome Sequencing, Assembly, and Annotation

The complete genomic library of *S. bungii* was established using an Illumina HiSeqNano DNA Sample Prep Kit (Illumina, San Diego, CA, USA), whereas the sequencing was performed using next-generation sequencing (NGS) via Illumina Hiseq2000 (Illumina, USA). To generate clean data, low-quality sequences were removed. About 40 million reads with a GC content of 43.65% were assembled to obtain a complete mitogenome using SPAdes v3.11.1 [19]. Thus, the complete mitochondrial genome sequence was used to predict the transcriptional direction of each gene component using the Improved de novo Metazoan Mitochondrial Genome Annotation (MITOS) platform [20]. The annotated mitochondrial genome sequence of *S. bungii* was submitted to GenBank (Accession: NC_056899.1).

2.3. Sequence Analysis

The mitochondrial ring structure was plotted by comparative genomics (CG) View Server [21], and 22 tRNA clover two-dimensional structures were predicted using tRNAscan-Se [22]. The composition skew was calculated according to the following formulae: AT-skew = (A − T)/(A + T) and GC-skew = (G − C)/(G + C) [23]. Next, a visual graph of the composition skew was created using the ggplot2 packages in R v.4.2.0. Moreover, the R script for the relative synonymous codon usage (RSCU) frequency graph was generated from PhyloSuite [24], which was then run in R v.4.2.0.

2.4. Phylogenetic Analysis

To clarify the phylogenetic position of *S. bungii*, the available complete mitogenomes were obtained from GenBank and were comprised of nine orders and 27 species (Table 1). *Stylochyrus rarior* (GenBank accession: CQ927176.2) from order Mesostigmata was used as the outgroup. A total of 27 species, including *S. bungii*, were employed to develop phylogenetic trees based on 13 PCGs.

Table 1. List of complete mitogenomes used in this study.

Class	Order	Family	Genus	Species	Accession
Diplopoda	Callipodida	Callipodidae	*Abacion*	*Abacion magnum*	NC_021932.1
	Glomeridesmida	Glomeridesmidae	*Glomeridesmus*	*Glomeridesmus* sp. ITV8918	MG905160.1
				Glomeridesmus spelaeus	MG372113.1
	Julida	Julidae	*Anaulaciulus*	*Anaulaciulus koreanus*	NC_034656.1
		Nemasomatidae	*Antrokoreana*	*Antrokoreana gracilipes*	NC_010221.1
	Playtdesmida	Andrognathidae	*Brachycybe*	*Brachycybe lecontii*	NC_021934.1
	Polydesmida	Paradoxosomatidae	*Asiomorpha*	*Asiomorpha coarctata*	KU721885.1
		Polydesmidae	*Epanerchodus*	*Epanerchodus koreanus*	NC_051495.1
		Xystodesmidae	*Appalachioria*	*Appalachioria falcifera*	NC_021933.1
			Xystodesmus	*Xystodesmus* sp. YD-2016	KU721886.1
	Sphaerotheriida	Sphaerotheriidae	*N/A*	*Sphaerotheriidae* sp. HYS-2012	NC_018361.1
	Spirobolida	Spirobolidae	*Narceus*	*Narceus annularus*	NC_003343.1
			Spirobolus	*Spirobolus bungii*	NC_056899.1
	Spirostreptida	Harpagophoridae	*Thyropygus*	*Thyropygus* sp. DVL-2001	NC_003344.1
		Odontopygidae	*Chaleponcus*	*Chaleponcus netus*	NC_062683.1
			Prionopetalum	*Prionopetalum kraepelini*	NC_062688.1
		Spirostreptidae	*Archispirostreptus*	*Archispirostreptus gigas*	NC_062689.1
			Macrolenostreptus	*Macrolenostreptus orestes*	NC_062682.1
			Pseudotibiozus	*Pseudotibiozus cerasopus*	NC_062681.1
			Tropostreptus	*Tropostreptus austerus*	NC_062687.1
				Tropostreptus droides	NC_062686.1
				Tropostreptus hamatus	MT394521.1
				Tropostreptus kipunji	MT394511.1
				Tropostreptus microcephalus	NC_062684.1
				Tropostreptus severus	NC_062685.1
				Tropostreptus sigmatospinus	MT394526.1
Arachnida	Mesostigmata	Ologamasidae	*Stylochyrus*	*Stylochyrus rarior*	CQ927176.2

All operations were performed with the PhyloSuite software package [24]. The sequences were aligned in batches using MAFFT software [25]. Ambiguously aligned areas were removed using Gblocks [26]. ModelFinder was utilized to partition the codons and identify the best substitution model for phylogenetic analyses [27]. Phylogenetic trees were constructed with Bayesian inference (BI) and maximum likelihood (ML). The ML phylogenies were inferred using IQ-TREE [28] under the model automatically selected by IQ-TREE ('Auto' option in IQ-TREE) for 5000 ultrafast [29] bootstraps, as well as the Shimodaira–Hasegawa-like approximate likelihood-ratio test [30]. BI analysis was performed using MrBayes v.3.2.6 [31] with four chains (one cold chain and three hot chains).

Two independent runs of 2,000,000 generations were conducted with sampling every 100 generations. The first 25% of trees were discarded as burn-in.

3. Results and Discussion

3.1. Mitogenome Structure and Organization

Akin to other well-characterized firefly mitochondrial genomes, the mitogenome of *S. bungii* was a double-stranded circular DNA molecule, which contained 37 typical mitochondrial genes (13 PCGs, 22 tRNAs, and two rRNAs) (Figure 1 and Table A1). Four PCGs (ND1, ND4L, ND4, and ND5), two rRNAs, and nine tRNAs (trnV, trnL(UAG), trnL(UAA), trnP, trnH, trnF, trnY, trnQ, and trnC) were transcribed from the major stand (J-stand), and the other genes from the minor strand (N-strand) (Figure 1 and Table 2). Fifteen intergenic spacers were observed between the mitochondrial regions with lengths between −6 and 40 bp. Among these intergenic spacers, the longest was 17 bp (found between trnQ and trnT) (Table 2).

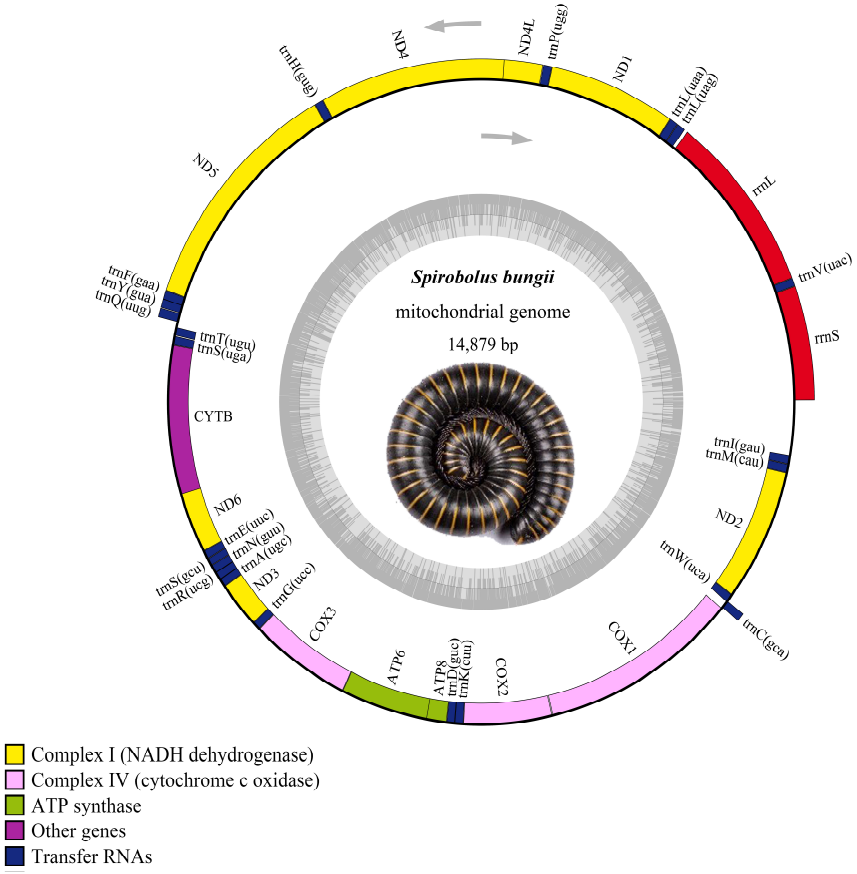

Figure 1. Circular map of the mitogenome of *S. bungii*. The circle shows the gene map of *S. bungii* where genes outside the map are coded on the major strand (J-strand), and those on the inside of the map are coded on the minor strand (N-strand). Genes are represented by differently colored blocks.

The complete mitochondrial genome was 14,879 bp in size, and its overall base composition was 26.60% for A, 32.62% for T, 28.44% for G, and 12.34% for C, with a GC content of 40.78% (Table 3), which was slightly higher than other Diplopoda species (Table A1) [15,32]. The AT-skew of *S. bungii* was negative, while the GC-skew was positive, which was opposed to *Narceus. annularus* in the same family Spirobolidae (Figure 2). Further, the GC-skews of all Polydesmida species were positive, while the AT-skews for all of this order were negative, which was completely opposed to the Spirostretida order (Figure 2).

Table 2. Mitogenomic organization of *S. bungii*.

Gene Name	Location		Size (bp)	Intergenic Nucleotides	Codon		Stand
	From	To			Start	Stop	
rrnS	12	814	803	11			J
trnV	815	873	59				J
rrnL	874	2143	1270				J
trnL1	2165	2227	63	21			J
trnL2	2228	2290	63				J
ND1	2291	3212	922		ATA	T	J
trnP	3213	3275	63				J
ND4L	3277	3558	282	1	ATG	TAG	J
ND4	3552	4893	1342	−7	ATG	T	J
trnH	4894	4956	63				J
ND5	4957	6658	1702		ATT	T	J
trnF	6659	6719	61				J
trnY	6716	6777	62	−4			J
trnQ	6780	6847	68	2			J
trnT	6888	6948	61	40			N
trnS	6953	7016	64	4			N
CYTB	7017	8133	1117		ATG	T	N
ND6	8126	8581	456	−8	ATT	TAA	N
trnE	8582	8642	61				N
trnS	8643	8699	57				N
trnN	8700	8762	63				N
trnR	8762	8823	62	−1			N
trnA	8823	8884	62	−1			N
ND3	8885	9230	346		ATT	T	N
trnG	9231	9293	63				N
COX3	9294	10,071	778		ATG	T	N
ATP6	10,072	10,747	676		ATG	T	N
ATP8	10,741	10,896	156	−7	ATT	TAA	N
trnD	10,897	10,958	62				N
trnK	10,958	11,023	66	−1			N
COX2	11,024	11,701	678		ATG	TAA	N
COX1	11,705	13,234	1530	3	CGA	TAA	N
trnC	13,240	13,302	63	5			J
trnW	13,295	13,356	62	−8			N
ND2	13,357	14,356	1000		ATA	T	N
trnM	14,357	14,419	63				N
trnI	14,420	14,483	64				N

Table 3. Composition and skewness in the mitochondrial genome of *S. bungii*.

Region	A%	T%	AT-Skew	G%	C%	GG-Skew
Whole mitogenome	26.60	32.62	−0.102	28.44	12.34	0.395
PCGs	24.53	32.22	−0.135	20.46	22.78	−0.054
rRNAs	31.02	35.12	−0.062	22.77	11.10	0.345
tRNAs	34.33	30.69	0.056	19.05	15.93	0.089

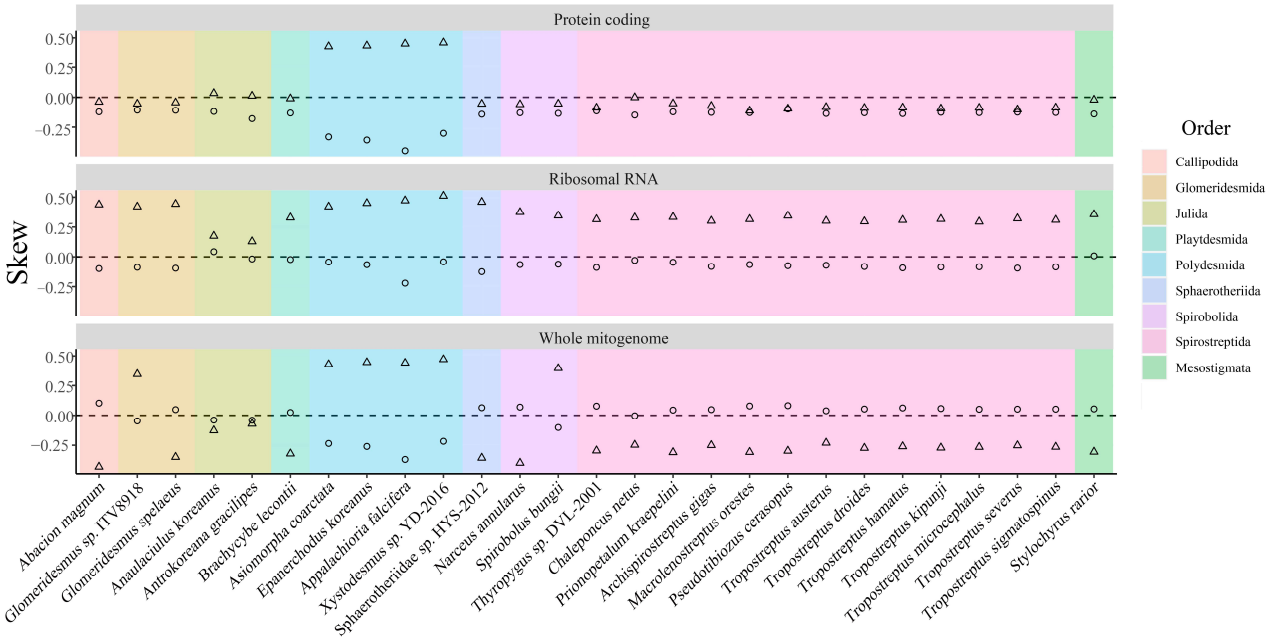

Figure 2. AT-skew (circle) and GC-skew (triangle) of 27 species used in this study.

3.2. The PCGs

The total length of the PCGs was 10,977 bp, which was consistent with other Diplopoda species (Table A1). The base composition of the PCGs was A = 24.53%, T = 32.22%, G = 20.46%, and C = 22.78% (Table 3). In contrast to the whole mitochondrial genome, the AT- and GC-skews were both negative, which were the same as the almost Spirostreptida species (Figure 2).

The gene arrangements of 13 PCGs were COX1, ND2, ND1, ND4L, ND4, ND5, Cytb, ND6, ND3, COX3, ATP6, ATP8, and COX2. Half of the PCGs began with a common ATG start codon, and most PCGs ended with a T end codon (Table 2). In the 13 PCGs, ND1, ND4L, ND4, ND5, CYTB, ND6, ND3, COX3, ATP6, ATP8, COX2, and ND2 used ATN (ATA/T/G/C) as the start codon, while COX1 was initiated by CGA. All PCGs stopped with TAA/G or with their incomplete single T form (Table 2). The single T as the stop codon has been found in other species [33–36].

The RSCU of the *S. bungii* mitogenome is presented in Figure 3, which indicates that Leu, Val, and Gly were the three most frequently utilized amino acids, and Cys had the lowest concentration (Figure 3B). Nine of the twenty-two amino acids (i.e., Pro, Thr, Leu1, Arg, Ala, Ser1, Ser2, Val, and Gly) had four codons, while the others had two (Figure 3A).

3.3. Transfer RNAs and Ribosomal RNAs

The typical sets of the 22 tRNAs were identified with sizes ranging from 57 bp (trnS) to 68 bp (trnQ) (Table 2). Moreover, the total length of the tRNAs was 1375 bp, with an A+T content of 65.02%, an AT-skew of 0.056, and a GC-skew of 0.089. Among all secondary structures of the 22 tRNA genes from the *S. bungii* mitochondrial genome, except for trnS1, all had a typical cloverleaf structure (Figure 4), as observed in other Diplopoda mitogenomes [10].

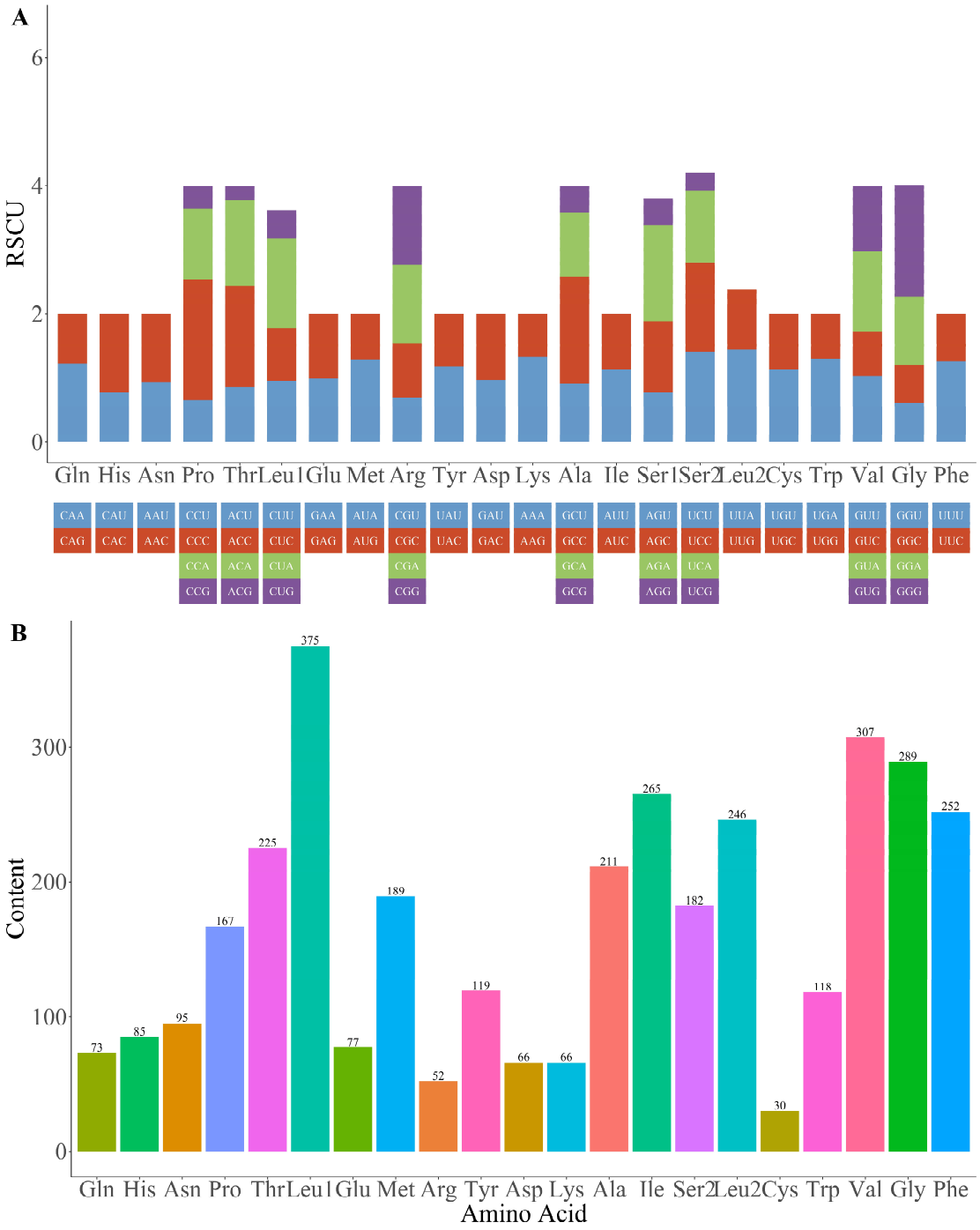

Figure 3. Relative synonymous codon usage (**A**) and codon distribution (**B**) in *S. bungii* mitogenome.

For *S. bungii*, the rrnL gene (length: 1270 bp) was encoded between trnV and trnL1, and the rrnS gene was 803 bp long. The total size of the two rRNAs was 2073 bp, with an A+T content of 66.14%, an AT-skew of −0.102, and a GC-skew of 0.345, which were higher than the other regions (Table A1). The rRNA AT-skews of all these species were positive, while the GC-skews were negative except for *Anaulaciulus gracilipes* (Figure 2).

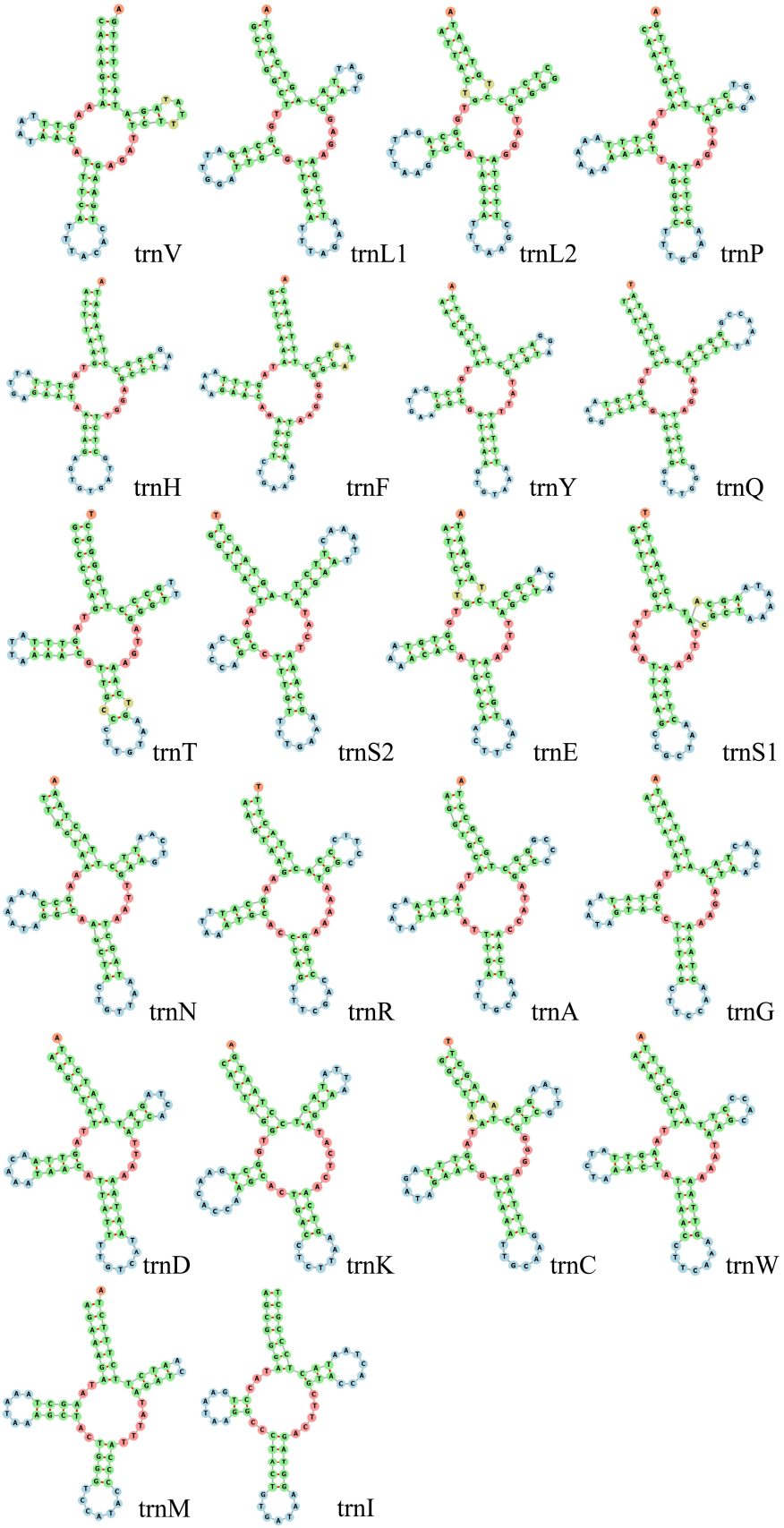

Figure 4. Secondary structure of 22 tRNA genes from the *S. bungii* mitochondrial genome.

3.4. Phylogenetic Analysis

Based on ML and BI analyses of nucleotide data of the 13 PCGs, we reconstructed the phylogenetic relationships of 26 species of Diplopoda, with *S. rarior* (Arachnida) as an outgroup. The two trees were similar to each other, with strongly supported branches (Figure 5). For the BI tree, Callipodida was clustered with Sphaerotheriida and Glomeridesmida, while it did not cluster with any species for the ML tree. However, *S. bungii* was most closely related to *N. annularus*, and the relationships between Callipodida, Spirobolida, Julida, and Spirostreptida were stable, which was congruent with a previous study of mitochondrial genomes [32].

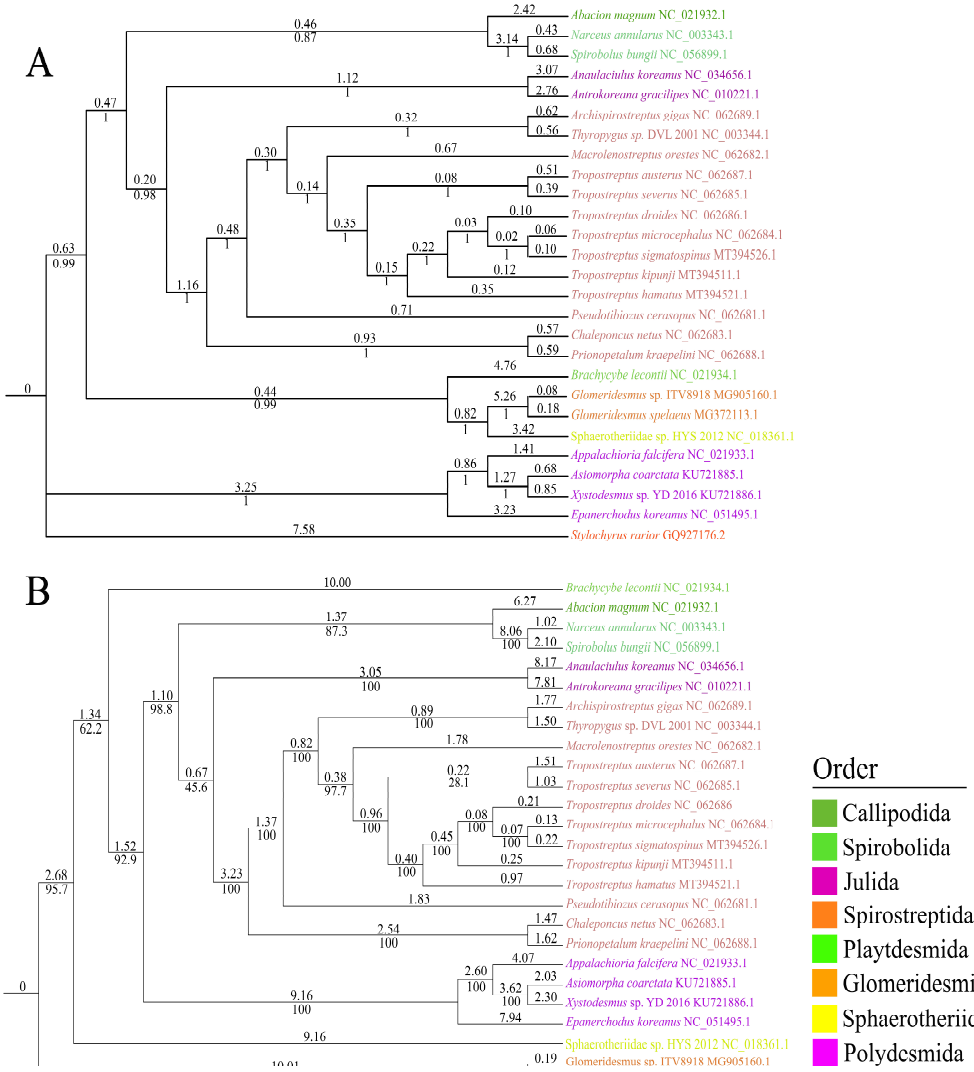

Figure 5. Mitogenomic phylogeny of 26 Diplopoda species and an outgroup (*Stylochyrus rarior*) based on 13 PCGs using Bayesian inference (**A**) and maximum likelihood (**B**) methods. The same colors of species in the tree indicated the same order.

3.5. Gene Arrangement among Diplopoda Classes

By comparing the gene arrangements of the mitogenomes between Diplopoda species, rearrangement occurred between and within orders (Figure 6). The positions of trnT for Julida differed from those of Spirobolida and Spirostreptida, which had similar gene arrangement patterns (Figure 6). Within Julida, the positions of trnC and trnW were inversed (Figure 6), which were found in fireflies [37]. An interesting phenomenon occurred where the gene orders of the mitogenomes between *S. bungii* and *N. annularus* were consistent, while they were transcribed in completely opposite directions (Figure 6). This was also

found in the Glomeridesmidae family of the Glomeridesmida order (Figure 6). Further, the positions of trnP in *Antrokoreana gracilipes* and *Anaulacilus koreanus*, which belonged to Julida order, were consistent but transcribed in opposite directions (Figure 6).

Figure 6. Gene arrangement image of Diplopoda mitogenomes.

4. Conclusions

The mitogenome of *S. bungii* was determined to be 14,879 bp in length, with a GC content of 40.78%. Additionally, based on a mitogenomic analysis of *S. bungii*, we found an intriguing phenomenon, where the AT- and GC-skews of the *S. bungii* mitogenome were opposed to most Diplopoda, while those of the 13 PCGs were consistent, except for Polydesmida. Consequently, this mitogenome, particularly the 13 PCGs, will assist with elucidating the genetic diversity, evolutionary origins, and genetic relationships of Diplopoda. The arrangement of genes in mitogenomes was remarkably variable across Diplopoda. Conversely, the mitogenome genes had consistent orders; however, for the Glomeridesmida and Spirobolida orders, they were transcribed in opposite directions. This indicated that the phenomenon was prevalent in Diplopoda, which will warrant additional investigations in the future. Furthermore, these results provide valuable data for the future resolution of phylogenetic relationships in this tribe.

Author Contributions: H.R. and H.L. conceived and designed this study. Y.F. analyzed the data. H.X. and Y.F. wrote the manuscript. H.X., G.C. and C.S. contributed reagents/materials/analysis tools. H.R. and H.L. contributed to the revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical review and approval was not required for the animal study because millipedes are common soil animals and are not listed in the IUCN Red List of Threatened Species.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are openly available in the US National Center for Biotechnology Information (NCBI database) (available online: https://www.ncbi.nlm.nih.gov/nuccore/NC_056899.1, accessed on 22 June 2022).

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Nucleotide composition indices in different regions of Diplopoda mitogenomes.

Region	Organism	Length (bp)	A%	T%	AT-Skew	G%	C%	GC-Skew
Whole mitogenome	*Abacion magnum*	15,160	36.67	29.90	0.102	9.54	23.86	−0.429
	Glomeridesmus sp. ITV8918	14,848	36.58	40.19	−0.047	15.62	7.60	0.345
	Glomeridesmus spelaeus	14,819	40.10	36.42	0.048	7.65	15.82	−0.348
	Anaulaciulus koreanus	14,916	36.06	39.03	−0.040	10.87	14.04	−0.127
	Antrokoreana gracilipes	14,747	29.76	32.33	−0.041	17.41	20.10	−0.072
	Brachycybe lecontii	15,115	39.22	37.41	0.024	7.92	15.38	−0.320
	Asiomorpha coarctata	15,644	25.80	41.65	−0.235	23.26	9.29	0.429
	Epanerchodus koreanus	15,581	27.81	47.30	−0.260	17.99	6.90	0.446
	Appalachioria falcifera	15,282	20.23	43.81	−0.368	25.90	10.06	0.441
	Xystodesmus sp. YD-2016	15,791	26.22	40.79	−0.217	24.25	8.73	0.471
	Sphaerotheriidae sp. HYS-2012	14,970	37.90	33.35	0.064	9.27	19.49	−0.355
	Narceus annularus	14,868	34.07	29.67	0.069	10.94	25.32	−0.397
	Spirobolus bungii	14,879	26.60	32.62	−0.102	28.44	12.34	0.395
	Thyropygus sp. DVL-2001	15,133	36.50	31.29	0.077	11.35	20.86	−0.295
	Chaleponcus netus	15,093	37.05	37.36	−0.004	9.63	15.95	−0.247
	Prionopetalum kraepelini	15,114	37.09	33.94	0.044	10.01	18.96	−0.309
	Archispirostreptus gigas	15,177	34.88	31.66	0.048	12.55	20.91	−0.250
	Macrolenostreptus orestes	15,367	36.64	31.33	0.078	11.10	20.93	−0.307
	Pseudotibiozus cerasopus	15,121	39.27	33.36	0.081	9.85	18.18	−0.297
	Tropostreptus austerus	15,261	34.66	32.09	0.038	12.81	20.46	−0.230
	Tropostreptus droides	15,172	36.36	32.67	0.053	11.26	19.70	−0.273
	Tropostreptus hamatus	15,150	35.71	31.52	0.062	12.13	20.64	−0.260
	Tropostreptus kipunji	15,170	36.66	32.72	0.057	11.15	19.45	−0.271
	Tropostreptus microcephalus	15,169	36.32	32.82	0.051	11.34	19.51	−0.265
	Tropostreptus severus	15,209	35.64	32.11	0.052	12.06	20.19	−0.252
	Tropostreptus sigmatospinus	15,172	36.37	32.80	0.052	11.34	19.49	−0.264
Protein coding	*Abacion magnum*	10,995	28.39	36.27	−0.122	16.99	18.32	−0.038
	Glomeridesmus sp. ITV8918	10,893	34.00	42.24	−0.108	11.24	12.52	−0.054
	Glomeridesmus spelaeus	10,860	33.88	42.14	−0.109	11.46	12.52	−0.044
	Anaulaciulus koreanus	11,034	32.83	41.73	−0.119	13.15	12.29	0.034
	Antrokoreana gracilipes	11,073	25.00	35.93	−0.179	19.78	19.29	0.012
	Brachycybe lecontii	11,013	32.64	42.58	−0.132	12.24	12.49	−0.010
	Asiomorpha coarctata	11,019	22.13	43.98	−0.331	24.20	9.69	0.428
	Epanerchodus koreanus	10,959	23.76	50.11	−0.357	18.74	7.38	0.435
	Appalachioria falcifera	10,998	17.46	45.63	−0.447	26.78	10.14	0.451
	Xystodesmus sp. YD-2016	11,028	22.92	42.69	−0.301	25.10	9.29	0.460
	Sphaerotheriidae sp. HYS-2012	11,049	30.12	40.07	−0.142	14.09	15.72	−0.055
	Narceus annularus	10,974	27.03	35.12	−0.130	17.81	20.04	−0.059
	Spirobolus bungii	10,977	24.53	32.22	−0.135	20.46	22.78	−0.054
	Thyropygus sp. DVL-2001	10,992	29.29	36.84	−0.114	15.45	18.41	−0.088
	Chaleponcus netus	11,016	31.42	42.46	−0.149	13.04	13.08	−0.001
	Prionopetalum kraepelini	11,013	30.67	39.23	−0.122	14.27	15.83	−0.052
	Archispirostreptus gigas	10,983	28.38	36.61	−0.127	16.23	18.77	−0.073
	Macrolenostreptus orestes	11,013	28.81	37.33	−0.129	15.01	18.85	−0.113
	Pseudotibiozus cerasopus	10,998	31.90	38.89	−0.099	13.21	16.00	−0.096
Protein coding	*Tropostreptus austerus*	10,983	27.95	36.76	−0.136	16.18	19.10	−0.083
	Tropostreptus droides	11,019	29.39	38.16	−0.130	14.76	17.68	−0.090
	Tropostreptus hamatus	11,040	28.25	37.26	−0.137	15.74	18.75	−0.087
	Tropostreptus kipunji	10,992	29.71	38.31	−0.126	14.47	17.51	−0.095
	Tropostreptus microcephalus	11,010	29.50	38.27	−0.129	14.67	17.55	−0.089
	Tropostreptus severus	11,022	28.88	37.16	−0.125	15.23	18.73	−0.103
	Tropostreptus sigmatospinus	11,007	29.52	38.16	−0.128	14.76	17.56	−0.087
Ribosomal RNA	*Abacion magnum*	2272	31.69	38.69	−0.099	21.26	8.32	0.438
	Glomeridesmus sp. ITV8918	1715	36.03	43.03	−0.088	14.87	6.06	0.421
	Glomeridesmus spelaeus	1715	35.28	42.74	−0.096	15.86	6.12	0.443
	Anaulaciulus koreanus	1924	39.35	36.07	0.043	14.45	10.14	0.175
	Antrokoreana gracilipes	2083	31.97	33.17	−0.018	19.68	15.17	0.129
	Brachycybe lecontii	2110	40.05	41.90	−0.023	11.90	6.02	0.328
	Asiomorpha coarctata	2016	33.13	36.21	−0.044	21.78	8.88	0.421
	Epanerchodus koreanus	2082	36.94	42.12	−0.066	15.18	5.76	0.450
	Appalachioria falcifera	2025	26.77	41.98	−0.221	23.01	8.25	0.472
	Xystodesmus sp. YD-2016	2007	33.13	36.07	−0.042	23.27	7.52	0.511

Table A1. *Cont.*

Region	Organism	Length (bp)	A%	T%	AT-Skew	G%	C%	GC-Skew
	Sphaerotheriidae sp. HYS-2012	2091	32.38	41.61	−0.125	18.99	7.03	0.460
	Narceus annularus	2075	32.14	36.58	−0.065	21.49	9.78	0.374
	Thyropygus sp. DVL-2001	2047	33.22	39.72	−0.089	17.78	9.28	0.314
	Chaleponcus netus	2059	37.30	39.58	−0.030	15.35	7.77	0.328
	Prionopetalum kraepelini	2048	36.04	39.50	−0.046	16.31	8.15	0.333
	Archispirostreptus gigas	2059	32.54	38.27	−0.081	18.99	10.20	0.301
	Macrolenostreptus orestes	2045	34.03	38.78	−0.065	17.85	9.29	0.315
	Pseudotibiozus cerasopus	2079	35.21	40.89	−0.075	16.07	7.84	0.344
	Tropostreptus austerus	2096	33.21	38.26	−0.071	18.56	9.97	0.301
	Tropostreptus droides	2049	33.43	39.39	−0.082	17.62	9.57	0.296
	Tropostreptus hamatus	2038	32.68	39.40	−0.093	18.25	9.67	0.308
	Tropostreptus kipunji	2048	33.64	39.94	−0.086	17.38	9.03	0.316
	Tropostreptus microcephalus	2049	33.38	39.53	−0.084	17.52	9.57	0.294
	Tropostreptus severus	2045	32.91	39.85	−0.095	18.00	9.24	0.321
	Tropostreptus sigmatospinus	2048	33.45	39.70	−0.085	17.58	9.28	0.309

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