Comparative Analysis of Mitogenomes among Five Species of Filchnerella (Orthoptera: Acridoidea: Pamphagidae) and Their Phylogenetic and Taxonomic Implications

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Simple Summary: Filchnerella belongs to Insecta, Orthoptera and Pamphagidae, of which there are 19 recorded species. The wings of Filchnerella are diverse, including three grasshopper wing types: longipennate, short wings and small wings. The previous studies of Filchnerella are more about the description of species morphology, and few about exploring the phylogenetic relationships with limited number of species and DNA fragments, which are insufficient to study the phylogeny of the entire genus, especially in order to understand the evolution of wing types in Filchnerella. To better understand the mitogenomic characteristics of Filchnerella and reveal its internal phylogenetic relationships, the complete mitochondrial genomes of Filchnerella suanensis, Filchnerella amplivertica, Filchnerella dingxiensis, Filchnerella pamphagoides and Filchnerella nigritibia were sequenced and comparatively analyzed in this study. The mitogenomes of these five Filchnerella species were found to be highly conserved. Phylogenetic analyses, based on mitogenome data of 16 species of Pamphagidae, using both the maximum likelihood (ML) and Bayesian inference (BI) methods, supported the monophyly of Filchnerella and produced valuable data for the phylogenetic study of the genus.

Abstract: Mitogenomes have been widely used for exploring phylogenetic analysis and taxonomic diagnosis. In this study, the complete mitogenomes of five species of Filchnerella were sequenced, annotated and analyzed. Then, combined with other seven mitogenomes of Filchnerella and four of Pamphagidae, the phylogenetic relationships were reconstructed by maximum likelihood (ML) and Bayesian (BI) methods based on PCGs+rRNAs. The sizes of the five complete mitogenomes are Filchnerella suanensis 15,656 bp, Filchnerella amplivertica 15,675 bp, Filchnerella nigritibia 15,661 bp, Filchnerella pamphagoides 15,661 bp and Filchnerella dingxiensis 15,666 bp. The nucleotide composition of mitogenomes is biased toward A+T. All tRNAs could be folded into the typical clover-leaf structure, except that tRNA Ser (AGN) lacked a dihydrouridine (DHU) arm. The phylogenetic relationships of Filchnerella species based on mitogenome data revealed a general pattern of wing evolution from long wing to increasingly shortened wing.

Keywords: Acridoidea; Pamphagidae; Filchnerella; mitogenomes; phylogeny; wing length

1. Introduction
The genus Filchnerella Karny, 1908, belongs to Insecta, Orthoptera, Acridoidea and Pamphagidae, and is the largest genus in the family Pamphagidae, with 19 known species [1], accounting for nearly 1/3 of all the Pamphagidae species in China. The genus is
endemic to China and is distributed in the arid northwestern provinces of Gansu, Qinghai, Ningxia and northern Shaanxi. The wing length is an important taxonomic character of Filchnerella [1]. The wings length of the genus includes longipennate (Tegmina very long, extending beyond the end of hind femora in male and reaching to or extending beyond the posterior of the third abdominal tergite in female and their length equal to the pronotum), short wings (Tegmina shortened, length distinctly greater than the length of pronotum, but the apex distinctly not reaching the end of hind femora in male, and distinctly shorter than metazona; the apex reaching, not reaching or slightly extending beyond the posterior margin of the second abdominal tergite in female) and small wings (Tegmina strongly abbreviated, length distinctly shorter than the length of pronotum in male and metazona in female). Different wing types are a widespread phenomenon in Orthoptera insects and have evolutionary consequences [2]. According to the perspective of evolutionary taxonomy, wings length is of great significance in the evolution of grasshoppers. The long wing is an ancestral characteristic, the shortened wing is an evolutionary characteristic and the wing-lessness is the newest characteristic, which is based on the overall evolutionary trend of Acridoidea [3]. Chen et al. [4] studied the phylogenetic implications in wing type evolution of Catantopidae. Does wing type evolution of species within one genus follow the above pattern? This lacks explicit reporting on grasshoppers. Filchnerella is a preferred material to study the question because of their diverse wing types.

The degree of wing development has a close relationship with insects’ movement ability. Mitochondria, as the main place of aerobic respiration in most eukaryotes [5], support the various life activities of individuals, such as flight. Insect mitochondrial genome is a circular molecule structure with the size of 15 to 18 kb, which contains 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribosomal RNA genes (rRNAs) and A+T-rich region [6–8]. Mitogenome has many unique advantages for some molecular systematic studies: the extraction of the mitogenome is easier than nuclear genes, and the genome is small, with a high copy number; there are few intercalated spacers, and strict matrilineal inheritance avoids the randomness of parental inheritance. In addition, although the sequence of the mitogenome is highly conserved and its structure is stable, the evolution rate of the mitogenome sequence is 5–10 times higher than that of nuclear genome. Therefore, mtDNA has become an important material for the study of phylogeny [9,10].

With the development of PCR technology and DNA sequencing technology, the usage of DNA sequences to study the phylogeny and evolution of insects has become a popular method [4,11–15]. There are also a few reports on the related studies of Pamphagidae. Flook et al. [16,17] studied the 12S rRNA and 16S rRNA sequence of three Pamphagidae species from Africa when studying the molecular phylogeny of the Orthoptera. Li et al. [18] studied the genetic differentiation among different populations of Haplotropis brunneriana and three other species of grasshoppers from China. Zhang et al. [19] carried out a molecular systematic analysis of some genera of Pamphagidae from China based on partial sequences of 16S rDNA. Zhang et al. [20] studied molecular phylogeny of Pamphagidae from China based on mitochondrial cytochrome oxidase II sequences. Zhang et al. [21] studied the complete mitochondrial genomes of three Pamphagidae grasshoppers: Asiotmethis zacharjini, Filchnerella helanshanensis and Pseudotmethis rubimarginis.

In Filchnerella, most existing studies are description of species based on morphology, while a few explored the phylogenetic relationship of Filchnerella using molecular methods. Li et al. [22] studied the phylogenetic relationships of six species of Filchnerella based on partial sequence of 16S rDNA. Zhang et al. [23] analyzed the molecular characteristics in the partial sequence of the mitochondrial COII gene of eight Filchnerella species. These studies only focused on seldom species and DNA fragments of Filchnerella, which is insufficient to study the phylogeny of the entire genus, especially in order to understand the evolution of wing types in the genus.

In this study, the mitogenomes of five species of Filchnerella were newly sequenced, annotated and analyzed. The phylogenetic relationship was reconstructed based on mi-
togenome data of twelve *Filchnerella* species, with four additional species of Pamphagidae as outgroups. It focuses on the evolution trend of wing length in *Filchnerella* and provides molecular data to the systematic study.

2. Materials and Methods

2.1. Sample and DNA Extraction

The grasshoppers used in the study are showed in Tables 1 and 2. The samples were originally conserved in 95% ethanol, and then transferred to 4 °C for cryopreservation. The Mitochondrial DNA were extracted from the hind femora muscle. Mitochondrial DNA was separated according to the method of Tamura and Aotsuka [24] with modifications [25,26].

| Species                  | Collecting Time | Collecting Sites | Collector              |
|--------------------------|-----------------|------------------|------------------------|
| *Filchnerella amplivertica* Li, Zhang & Yin, 2009 | July 2003       | Zhongwei, Ningxia | LI Xin-Jiang, WANG Wen-Qiang |
| *Filchnerella pamphagoides* Karny, 1908            | September 2003  | Lanzhou, Gansu   | ZHANG Dao-Chuan, LI Xin-Jiang |
| *Filchnerella sunanensis* Liu, 1982                | July 2006       | Sunan, Gansu     | LI Xin-Jiang, ZHENG Jin-Yu |
| *Filchnerella nigritibia* Zheng, 1992              | July 2006       | Dawukou, Ningxia | ZHANG Dao-Chuan, LI Xin-Jiang |
| *Filchnerella dingxiensis* Zhang, Xiao & Zhi, 2011 | July 2006       | Dingxi, Gansu    | ZHANG Dao-Chuan, ZHI Yong-Chao |

| Species                  | Collecting Time | Collecting Sites | Collector              |
|--------------------------|-----------------|------------------|------------------------|
| *Filchnerella nigritibia* Zheng, 1992                   | 15,661          | MZ433420         | This study             |
| *Filchnerella amplivertica* Li, Zhang & Yin, 2009       | 15,657          | MZ433418         | This study             |
| *Filchnerella pamphagoides* Karny, 1908                 | 15,661          | MZ433417         | This study             |
| *Filchnerella sunanensis* Liu, 1982                     | 15,656          | MZ433421         | This study             |
| *Filchnerella dingxiensis* Zhang, Xiao & Zhi, 2011      | 15,666          | MZ433419         | This study             |
| *Filchnerella becki* Ramme, 1931                        | 15,658          | NC_024923        | [27]                   |
| *Filchnerella helanshanensis* Zheng, 1992               | 15,657          | NC_020329        | [21]                   |
| *Filchnerella qilianshanensis* Xi & Zheng, 1984         | 15,661          | NC_046558        | Unpublished            |
| *Filchnerella tenggerensis* Zheng & Fu, 1989            | 15,659          | NC_046559        | Unpublished            |
| *Filchnerella rubrimargina* Zheng, 1992                 | 15,661          | NC_052733        | Unpublished            |
| *Filchnerella yongdengensis* Xi& Zheng, 1984            | 15674           | MK_903560        | [2]                    |
| *Filchnerella kukunoris* Bey-Bienko, 1948               | 15662           | MK_903590        | [2]                    |
| *Humaphlotropis calaishanensis* Li, Cao & Yin, 2014     | 15,659          | NC_023535        | [28]                   |
| *Thrinchus schrenkii* Fischer von Waldheim, 1846        | 15,672          | NC_014610        | [29]                   |
| *Asiotmethis jubatus* (Uvarov, 1926)                    | 15,669          | NC_025904        | [30]                   |
| *Asiotmethis zacharjini* (Bey-Bienko, 1926)             | 15,660          | NC_020328        | [21]                   |

2.2. PCR Amplification and Sequencing

The complete mitogenomes sequences of other species of *Filchnerella* previously obtained in our laboratory and the complete mitogenomes sequences of Pamphagidae downloaded from GenBank were used as reference sequences for identifying conservative regions in ClustalX1.83 [31]. Primers used in the present study including the general primer, referring to Zhi et al. [29], and specific primer designed using Primer Premier 5.0(PREMIER Biosoft, San Francisco, CA, USA) [32] and DNAMAN 6.0(Lynnon Biosoft., San Ramon, CA, USA) [33]. PCR reactions [28–30] were carried out under the following conditions: 5 min initial denaturation at 94 °C, followed by 30 cycles of 30 s at 94 °C, annealing at 50 °C for 30 s, elongation at 72 °C for 30 s and a final elongation for 5 min at 72 °C. All samples were submitted to Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) for sequencing.

2.3. Sequence Assembly, Annotations and Analysis

The sequences files obtained by sequencing were opened with Seqman software to proofread for repeats at the beginning and end of the sequence. Then, DNAMAN 6.0 [33] was used to concatenate all the sequences obtained. The tRNAscan-SE 1.21 was used to
identify tRNA in the concatenate complete mitochondrial genome sequence, including the relative position, length, anticodon and secondary structure of tRNA. A+T rich regions and rRNA genes were identified by comparing with published mitochondrial genome sequences of grasshoppers from the family Pamphagidae. Relative synonymous codon usage (RSCU) of PCGs was calculated in MEGA X [34]. The ratio of nonsynonymous substitution (Ka) to synonymous substitution (Ks) for all PCGs was calculated using DnaSP 5.0 [35].

2.4. Phylogenetic Analysis

Phylogenetic analyses were performed on the dataset of 13 PCGs and two rRNAs from 16 complete mitogenomes of Pamphagidae, including the five mitogenomes of *Filchnerella* that were sequenced in the present study. Four species in three other Pamphagidae genera, *Humaphilotropis ciliaisianensis*, *Thrinchus schrenkii*, *Asiotmethis jubatus* and *Asiotmethis zacharjini*, were used as outgroups. The combined sequence of protein-coding and rRNA encoding genes was aligned by ClustalX1.83 (Conway Institute UCD Dublin, Dublin, Ireland) [31]. ModelFinder (Research School of Biology, Australian National University, Canberra, Australia) [36] was used to select the best-fit model using AICc criterion. Maximum likelihood (ML) analysis was conducted using IQ-TREE (http://www.iqtree.org/, accessed on 20 March 2021) [37] in PhyloSuite v1.2.2 (Key laboratory of Aquaculture Disease Control, Wuhan, Hubei, China) [38]. Node confidence was assessed with 1000 bootstrap replicates. Bayesian inference (BI) analysis was executed with 10 million generations, sampling trees every 1000 generations in MrBayes 3.1.4 (http://nbisweden.github.io/MrBayes/index.html, accessed on 20 March 2018) [39], and selected GTR model (iset nst = 6 rates = invgamma). The consensus tree was calculated after discarding the first 2500 trees (25%) as the burn-in phase.

3. Results and Discussion

3.1. Genome Structure

The complete mitogenomes of *Filchnerella sunanensis* Liu, 1982; *Filchnerella amplivertica* Li, Zhang & Yin, 2009; *Filchnerella nigrilabia* Zheng, 1992; *Filchnerella pamphagoides* Karny, 1908; and *Filchnerella dingxiensis* Zhang, Xiao & Zhi, 2011, were sequenced, and their length was 15,656 bp, 15,657 bp, 15,661 bp, 15,661 bp and 15,666 bp, respectively (Table 2). Their structures are the same as those of insects [4,40]. Each mitogenome was found to be composed of circular double-stranded molecules, containing the typical set of 37 genes (13 typical protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribosomal RNA genes (rRNAs)) and an A+T rich region (Figure 1). Most of these genes were located on the J-strand (9 PCGs and 14 tRNAs), whereas the other genes (4 PCGs, 8 tRNAs and 2 rRNAs) were located on the N-strand (Table 3).

3.2. Nucleotide Composition

The nucleotide composition of five grasshopper species revealed a strong A+T bias in the whole mitogenome (Table 4). The A+T content ranged from 72.3% (*F. nigrilabia*) to 72.9% (*F. sunanensis*). A+T % was the highest in the A+T-rich region (83.4%) and the lowest in tRNA genes (69.6%), which is consistent with the characteristic of base composition bias in insect mitogenomes [4,22,23,40]. Strand bias was demonstrated in nucleotide compositional skew. The skew statistics indicated that the whole mitogenome, the rRNAs, the tRNAs and the A+T rich region of the five species of *Filchnerella* were negative for the GC-skew and positive for the AT-skew. The PCGs of the five species of *Filchnerella* were negative for the GC-skew and negative for the AT-skew.
Figure 1. The circular maps of five species of grasshopper mitogenomes. Genes are characterized by different color blocks. The J-strand is visualized on the outer circle and the N-strand on the inner circle. The species from inside to outside are as follows: F. sunanensis, F. amplivertica, F. nigritibia, F. pamphagoides and F. dingxiensis.
Table 3. Annotations for the five species grasshopper mitogenomes.

| Gene          | Coding Strand | Nucleotide Number      | Size (bp) | Intergenic Length | Anticodon | Initiation Codon | Termination Codon |
|---------------|---------------|------------------------|-----------|-------------------|-----------|------------------|-------------------|
| tRNA<sub>Ile</sub> | J             | 1–68/..././.           | 68/..././. | 0/..././.         | GAT/..././. | ATG/..././.       | TAG/..././.        |
| tRNA<sub>Gln</sub> | N             | 69–137/..././.         | 69/..././. | –1/..././.        | TTG/..././. |                   |                   |
| tRNA<sub>Met</sub> | J             | 137–205/..././.        | 69/..././. | 0/..././.         | CAT/..././. |                   |                   |
| ND2           | J             | 206–1228/..././.       | 1025/..././.| 2/..././.        |           |                   |                   |
| tRNA<sub>Trp</sub> | J             | 1231–1297/..././.1231–1298/. | 67/.../68/. | –8/..././.        | TCA/..././. |                   |                   |
| tRNA<sub>Cys</sub> | N             | 1290–1354/..././1291–1355/. | 65/..././. | 8/11/9/8/        | GCA/..././. |                   |                   |
| tRNA<sub>Tyr</sub> | J             | 1360–1428/1364–1429/. | 66/..././. | 1/..././.         | GTA/..././. |                   |                   |
| COI           | J             | 1430–2963/1431–2964/. | 1534/..././ | 0/..././.        |           |                   |                   |
| tRNA<sub>Leu(UUR)</sub> | J             | 2964–3029/2965–3030/. | 66/..././. | 4/.../5/4        | TAA/..././. |                   |                   |
| COII          | J             | 3034–3717/3035–3718   | 684/..././.| 3/..././.        | ATG/..././. |                   | TAA/..././T        |
| tRNA<sub>Asp</sub> | J             | 3721–3789/3722–3791/. | 69/70/.71/ | 2/..././.        | GTC/..././. |                   |                   |
| tRNA<sub>Ala</sub> | J             | 3792–3862/3794–3864/3794–3864 | 71/..././. | 17/..././.       | CTT/..././. |                   |                   |
| ATP8          | J             | 3880–4041/3882–4043/. | 162/..././.| –7/..././.       | ATC/..././. | TAA/..././.       |                   |
| ATP6          | J             | 4035–4712/4037–4714/. | 678/..././.| 3/.../4/        | ATG/..././. | TAA/..././.       |                   |
| COIII         | J             | 4716–5507/4718–5509/. | 792/..././.| 3/..././.        | TAA/..././. |                   |                   |
| tRNA<sub>Gly</sub> | J             | 5511–5577/5513–5580/. | 67/68/.67/ | 0/..././.        | TCC/..././. |                   |                   |
| ND3           | J             | 5578–5931/5581–5932/5581–5934 | 354/352/ | –2/0/.2/        | ATC/..././. | TAG/T            |                   |
| tRNA<sub>Arg</sub> | J             | 5930–5995/5933–5998/5934–5999/5935–6000 | 66/..././. | 0/3/..././.     | TGC/..././. |                   |                   |
| tRNA<sub>Glu</sub> | J             | 5996–6059/6002–6064/6002–6065/6003–6066/6004–6067 | 64/63/64/ ./ | 2/1/.2/        | TCG/..././. |                   |                   |
| tRNA<sub>Aux</sub> | J             | 6062–6126/6066–6130/6067–6131 | 65/..././. | 0/..././.        | GTT/..././. |                   |                   |
| tRNA<sub>Ser</sub> | J             | 6127–6193/6131–6197/6132–6198 | 67/..././. | 1/..././.        | GCT/..././. |                   |                   |
| tRNA<sub>Glu</sub> | J             | 6195–6263/6199–6266/6200–6267/6202–6268/6203–6269 | 69/68/.67/ | 1/..././.        | TTC/..././. |                   |
| Gene       | Coding Strand | Nucleotide Number | Size (bp) | Intergenic Length | Anticodon | Initiation Codon | Termination Codon |
|------------|---------------|-------------------|-----------|-------------------|-----------|------------------|-------------------|
| tRNA<sup>Phe</sup> | N             | 6265–6329/6268–6333/6269–6334 | 65/66/././. | 0./././. | GAA./././. |
|            |               | 6330–8046/6334–8050/6335–8051 | 1717./././. | 15./././. | ATT./././. | T./././. |
| tRNA<sup>His</sup> | N             | 8062–8131/8066–8132/8067–8133 | 70/67./././ | 0./–1./. | GTG./././. |
|            |               | 8068–8135/8069–8136 |           |          |          |          |
| ND4        | N             | 8132–9465/8133–9466/8133–9467 | 1334././.1335./1. | –7././. | ATG./././. | TA./.TAG/. |
| ND4L       | N             | 9459–9752/9460–9753/9461–9754 | 294./././. | 2./././. | ATG./././. | TAA./. |
| tRNA<sup>Thr</sup> | J             | 9755–9822/9756–9823/9757–9826 | 68././70/68./ | 0./././. | TGT./././. |
| tRNA<sup>Pro</sup> | N             | 9823–9888/9824–9889/9827–9893 | 66./././7. | 2./././. | TGG./././. |
|            |               | 9828–9894 |          |          |          |          |
| ND6        | J             | 9891–10412/9892–10413/9896–10417/10417/9897–10418 | 522./././ | 3./././. | GTG./././. | TAA./. |
| Cytb       | J             | 10416–11558/10417–11557/10421–11563 | 1143/1141/1143./ | –2/0–2./ | ATG./././. | TAG/T | TAG/. |
| tRNA<sup>Ser (UCN)</sup> | J          | 11557–11626/11558–11627/11562–11631 | 70./././7. | 26././25 | TGA./././. |
|            |               | 11563–11631/1163–11632 |          |          |          |          |
| NDI        | N             | 11653–12597/11654–12598/11658–12602 | 945./././ | 3././4. | ATA./././. | TAG./. |
| tRNA<sup>Lon (UCN)</sup> | N           | 12601–12666/12602–12667/12606–12671 | 66./././ | 0./3/0 | TAG./././. |
|            |               | 12607–12672 |          |          |          |          |
| lrRNA      | N             | 12667–13986/12668–13987/12672–13991 | 1320/./. | 0./2/0 |          |          |
|            |               | 13267–13988/12673–13993 |          |          |          |          |
| tRNA<sup>Val</sup> | N           | 13984–14056/13988–14058/13992–14062 | 70./71./ | 0./. | TAC./. |
|            |               | 13991–14061/13994–14064 |          |          |          |          |
| srRNA      | N             | 14057–14908/14059–14909/14063–14914 | 852/851/852/853 | 0./. |          |          |
|            |               | 14062–14914/14065–14917 |          |          |          |          |
| A+T rich   | N             | 14909–15656/14910–15657/14915–15661 | 748./747/7. |          |          |          |

**Note:** N and J indicate that the gene was located in the minor (N) and major (J) strand. ./.: same as the one previous to it. The species are as follows: F. sunanensis, F. ampliortica, F. nigritibia, F. Pamphagoides and F. dixxiensis.
| Species       | Regions          | T%  | C%  | A%  | G%  | Size (bp) | A+T% | AT-Skew | GC-Skew |
|--------------|------------------|-----|-----|-----|-----|-----------|------|---------|---------|
|              | All genes        | 30.6| 16.1| 42.3| 11.0| 15,656.0  | 72.9 | 0.16    | −0.19   |
|              | rRNA genes       | 29.7| 16.9| 43.2| 10.3| 2172.0    | 72.9 | 0.19    | −0.24   |
|              | tRNA genes       | 31.1| 16.5| 38.7| 13.6| 1482.0    | 69.8 | 0.11    | −0.10   |
|              | A+T-rich region  | 37.7| 9.4 | 45.6| 7.4 | 748.0     | 83.3 | 0.09    | −0.12   |
|              | PCGs             | 41.4| 14.0| 31.2| 13.5| 11,182.0  | 72.6 | −0.14   | −0.02   |
|              | All codons       |     |     |     |     |           |      |         |         |
|              | 1st              | 35  | 13.4| 31.7| 20.3| 3729.0    | 66.7 | −0.05   | 0.20    |
|              | 2nd              | 45  | 20.4| 19.7| 14.6| 3727.0    | 64.7 | −0.39   | −0.17   |
|              | 3rd              | 44  | 8.1 | 42.1| 5.6 | 3726.0    | 86.1 | −0.02   | −0.18   |
|              | F. sunamensis    |     |     |     |     |           |      |         |         |
|              | 1st              | 28  | 15.8| 35.1| 20.6| 2298.0    | 63.1 | 0.11    | 0.13    |
|              | 2nd              | 43  | 22.6| 20.5| 13.5| 2297.0    | 63.5 | −0.35   | −0.25   |
|              | 3rd              | 32  | 12.0| 53.2| 2.4 | 2297.0    | 85.2 | 0.25    | −0.67   |
|              | Total            | 34.8| 16.8| 36.3| 12.2| 6892.0    | 71.1 | 0.02    | −0.16   |
|              | All genes        | 30.3| 16.4| 42.1| 11.2| 15,657.0  | 72.4 | 0.16    | −0.19   |
|              | rRNA genes       | 29.7| 16.9| 43.2| 10.3| 2171.0    | 72.9 | 0.19    | −0.24   |
|              | tRNA genes       | 31.4| 16.5| 38.4| 13.8| 1482.0    | 69.8 | 0.10    | −0.09   |
|              | A+T-rich region  | 36.9| 9.9 | 46.5| 6.7 | 748.0     | 83.4 | 0.12    | −0.19   |
|              | PCGs             | 41.1| 14.3| 30.7| 13.9| 11,178.0  | 71.8 | −0.14   | −0.01   |
|              | All codons       |     |     |     |     |           |      |         |         |
|              | 1st              | 34  | 13.6| 31.2| 20.8| 3729.0    | 65.2 | −0.04   | 0.21    |
|              | 2nd              | 45  | 20.5| 19.6| 14.7| 3725.0    | 64.6 | −0.39   | −0.16   |
|              | 3rd              | 44  | 8.8 | 41.4| 6.2 | 3724.0    | 85.4 | −0.03   | −0.17   |
| Species   | Regions       | T%   | C%   | A%   | G%   | Size (bp) | A+T%  | AT-Skew | GC-Skew |
|-----------|---------------|------|------|------|------|-----------|-------|---------|---------|
|           |               |      |      |      |      | Genes on J-strand |      |         |         |
|           |               |      |      |      |      | Genes on N-strand |      |         |         |
|           |               |      |      |      |      | All genes          |      |         |         |
|           |               |      |      |      |      | rRNA genes         |      |         |         |
|           |               |      |      |      |      | tRNA genes         |      |         |         |
|           |               |      |      |      |      | A+T-rich region    |      |         |         |
|           |               |      |      |      |      | PCGs                |      |         |         |
| F. amplivertica |           |      |      |      |      |               |      |         |         |
|           | 1st           | 28   | 16.2 | 34.6 | 21.1 | 2298.0     | 62.6  | 0.11    | 0.13    |
|           | 2nd           | 43   | 22.7 | 20.3 | 13.8 | 2295.0     | 63.3  | −0.36   | −0.24   |
|           | 3rd           | 31   | 13.4 | 52.1 | 3.4  | 2295.0     | 83.1  | 0.25    | −0.60   |
|           | Total         | 34.2 | 17.4 | 35.7 | 12.8 | 6888.0     | 69.9  | 0.02    | −0.15   |
|           | 1st           | 44   | 9.6  | 25.9 | 20.4 | 1431       | 69.9  | −0.26   | 0.36    |
|           | 2nd           | 48   | 17.1 | 18.5 | 16.3 | 1430       | 66.5  | −0.44   | −0.02   |
|           | 3rd           | 64   | 1.3  | 24.1 | 10.8 | 1429       | 88.1  | −0.45   | 0.79    |
|           | Total         | 52.0 | 9.3  | 22.8 | 15.8 | 4290       | 74.8  | −0.39   | 0.26    |
|           | F. nigritibia  |     |      |      |      |               |      |         |         |
|           |               |      |      |      |      | All codons      |      |         |         |
|           |               |      |      |      |      | Genes on J-strand |      |         |         |
|           | 1st           | 28   | 16.4 | 34.5 | 21.1 | 2298.0     | 62.5  | 0.10    | 0.13    |
|           | 2nd           | 43   | 22.9 | 20.3 | 13.8 | 2297.0     | 63.3  | −0.36   | −0.25   |
|           | 3rd           | 31   | 13.3 | 52.4 | 3.3  | 2297.0     | 83.4  | 0.26    | −0.60   |
|           | Total         | 34.0 | 17.5 | 35.8 | 12.7 | 6892.0     | 69.8  | 0.03    | −0.16   |
|           | 1st           | 44   | 9.4  | 26.0 | 20.1 | 1431       | 70.0  | −0.26   | 0.36    |
|           | 2nd           | 48   | 17.1 | 18.7 | 16.2 | 1430       | 66.7  | −0.44   | −0.03   |
|           | 3rd           | 63   | 1.4  | 24.4 | 10.8 | 1430       | 87.4  | −0.44   | 0.77    |
|           | Total         | 52.0 | 9.3  | 23.0 | 15.7 | 4291       | 75.0  | −0.39   | 0.26    |
| Species          | Regions          | T%    | C%    | A%    | G%    | Size (bp) | A+T%  | AT-Skew | GC-Skew |
|------------------|------------------|-------|-------|-------|-------|-----------|-------|---------|---------|
| All genes        | T%               | C%    | A%    | G%    | Size (bp) | A+T%  | AT-Skew | GC-Skew |
| F. pamphagoides  | 30.5             | 16.3  | 42.0  | 11.1  | 15,661.0 | 72.5  | 0.16    | −0.19   |
|                  | rRNA genes       | 30.0  | 17.2  | 42.1  | 2167.0  | 72.1  | 0.17    | −0.23   |
|                  | tRNA genes       | 30.9  | 16.4  | 38.9  | 1485.0  | 69.8  | 0.11    | −0.09   |
|                  | A+T-rich region  | 36.1  | 10.2  | 46.2  | 747.0   | 82.3  | 0.12    | −0.15   |
|                  | PCGs             | 41.3  | 14.1  | 30.9  | 11,183.0| 72.2  | −0.14   | −0.01   |
|                  | A+T-rich region  | 36.1  | 10.2  | 46.2  | 747.0   | 82.3  | 0.12    | −0.15   |
|                  | PCGs             | 41.3  | 14.1  | 30.9  | 11,183.0| 72.2  | −0.14   | −0.01   |
|                  | All genes        | 30.5  | 16.3  | 42.0  | 11.1  | 15,661.0 | 72.5  | 0.16    | −0.19   |
|                  | rRNA genes       | 30.0  | 17.2  | 42.1  | 2167.0  | 72.1  | 0.17    | −0.23   |
|                  | tRNA genes       | 30.9  | 16.4  | 38.9  | 1485.0  | 69.8  | 0.11    | −0.09   |
|                  | A+T-rich region  | 36.1  | 10.2  | 46.2  | 747.0   | 82.3  | 0.12    | −0.15   |
|                  | PCGs             | 41.3  | 14.1  | 30.9  | 11,183.0| 72.2  | −0.14   | −0.01   |
|                  | All genes        | 30.5  | 16.3  | 42.0  | 11.1  | 15,661.0 | 72.5  | 0.16    | −0.19   |
|                  | rRNA genes       | 30.0  | 17.2  | 42.1  | 2167.0  | 72.1  | 0.17    | −0.23   |
|                  | tRNA genes       | 30.9  | 16.4  | 38.9  | 1485.0  | 69.8  | 0.11    | −0.09   |
|                  | A+T-rich region  | 36.1  | 10.2  | 46.2  | 747.0   | 82.3  | 0.12    | −0.15   |
|                  | PCGs             | 41.3  | 14.1  | 30.9  | 11,183.0| 72.2  | −0.14   | −0.01   |
|                  | All genes        | 30.5  | 16.3  | 42.0  | 11.1  | 15,661.0 | 72.5  | 0.16    | −0.19   |
|                  | rRNA genes       | 30.0  | 17.2  | 42.1  | 2167.0  | 72.1  | 0.17    | −0.23   |
|                  | tRNA genes       | 30.9  | 16.4  | 38.9  | 1485.0  | 69.8  | 0.11    | −0.09   |
|                  | A+T-rich region  | 36.1  | 10.2  | 46.2  | 747.0   | 82.3  | 0.12    | −0.15   |
|                  | PCGs             | 41.3  | 14.1  | 30.9  | 11,183.0| 72.2  | −0.14   | −0.01   |

Table 4. Cont.
Table 4. Cont.

| Species          | Regions | T%  | C%  | A%  | G%  | Size (bp) | A+T% | AT-Skew | GC-Skew |
|------------------|---------|-----|-----|-----|-----|-----------|------|---------|---------|
|                  |         |     |     |     |     |           |      |         |         |
| **F. dingxiensis** |         |     |     |     |     |           |      |         |         |
| **Genes on J-strand** |         |     |     |     |     |           |      |         |         |
| 1st              | 28.4    | 15.7| 35.4| 20.5| 2300.0| 63.7      | 0.11 | 0.13    |         |
| 2nd              | 43.3    | 22.6| 20.5| 13.7| 2297.0| 63.8      | −0.36| −0.25   |         |
| 3rd              | 32.8    | 12.5| 52.3| 2.5 | 2296.0| 85.1      | 0.23 | −0.67   |         |
| Total            | 34.8    | 16.9| 36.0| 12.2| 6893.0| 70.9      | 0.02 | −0.16   |         |
| **Genes on N-strand** |         |     |     |     |     |           |      |         |         |
| 1st              | 44.4    | 9.4 | 26.0| 20.1| 1431.0| 70.4      | −0.26| 0.36    |         |
| 2nd              | 47.8    | 17.2| 18.4| 16.6| 1430.0| 66.2      | −0.44| −0.02   |         |
| 3rd              | 63.0    | 1.4 | 24.8| 10.8| 1430.0| 87.8      | −0.44| 0.77    |         |
| Total            | 51.7    | 9.3 | 23.1| 15.9| 4291.0| 74.8      | −0.38| 0.26    |         |

**Note:** rRNA genes: ribosomal RNA genes; tRNA genes: transfer RNA genes; PCGs: protein-coding genes; 1st, 2nd, 3rd: the 1st, 2nd, 3rd codon position of the PCGs.
3.3. Protein-Coding Genes and Codon Usage

The 13 PCGs within five species of Filchnerella ranged from 162 bp (atp8) to 1717 bp (nd5) in size (Table 3). The size and arrangement are conserved. The biased usage of A/T could also be reflected in codon frequencies. The A+T content of all protein codons is higher than that of G+C, and each base of the codon indicated that the A+T content of the last site was higher than that of the first two sites. This is consistent with the characteristics of high A+T bias of base composition and selection pressure from G+C to A+T in insect mitochondrial genomes. All the initiation codons in the mitogenomes of the five species of Filchnerella were ATN, and ATG was the most frequently used initiation codon. Most termination codons were TAN, and TAA were the most frequently used termination codon.

Relative synonymous codon usage (RSCU) of five species of Filchnerella is shown in Figure 2, and for most amino acids, the usages of synonymous codons are biased. In addition, the synonymous codon preferences are conserved. In the five species of Filchnerella, Ala, Arg, Gly, Leu, Pro, Ser, Thr and Val are the most frequently encoded amino acids. Similarly, the biased usage of A+T nucleotides is also reflected by RSCU. The most frequently used codons are TTA, ATT, TTT and ATA, indicating the preference of nucleotide A/T in the five species of Filchnerella.

3.4. Transfer and Ribosomal RNA Genes

The size of 22 transfer RNAs (tRNA) of five Filchnerella grasshoppers ranged from 63 bp to 72 bp (Table 3). Among them, 21 tRNAs could be folded into the typical clover-leaf structure, except that tRNA Ser (AGN) lacked a dihydrouridine (DHU) arm (Figure 3). The lengths of the acceptor stem (7 bp) and anticodon loop (7 bp, except for the trnA of F. amplivertica (9 bp)) are conserved. The classic secondary structures comprised a DHU arm (2–4 bp), a TΨC arm (3–6 bp) and an anticodon arm (3–6 bp). However, the extra arm and loops of DHU and TΨC were more variable, with obvious nucleotide substitutions and length variation. Additionally, noncanonical match of G-U and mismatches of U-U, U-C, A-A, A-G and A-C were scattered throughout tRNA stems. Among the five Filchnerella grasshoppers, mitochondrial, rrnL-encoding and rrnS-encoding genes were oriented on the N-strand and located at the conserved positions between trnL1 (CUN) and trnV, and between trnV and the A+T-rich region, respectively. The rrnL ranged from 1314 bp (F. pamphagoides) to 1321 bp (F. dingxiensis) in size, while the rrnS varied from 851 bp (F. amplivertica) to 854 bp (F. pamphagoides). Therefore, there was no substantial size variation between rRNAs within five Filchnerella mitogenomes.

3.5. Ka/Ks of 12 Grassoppers of Filchnerella

To characterize the evolutionary patterns of 13 PCGs, the Ka/Ks across the 12 Filchnerella mitogenomes were calculated. As shown in Figure 4, similar to previous studies in insects [41,42], the Ka/Ks value for nad4L is the highest, followed by the nad6 and atp8; the lowest value is for cox1. The Ka/Ks values for all PCGs are <1, indicating that they are not neutral and are evolving under purifying selection. The gene nad4L (Ka/Ks = 0.32812) exhibits the highest rate, which is suggested to be under the least selection pressure [43] and the fastest evolving gene among the mitochondrial PCGs in Filchnerella. The gene cox1 (Ka/Ks = 0.02182) exhibits the smallest rate, which has been regarded as under strong purifying selection [43–45].
Figure 2. Relative synonymous codon usage (RSCU) of five grasshoppers of Filchnerella.
1. Introduction

The genus **Filchnerella** Karny, 1908, belongs to Insecta, Orthoptera, Acridoidea and Pamphagidae, and is the largest genus in the family Pamphagidae, with 19 known species [1], accounting for nearly 1/3 of all the Pamphagidae species in China. The genus is endemic to China and is distributed in the arid northwestern provinces of Gansu, Qinghai, Ningxia and northern Shaanxi. The wing length is an important taxonomic character of **Filchnerella** [1]. The wings length of the genus includes longipennate (Tegmina very long, extending beyond the end of hind femora in male and reaching to or extending beyond the posterior of the third abdominal tergite in female and their length equal to pronotum), short wings (Tegmina shortened, length distinctly greater than the length of pronotum, but the apex distinctly not reaching the end of hind femora in male, and distinctly shorter than metazona; the apex reaching, not reaching or slightly extending beyond

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**Figure 3.** Secondary structures of 22 tRNAs identified in the mitogenome of *F. sunanensis*. All of the genes are shown in order of occurrence. Watson–Crick base pairings and mismatches are represented by dashes (−) and pluses (+). The conserved and variable sites among the other 4 species of grasshoppers are indicated with black and red hollow circles, respectively.
3.5. Ka/Ks of 12 Grassoppers of Filchnerella

To characterize the evolutionary patterns of 13 PCGs, the Ka/Ks across the 12 individuals were calculated. As shown in Figure 4, similar to previous studies in similar organisms, the Ka/Ks values for all PCGs are <1, indicating that they are not in a state of purifying selection. The gene nad4L has the highest rate, which is suggested to be under the least selection pressure [43] and is the highest, followed by the nad6 gene, which is the highest, followed by the nad3 gene. The gene atp8 exhibits the smallest rate, which has been regarded as under strong purifying selection [43–45].

Figure 4. Synonymous (Ka) and non-synonymous (Ks) substitutional rates and the ratios of Ka/Ks of PCGs in 13 grasshoppers of Filchnerella.

3.6. Phylogenetic Analysis

The same tree topologies were recovered from both BI and ML analyses with high bootstrap values (BS) and Bayesian posterior probability values (PP) in the most clades (Figure 5). The included Filchnerella species were shown to form a monophyletic clade. Although the BS value (50%) is relatively low and the branching structure may be unstable, the PP value (0.99) is very high, which strongly supports this topology structure. Therefore, this branch structure is still credible, which is consistent with the traditional taxonomic result [1].

The tree showed that Filchnerella can be divided into three major clades. F. sunanensis separated first at the base of the tree, forming an independent clade A. Clade B is the sister to clade C, with high BS (100%) and PP (1). Nine species Filchnerella were clustered into clade B. F. pamphagides and F. dingxiensis were clustered into clade C.

Clade A (F. sunanensis) separated first from Filchnerella, which is consistent with Li et al. [22]. F. sunanensis has the longest tegmina in Filchnerella (extends far beyond the end of hind femora in male), which is the most ancestral species in traditional evolutionary taxonomy [3]. It is supported by molecular systematics results in the present study.

In clade B, first, F. qilianshanensis (tegmina extending just beyond the end of hind femora in male) and F. kukanoris (tegmina extending the base of epiproct) were clustered together at the base of the clade. Then, F. tenggerensis, F. beicki (tegmina extending the base of epiproct), F. helanshanensis (tegmina not reaching the 1/2 of hind femora), F. amplivertica (tegmina not reaching the base of epiproct), F. nigrilibia (tegmina extending the 1/3 of hind femora), F. yongdengensis (tegmina extending the 1/2 of hind femora) and F. rubrimargina (tegmina not reaching the 1/2 of hind femora) separate from the ancestor of clade B in order. Except for F. helanshanensis and F. yongdengensis, the evolutionary trend of wing length in clade B follows the direction of wing length evolution in grasshoppers (from long wing to short wing) [3]. Therefore, although only females were found, we also can infer that the length of tegmina of F. tenggerensis in males extends the base of the epiproct.
together at the base of the clade. Then, *F. tenggerensis*, *F. beicki* (tegmina extending the base of epiproct), *F. helanshanensis* (tegmina not reaching the 1/2 of hind femora), *F. amplivertica* (tegmina not reaching the base of epiproct), *F. nigritibia* (tegmina extending the 1/3 of hind femora), *F. yongdengensis* (tegmina extending the 1/2 of hind femora) and *F. rubrimargina* (tegmina not reaching the 1/2 of hind femora) separate from the ancestor of clade B in order. Except for *F. helanshanensis* and *F. yongdengensis*, the evolutionary trend of wing length in clade B follows the direction of wing length evolution in grasshoppers (from long wing to short wing) [3]. Therefore, although only females were found, we also can infer that the length of tegmina of *F. tenggerensis* in males extends the base of the epiproct.

Clade C comprises 2 species: *F. pamphagoides* and *F. dingxiensis*, and both are small wings whose tegmina is strongly abbreviated, and the length is distinctly shorter than the length of the pronotum in males. According to the traditional taxonomic view, clade C should separate from clade B, but it is a parallel evolution with clade B in the present study. This may be related to the unique origin of the small wings. The phylogenetic relationship of the 12 species of *Filchnerella* grasshoppers that resulted from this study shows similarities compared with those based on morphology and molecular data revealed in previous studies [22,23,46].

4. Conclusions

In this study, the complete mitochondrial genome of *F. sunanensis*, *F. amplivertica*, *F. nigritibia*, *F. pamphagoides* and *F. dingxiensis* were sequenced, annotated and analyzed. It was found that their structures are the same as those of Acridoidea. The nucleotide composition of five grasshopper species revealed a strong A+T bias in the complete mitogenome. Relative synonymous codon usage (RSCU) of five species of *Filchnerella* shows that the usages of synonymous codons are biased for most amino acids. All tRNAs could be folded into the typical clover-leaf structure, except that tRNA Ser (AGN) lacked a dihydrouridine (DHU) arm and there was no substantial size variation between rRNAs within five *Filchnerella* mitogenomes. The Ka/Ks values for all PCGs are <1, indicating that they are evolving under purifying selection. These five newly sequenced mitogenomes...
of genus *Filchnerella* can provide valuable data for future studies of phylogenetic relationships of Pamphagidae. The phylogenetic tree showed that the evolutionary relationships within *Filchnerella* were monophyletic clade, and the evolutionary trend of *Filchnerella* well demonstrated the direction of wing length evolution.

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