The Complete Mitochondrial Genome of the Booklouse, *Liposcelis decolor*: Insights into Gene Arrangement and Genome Organization within the Genus *Liposcelis*

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Abstract

Booklice in the genus *Liposcelis* are pests of stored grain products. They pose a considerable economic threat to global food security and safety. To date, the complete mitochondrial genome has only been determined for a single booklouse species *Liposcelis bostrychophila*. Unlike most bilateral animals, which have their 37 mt genes on one circular chromosome, ~15 kb in size, the mt genome of *L. bostrychophila* has two circular chromosomes, 8 and 8.5 kb in size. Here, we report the mt genome of another booklouse, *Liposcelis decolor*. The mt genome of *L. decolor* has the typical mt chromosome of bilateral animals, 14,405 bp long with 37 genes (13 PCGs, 22 tRNAs and 2 rRNAs). However, the arrangement of these genes in *L. decolor* differs substantially from that observed in *L. bostrychophila* and other insects. With the exception of atp8-atp6, *L. decolor* differs from *L. bostrychophila* in the arrangement of all of the other 35 genes. The variation in the mt genome organization and mt gene arrangement between the two *Liposcelis* species is unprecedented for closely related animals in the same genus. Furthermore, our results indicate that the two-chromosome mt genome organization observed in *L. bostrychophila* likely evolved recently after *L. bostrychophila* and *L. decolor* split from their most recent common ancestor.

Introduction

Insect mitochondrial (mt) genomes usually consist of a single circular chromosome (13–20 kb) containing 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs) and two ribosomal RNA genes (rRNAs), which is typical of bilateral animals [1]. The mt genome usually contains one large non-coding element called the A+T-rich or control region, which contains the sites for genome replication and the initiation of gene transcription [1]. Due to several unique features, including conserved gene content, maternal inheritance, and rapid nucleotide evolution, mt genome sequences have been used to facilitate the understanding of animal evolution [2]. Currently, more than 300 insect mt genomes have been sequenced [3]. Various types of gene rearrangements have been observed in insect mt genomes. Rearrangements of tRNA genes are the most common whereas rearrangements of protein-coding and rRNA genes are less common [4–6]. Studies in recent years also revealed variations of mt genome organization in bilateral animals [1]. For instance, mt genomes that consist of multiple chromosomes have been reported in parasitic lice [7–9], booklice [3], rotilera [10] and nematodes [11–13]. Variations in mt genome organization may provide a novel perspective for understanding animal evolution [14–16], in addition to genome sequences [3,17], RNA secondary structures [7,18–20], and gene rearrangements [5,18,21,22].

Several types of atypical mt genome organization have been reported in psocodean insects (superorder Psocodea) in recent years. Psocodea contains two orders of insects: Psocoptera (booklouse and barklice) and Phthiraptera (chewing and sucking lice). The mt genomes of human lice, *Pediculus humanus*, *P. capitis* and *Phthirus pubis*, consist of 14 to 20 mini-chromosomes, each one is 1.8 to 4 kb in size and contains one to five genes [7,9]. The chewing louse, *Coloecra sp.*, has a typical mt chromosome with 37 genes and a circular mt DNA molecule that is approximately half the size of the typical mt chromosome [8]. The booklouse, *Liposcelis bostrychophila*, has a bipartite mt genome with two chromosomes: one chromosome is ~8 kb in size and has 16 genes and the other is ~8.5 kb in size and has 22 genes [3]. Extensive gene rearrangement has been found in the mt genomes of most of the 12 Psocodea species that have been completely or nearly completely sequenced to date, including the booklouse, *L. bostrychophila* [3].

During the last two decades, the booklice of the genus *Liposcelis* have emerged as serious pests of stored commodities worldwide [23,24]. The genus *Liposcelis* has 126 known species worldwide and includes four groups (A, B, C, and D) [25,26]. The booklouse, *L. decolor*, investigated in the current study, belongs to group C, while...
L. bostrychophila belongs to group D. Many previous studies have indicated that there is great variation among the four Liposcelis groups with respect to morphology, physiology, biochemistry and molecular biology [25,27,28]. In particular, analyses of ITS (internal transcribed spacers) sequences indicated that Liposcelis species of groups C and D have the highest nucleotide divergence among the four groups [25].

To understand whether the bipartite mt genome organization observed in L. bostrychophila occurred in other booklice of the genus Liposcelis, we sequenced the mt genome of L. decolor. We found that, unlike L. bostrychophila, L. decolor has the typical mt chromosome of bilateral animals. However, the arrangement of mt genes in L. decolor differs substantially from that in L. bostrychophila and other known insects. Our results showed, for the first time, a high level of variation in both mt genome organization and mt gene arrangement between closely related animal species in the same genus.

Materials and Methods

Ethics statement

No specific permits were required for the insects collected in this study. The sampling locations were not privately owned or protected in any way and the collection did not involve endangered or protected species.

Sample collection, DNA extraction, PCR and sequencing

L. decolor individuals were collected at grain storage facilities in Binzhou, Shandong Province, China in 2010, and identified to species according to their morphological characteristics [28–30]. Subsequently, the ITS sequence [25] and sequences of partial rrnL and cox1 genes [31] were used to confirm the species identification. The ITS sequence obtained has been deposited in GenBank under accession number KF874610. An L. decolor colony was maintained in the lab on a diet of whole wheat flour, skim milk, and yeast powder (10:1:1) in an incubator at 27 ± 0.5°C, 75–80% relative humidity and a scotoperiod of 24 hours. Voucher specimens (#Ps-01-01-03) were deposited at the Insect Collection, Southwest University, Chongqing, China. Total genomic DNA was extracted using a Tissue/Cell gDNA Mini Kit (Watson Biotechnologies, Shanghai, China) and stored at −20°C. Parts of cox1, cox3, cob, rrsS, rrnL, and nad5 genes were amplified by PCR with conserved insect primers (Table S1) [32]. Species-specific primers were then designed for long PCR (Table S1).

Figure 1. Mitochondrial genome of Liposcelis decolor. Transcriptional orientation is indicated with arrows. Protein-coding genes, ribosomal RNA genes and transfer RNA genes are shown in orange, blue and green respectively. tRNA genes for the two serine and two leucine tRNAs: S1 = AGN, S2 = UCN, L1 = CUN, and L2 = UUR. The non-coding regions larger than 60 bp are indicated in black. CR = putative control region. Arrows and purple curves indicate primers and PCR fragment, respectively. See Table S1 for sequence of PCR primers. doi:10.1371/journal.pone.0091902.g001

Figure 2. PCR amplification and verification of mitochondrial DNA of Liposcelis decolor. Long-PCR amplification of two fragments to verify the single circular mt genome in L. decolor. Lane C1, negative control without the forward primer d1 or d3; lane C2, negative control without the reverse primer d2 or d4; lane C3, negative control without the DNA template. Lane M: 1 kb marker (Biomed). “d1-d2”, the product of PCR with primers d1 and d2, etc. Primer details are given in Table S1. doi:10.1371/journal.pone.0091902.g002
Each long PCR reaction was performed in a 25 μL volume, containing 1 μL each of forward primer (10 μM) and reverse primer (10 μM), 4 μL of dNTPs (each 2.5 mM), 1 μL of template DNA (~300 ng/μL), 2.5 μL MgCl₂ (25 mM), 2.5 μL of 10× LA PCR reaction buffer, 12.75 μL ddH₂O and 0.25 μL LA Taq DNA polymerase (5 U/μL, Takara). All reactions were carried out using C1000™ thermal cyclers (Bio-RAD, Hercules, CA, USA), following by ampicillin selection, then sequenced with M13 primers. Longer PCR products (> 6 kb) were directly sequenced with both forward and reverse PCR primers and internal primers by primer walking. All amplification products were sequenced with an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster city, CA, USA) at the Beijing Genomics Institute (BGI) in Beijing, China.

**Sequence assembly, annotation and analysis**

SeqMan (DNAStar) was used to assemble the two overlapping nucleotide sequences, which were further confirmed by manually inspection. The protein-coding and rRNA genes were identified using the program ORF Finder [http://www.ncbi.nlm.nih.gov/gorf/gorf.html] and BLAST searches against the GenBank database, respectively. Subsequently, all of these genes were further confirmed by alignment with homologous genes from those of other louse and booklouse species. The transfer RNA genes were identified by their cloverleaf secondary structure using tRNAscan-SE 1.21 [34] with Search Mode = "EufindtRNA-Cove", Genetic Code = “Invertebrate Mito” and Cove score cutoff = 0.1. The stem-loop secondary structure of the putative control regions was folded using the MiFold Server [35] under the RNA folding option with default parameters. The base composition and codon usage were determined using the ARWEN [33] with default parameters and tRNAscan-SE 1.21 [34] with Search Mode = "EufindtRNA-Cove", Genetic Code = “Invertebrate Mito” and Cove score cutoff = 0.1. The stem-loop secondary structure of the putative control regions was folded using the MiFold Server [35] under the RNA folding option with default parameters. The base composition and codon usage were analyzed with BioEdit [http://bioedit.net/bioedit.html] and DAMBE 5.3.9 [36]. Sequences of mt genomes of other lice were retrieved from GenBank and MitoZoa (Table S2) [37].

**Phylogenetic analyses**

Phylogenetic analyses were conducted with the 11 Psocodea mt genome sequences currently available in GenBank including the new booklouse sequence obtained in this study. The mt genome sequence of the fruit fly, *Drosophila melanogaster*, served as an outgroup. Sequences of *atp8*, *nad4L*, and tRNA genes were too short and too variable to be correctly aligned among the psocodean species; these genes were thus excluded from the phylogenetic analyses. The *nad6* was also excluded as this gene has not been identified in the human pubic louse, *P. pubis* [7]. The amino acid sequences from each protein-coding gene and the nucleotide sequence of each rRNA gene were aligned with MAFFT v7 [38]. The nucleotide sequences of each protein-coding gene were aligned based on the corresponding amino acid alignments using PAL2NAL [39] to ensure the correct reading frame; the poorly aligned sites were removed with GUIDANCE[40] using the default setting. Then, positions with gap in more than half of the species were removed. Substitution saturations of the nucleotide sequences were examined using DAMBE 5.3.9 following Xia et al. [41]. Whole PCG sequences were chosen to enter the next step if *I*ₘ (index of substitution saturation) is significantly lower than *I*ₑ (critical value for symmetrical tree topology) (*P* < 0.05). All of the protein-coding and rRNA genes, except *nad5* and *nad6*, passed this test. Consequently, the third codon positions of *nad5* and *nad6* were excluded from phylogenetic analyses. The best fit models for the alignment of nucleotide sequence and amino acid sequence were determined using the Akaïke Information Criterion in jModelTest 0.1.1 [42] and ProtTest 3 [43], respectively. Specifically, the GTR+I+G model and MrREV+I+G model were chosen for the nucleotide sequence dataset and the amino acid sequence dataset, respectively. Phylogenetic trees were estimated via Bayesian inference (BI) method using MrBayes v3.12 [44]. Four independent Markov chains were simultaneously run for 2,000,000 generations with a heating scheme (temp = 0.2). Trees were
sampled every 100 generations (sample-freq = 100) and the first 25% of the generations were discarded as burn-in and the remaining samples were used to compute the consensus tree. Stationarity was considered to be reached when the average standard deviation of split frequencies was below 0.01 [45].

Results and Discussion

Mitochondrial genome of Liposcelis decolor

The mt genome of L. decolor has one typical circular chromosome, unlike the booklouse, L. bostrychophila (Figure 1). The size and the circular organization of the mt chromosome of L. decolor was confirmed by two overlapping PCR amplicons, 9.1 kb (d1-d2 from cox3 to rrnS) and 5.5 kb in size (d3-d4 from rrnS to cox3) respectively (Figure 1). The two amplicons overlapped by 33 bp in cox3 and 92 bp in rrnS.

Sequencing and assembly of these two PCR amplicons revealed that the mt genome of L. decolor is 14,405 bp in length and encodes 37 genes that are typically found in metazoan mt genomes (Figure 1 and Table S1) (GenBank accession number: JX870621). All of the protein-coding genes (PCGs) initiate translation at an ATN codon, except for the TTG codon used in cox1. TAA and TAG serve as stop codons for all of the PCGs (Table S4). Eleven of the 13 PCGs had average length for Psocodean species, but nad4L is shorter and the nad4L is longer than in other Psocodean species (Figure S1). All 22 tRNA coding genes usually found in the mt genomes of metazoans are present in L. decolor (Figure 3); all have the conventional cloverleaf shaped secondary structure except trnS, which lacks the D-arm, as in other insects. There are three non-coding regions longer than 60 bp in the mt genome of L. decolor. The longest non-coding region (118 bp) lies between cob and trnY, has an A+T content of 82.20%; three stem-loop secondary structures can be found in this region (Figure 4A). An 80 bp non-coding region lies between nad2 and nad4 with an 88.75% A+T content but has no stem-loop secondary structure. A 69 bp non-coding region is between trnG and trnL1 with a 92.75% A+T content and has two stem-loops (Figure 4B). The 118-bp region is considered to be most likely the putative control region (Figure 1) due to it is longer sequence in the mt genome and the presence of typical loop secondary structure.

The A+T content of the L. decolor mt genome is 75.23%. This is typical of psocodean insects but is higher than that of L. bostrychophila, 68.63% (Figure S2 and Table S5). The higher A+T content of L. decolor is present in all regions, both genes and non-coding regions (Table S5). The difference of A+T content between the two booklice is reflected further in the codon usage: the relative synonymous codon usages (RSCU) of the two booklice showed that L. decolor used more NNA and NNT codon than L. bostrychophila (Figure 5). The nucleotide composition of mt genome is usually conserved within a genus; however, it varies between L. decolor and L. bostrychophila. This variation may be

Figure 5. Relative synonymous codon usage (RSCU) for protein coding genes of Liposcelis decolor and L. bostrychophila. Abbreviations of tRNA genes are according to the single letter according to the IPUC-IUB one-letter amino acid codes.
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Abbreviations

of tRNA genes are according to the single letter according to the IPUC-IUB one-letter amino acid codes.
related to mt genome fragmentation, because all of the psocodean fragmented mt genomes have a lower A+T content (Figure S2).

Mitochondrial gene rearrangement in *L. decolor*

The mt gene arrangement in *L. decolor* differs substantially from that of the hypothetical ancestor of insects and from that of the booklouse, *L. bostrychophila* (Figure 6). With the only exception of...
atp8-atp6, there is no gene boundary or gene block shared between L. decolor and L. bostrychophila, even though these two booklice belong to the same genus. Atp8-atp6 is a highly conserved ancestral gene boundary for animals and is assumed to be constrained by the function of a bicistronic atp8-atp6 transcript [46]. The mt gene arrangement is usually conserved within the same genus. For example, the two Pediculus species of human lice, both have extensively fragmented mt genomes but have the same mt gene arrangement [7]. Furthermore, Coloceras sp. and Campanulotes bidentatus, also have the same gene arrangement, except for a difference in the location of their trnQ. The extent of the variation in the mt gene arrangement between the two species of Liposcelis booklouse is unprecedented for animals within the same genus, indicating that gene rearrangement occurred frequently after these two booklice diverged from each other. The genus Liposcelis is divided into four groups (A, B, C, D) phylogenetically [25,26]; L. decolor and L. bostrychophila are in different groups, C and D respectively, although they often co-occur in a wide range of stored products in the same ecosystems. Previous studies have revealed that substantial variation exists among the Liposcelis groups at both morphological and molecular levels [25,30,31]. Whether or not the multipartite mt genome observed in L. bostrychophila occurred only in species of group D remains to be investigated.

Phylogenetic relationship of L. decolor to other species in the Psocodea

Bayesian inference was used to determine phylogenetic relationships among 11 species of Psocodea from the orders Psopectera and Phthiraptera, with nucleotide and deducted amino acid sequences of mt genomes (Figure 7 and Figure S3). Although L. decolor and L. bostrychophila differ in both mt genome organization and mt gene arrangement, these two booklice are more closely related to each other than to other species in the Psocodea. The two Liposcelis species formed a sister clade to the parasitic lice (order Phthiraptera). This was also indicated by previous studies [3,30,47]. The close relationships between the two booklice, and between the booklice and the parasitic lice are strongly supported [3,30,47]. The checked bars designate the fragmented mt genomes observed in the blood-sucking lice (suborder Anoplura).

Supporting Information

Figure S1 Size of mitochondrial protein-coding genes and rRNA genes of the Psocodea. Lower horizontal bar, non-outlier smallest observation; lower edge of rectangle, 25 percentile; the central horizontal bar, median; upper edge of rectangle, 75 percentile; upper horizontal bar, non-outlier largest observation; small circle, outlier. Species are abbreviated as following: Ld, Liposcelis decolor; Lb, Liposcelis bostrychophila; Ls, Lepidopsocidae sp. RS-2001; Bm, Bothriometopus macrocnemis; Cs, Campanulotes bidentatus; Cs, Coloceras sp. SLC-2011; Hm, Heterodoxus macropus; Ib, Ibirococus biquangipus; Pc, Pediculus capitis; Ph, Pediculus humanus; Pf, Phthirus pubis; Ae, Anaticola crassicornis; Dm, Damalinia meyeri; Ps, Philopterus sp. SLC-2011; Q5, Quadriaceta sp. SLC-2011; Bp, Brachionus plicatilis. (TIF)

Figure S2 A+T contents of the mitochondrial genomes of the Psocodea. The checked bars designate the fragmented genomes. The mt genomes sequenced incompletely are marked by asterisks. Species are abbreviated as following: Ld, Liposcelis decolor; Lb, Liposcelis bostrychophila; Ls, Lepidopsocidae sp. RS-2001; Bm, Bothriometopus macrocnemis; Cs, Campanulotes bidentatus; Cs, Coloceras sp. SLC-2011; Hm, Heterodoxus macropus; Ib, Ibirococus biquangipus; Pc, Pediculus capitis; Ph, Pediculus humanus; Pf, Phthirus pubis; Ae, Anaticola crassicornis; Dm, Damalinia meyeri; Ps, Philopterus sp. SLC-2011; Q5, Quadriaceta sp. SLC-2011; Bp, Brachionus plicatilis. (TIF)

Figure S3 Alignments of mitochondrial gene sequences used for phylogenetic analyses. A. Nucleotide sequence alignment (gene order of this alignment: cox1-3, atp6, cob, nad1-3, nad5-6, rns and rnl2; sequences of nad3 and nad6 just with codon positions 1 and 2); B. Amino acid sequence alignment (gene order of this alignment: cox1-3, atp6, cob, nad1-3 and nad5-6). Species are abbreviated as following: Ld, Liposcelis decolor; Lb, Liposcelis bostrychophila; Ls, Lepidopsocidae sp. RS-2001; Bm, Bothriometopus macrocnemis; Cs, Campanulotes bidentatus; Cs, Coloceras sp. SLC-2011; Hm, Heterodoxus macropus; Ib, Ibirococus biquangipus; Pc, Pediculus capitis; Ph, Pediculus humanus; Pf, Phthirus pubis; Dm, Drosophila melanogaster. (DOC)

Table S1 PCR primers used for the amplification of the mitochondrial genome of Liposcelis decolor. (DOC)

Table S2 GenBank accession numbers of all the insects mentioned in this study. (DOC)

Table S3 Summary of the mitochondrial genome of Liposcelis decolor.  *Genes located in the different strand from that of cox1 are underlined. binc = intergenic nucleotides, indicating gap nucleotides (positive value) and overlapping nucleotides (negative value) of two adjacent genes. aAT-skew = (A−T)/(A+T); bGC-skew = (G−C)/(G+C); cCR = control region (putative). (DOC)

Table S4 Start and stop codons of mitochondrial protein-coding genes of the Psocodea. Species are abbreviated as follows: Ld, Liposcelis decolor; Lb, Liposcelis bostrychophila; Ls, Lepidopsocidae sp. RS-2001; Bm, Bothriometopus macrocnemis; Cs, Campanulotes bidentatus; Cs, Coloceras sp. SLC-2011; Hm, Heterodoxus macropus; Ib, Ibirococus biquangipus; Pc, Pediculus capitis; Ph, Pediculus humanus; Pf, Phthirus pubis; Ae, Anaticola crassicornis; Dm, Damalinia meyeri; Ps, Philopterus sp. SLC-2011. (DOC)

Table S5 Nucleotide compositions of the mitochondrial genomes of Liposcelis decolor and L. bostrychophila. (DOC)

Author Contributions

Conceived and designed the experiments: SCC DDW JW. Performed the experiments: SCC DDW WD. Analyzed the data: SCC DDW RS. Contributed reagents/materials/analysis tools: JW. Wrote the paper: SCC DDW RS JW.

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