



## A novel mitochondrial genome fragmentation pattern in *Liposcelis brunnea*, the type species of the genus *Liposcelis* (Psocodea: Liposcelidae)

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### ABSTRACT

Booklice in the genus *Liposcelis* (Psocodea: Liposcelidae) are essential storage pests worldwide. Fragmented mt genomes have been identified in the *Liposcelis* species together with the typical mitochondrial (mt) genome, which is a single circular chromosome with 37 genes. Gene rearrangement, pseudogenes, and repeat regions (RRs) are very common among fragmented mt genomes. We sequenced the mt genome of the booklouse *L. brunnea*, the type species of the genus *Liposcelis*. We identified 37 genes in the mt genome of *L. brunnea*, which was fragmented into three chromosomes. The chromosomes I, II, III were 7.3 kb, 5.5 kb, and 5.3 kb in size with 9, 19, and 15 genes, respectively. In addition, 16 pseudogenes and four repeat regions were present in three chromosomes. Gene rearrangement in the mt genome of *L. brunnea* was obvious compared to that in other mt genomes in the genus *Liposcelis*. We found a possible correlation among mt genome rearrangement, the morphological classification standard, and phylogenetic relationships. In summary, a three-chromosome mt genome in an insect was identified for the first time, which may aid in understanding mt genome fragmentation, gene rearrangement, and evolution.

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### 1. Introduction

The family Liposcelidae contains about 200 species of booklice in 9 genera [1]. Unlike their relatives barklice and parasitic lice, booklice are usually found in mildewed papers or books, although they have diverse life habits from troglitic to epizoic. The largest genus of Liposcelidae is *Liposcelis* with 126 species to date [2]. Some booklice in the genus *Liposcelis*, for example *L. bostrychophila*, *L. entomophila*, and *L. brunnea*, are common storage pests, infesting stored food commodities [3–6].

The typical mitochondrial (mt) genome of bilateral animals is a circular chromosome that is about 16 kb in size, with 13 protein-coding genes (PCGs), two ribosome RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes [7]. However, the regular one-chromosome mt genome was replaced by several linear or circular chromosomes in cnidarians [8–10], thrips [11], nematodes [12,13], parasitic lice [14–22], booklice [23,24]. In 2009, an extremely fragmented mt genome was found in the human body louse *Pediculus humanus*, whose typical one-circle chromosome was split into 20 minichromosomes [15]. Seventeen parasitic lice of eutherian mammals have subsequently been found to possess extensively fragmented mt genomes: the minichromosome

numbers range from 9 to 20; each minichromosome is 0.8–4 kb in size with 1–8 genes [16,21]. Meanwhile, no mt genome fragmentation has been found in any of the eight species of bird lice. In the genus *Liposcelis*, mt genome fragmentation was first reported in the booklouse *L. bostrychophila* (Beibei, China, BB hereafter) [23]. The mt genome of *L. bostrychophila* was fragmented into two chromosomes: chromosome I (8.5 kb, 22 genes) and chromosome II (7.9 kb, 16 genes). In a recent study [25], the intra-specific mt genome diversity in *L. bostrychophila* was enriched by the mt genomes of six strains of *L. bostrychophila* (BJ, XSG, HLM, SY, KA, CA). Three groups of the strains were finally generated based on their gene rearrangement and sequence similarities: BJ and XSG in Group 1; HLM and SY in Group 2; and KA, CA, and BB in Group 3. The mt genomes of *L. entomophila* and *L. paeta* were fragmented into two chromosomes, while *L. sculptimacula* and *L. decolor* both had typical mt genomes [26,27]. A *Liposcelis* sp. that was proved to be a cryptic species of *L. bostrychophila* was found to have two mt genome fragmentation types: five chromosomes and seven chromosomes [28]. In summary, regular and fragmented mt genomes coexist in the genus *Liposcelis*.

Pseudogenes can be downstream products of mt genome fragmentation, and they have been found in species with fragmented mt genomes [15,29]. For example, mt *cox1* pseudogenes are present in many linear mt genomes from the family Hydridae in Medusozoa [8];

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eleven pseudogenes of PCGs were found in the two-chromosomal mt genome of the cyst nematode *Globodera ellingtonae* [13]; a rat louse, *Polyplax spinulosa* [19], as well as two pig lice [30], *Haematopinus suis* and *Haematopinus apri*, were found to have tRNA pseudogenes in their extended fragmented mt genomes. In booklice, three to five pseudogenes were found in the mt genome of *L. bostrychophila* strains [24]. Repeat regions (RRs) including non-coding regions and tRNA genes, 881–986 bp in size, are present in both mt chromosomes in every *L. bostrychophila* strain. Numerous pseudogenes and non-coding regions are present in *L. paeta* and *L. entomophila*, which in total account for 10% and 30% of their mt genome lengths, respectively [23]. Gene rearrangement is also common in booklice. The mt gene boundaries of five *Liposcelis* species were so unstable that almost every gene boundary changed in different species [27].

To further explore the mt genome evolution in the genus *Liposcelis*, we sequenced the mt genome of the booklouse *Liposcelis brunnea* Motschulsky, 1852 [31,32], the type species of the genus *Liposcelis*. We found that the mt genome of *L. brunnea* was fragmented into three chromosomes, 5 kb to 7 kb in size, with 9 to 19 genes. All 37 regular mt genes were annotated as well as 16 pseudogenes and 4 RRs. We analyzed the mt genome fragmentation, gene rearrangement, and phylogeny as well as their potential relationships with classical morphological taxonomy in the genus *Liposcelis*. Our results provide valuable information for deeper investigations into the mt genome evolution of booklice.

## 2. Materials and methods

### 2.1. Sample collection, DNA extraction, sequence amplification, and sequencing

The booklouse *L. brunnea* was collected from grain storage facilities in Prague, Czech Republic (Cz strain hereafter). The samples were identified to be *L. brunnea* based on morphology [33]. The sequencing samples were stored in 100% ethanol at  $-80^{\circ}\text{C}$  before DNA extraction. Genomic DNA was extracted from 15 booklice using a DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's instructions.

Partial sequences of the *cox1*, *rrnS*, and *rrnL* genes were amplified by polymerase chain reaction (PCR) using the universal primer pairs LCO1490-HCO2198 [34], 12SF-12SR [35], and 16Sar-16Sbr [36], which target conserved gene regions among arthropods. The PCR amplification was performed in a 25- $\mu\text{L}$  reaction volume containing 12.5  $\mu\text{L}$  of  $2 \times \text{Taq Mix}$  (Tiangen, Beijing, China), 1  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ), 1  $\mu\text{L}$  genomic DNA, and 9.5  $\mu\text{L}$  ddH<sub>2</sub>O. The PCR cycling conditions were  $94^{\circ}\text{C}$  for 3 min, followed by 35 cycles of  $94^{\circ}\text{C}$  for 1 min,  $50^{\circ}\text{C}$  for 1 min,  $72^{\circ}\text{C}$  for 1 min, and finally  $72^{\circ}\text{C}$  for 10 min. The size of PCR amplicons was estimated by agarose gel electrophoresis (1%). Negative controls were run in all PCR experiments. The PCR products were sequenced using the Sanger sequencing method at Beijing Genomics Institute (BGI). Afterwards, the three partial sequences from the *cox1*, *rrnS*, and *rrnL* genes were deposited in GenBank under the accession numbers MG820270, MG792324, and MG792019. A sequencing library was constructed with the total DNA of *L. brunnea* following the manufacturer's instructions (Illumina Inc.). The library was then sequenced on an Illumina HiSeq 2500 sequencer at the BerryGenomics company. The insert size was 450 bp, and 250 bp from each end of the insert was sequenced (pair-end sequencing).

### 2.2. Sequence assembly, long-PCR confirmation, and annotation

Raw data were filtered using Trimmomatic v0.32 [37] before data assembly. The subsequent clean sequence reads were assembled into three partial sequences using Geneious 10.1.3 [38] with the following parameters: 1) minimum overlap identity, 98%; 2) maximum of 5% mismatches per read; 3) maximum ambiguity, 2; and 4) up to 100 iterations. After the assembly, three contigs were obtained. We connected the start and end of the three obtained contigs after trimming. The

clean sequence reads were then assembled into three circled contigs to confirm the results using the following standards: the coverage was smooth across the chromosome and coverage was correct in the end-start connection position (an example is shown in Fig. S1).

The assembly results indicated that the mt genome of *L. brunnea* might consist of three circular chromosomes: chromosome I (assembled from the conserved *cox1* sequence), chromosome II (from the conserved *rrnS* sequence), and chromosome III (from the conserved *rrnL* sequence). For further confirmation, we designed two pairs of long-PCR primers for each chromosome with overlapping parts on both sides to cover the entire chromosome (Table S1). Long-PCRs were performed to obtain all the amplicons. All six of the long-PCR amplicons were sequenced using the primer walking method at BGI. The sequences were proofread and assembled into contigs using Geneious 10.0.5. All sequencing results from primer walking were matched to the sequences of the three chromosomes. Each long-PCR amplification was performed in a 25- $\mu\text{L}$  reaction volume containing 2.5  $\mu\text{L}$   $10 \times \text{LA Taq Buffer}$ , 0.125  $\mu\text{L}$  TaKaRa LA Taq (5 U/ $\mu\text{L}$ ), 4  $\mu\text{L}$  dNTP mixture, 1  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ), 2.5  $\mu\text{L}$  MgCl<sub>2</sub>, 1  $\mu\text{L}$  genomic DNA, and 12.875  $\mu\text{L}$  ddH<sub>2</sub>O. The PCR cycling conditions were  $94^{\circ}\text{C}$  for 1 min, followed by 40 cycles of  $98^{\circ}\text{C}$  for 10 s,  $68^{\circ}\text{C}$  for 9–15 min, and finally  $72^{\circ}\text{C}$  for 6 min. The long-PCR amplicon size was estimated using agarose gel electrophoresis (1%).

The PCGs and rRNA genes were identified using MITOS [39] and BLAST searches of the NCBI database and then verified by alignment with homologous genes from other booklice. ARWEN [40], tRNAscan [41], and MITOS were used to identify tRNA genes. The nucleotide composition of the mt genome was determined using Geneious 10.1.3.

### 2.3. Gene cluster extraction

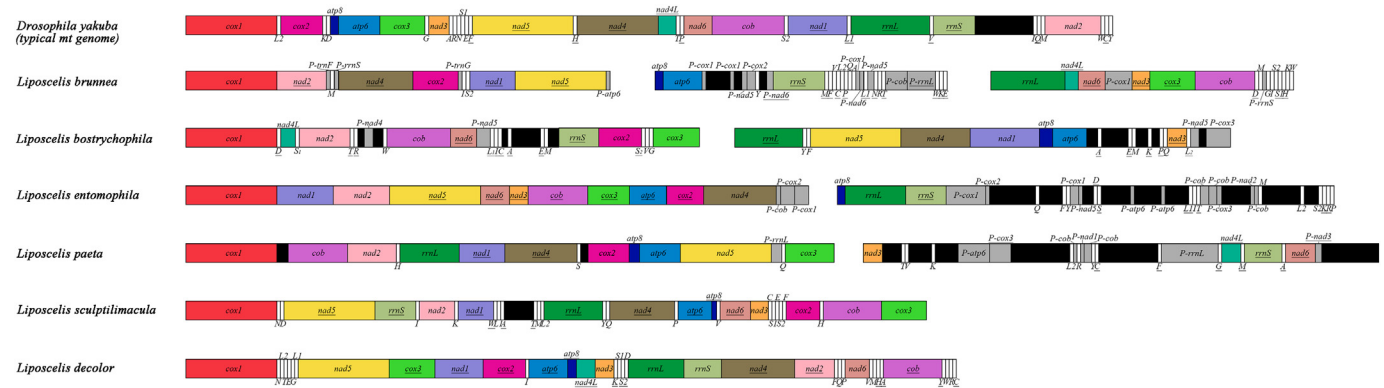
The neighborhood relations (adjacency) between genes are essential for ancestral genome structure reconstruction and are applied in software designed for ancestral genome reconstruction, for example ANGES [42], FPSAC [43], and DeCoSTAR [44]. However, they are not applicable for ancestral reconstruction of multiple circular chromosomes. Shared gene clusters with neighborhood relationships were obtained manually by comparing the mt gene arrangement among *Liposcelis* species (Fig. 1). Because of the high mobility of tRNA genes, only PCGs and rRNA genes were considered in this procedure. We applied the strict guideline that only those gene clusters with the same genes and same relative coding direction were counted to avoid any other influences. We used the mt genome of the *L. bostrychophila* BJ strain as the reference sequence for this species. The *Liposcelis* sp. reported by Perlman was excluded from this process as well as from the phylogenetic analysis because of its undefined morphology and taxonomic status [45,46].

### 2.4. Pseudogene and RR analyses

Pseudogenes have been identified in the booklice *L. bostrychophila*, *L. entomophila*, and *L. paeta*, which all have fragmented mt genomes [23,24]. To assess the pseudogene status in *L. brunnea*, all 37 mt gene sequences were extracted, and their homologous sequences were obtained using the BLAST program (NCBI-BLAST, [www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) against the whole mt genome sequence. All homologous sequences with more than 95% similarities relative to the intact coding regions were named pseudogenes. RRs were defined as those identical regions including both coding and non-coding sequences among different chromosomes. We used all possible combinations for BLAST searches and obtained four RRs.

### 2.5. Sequence alignment and phylogenetic analyses

We inferred the phylogenetic relationships of the genus *Liposcelis*, including *L. brunnea* and five other booklice (*L. bostrychophila*, *L. decolor*, *L. paeta*, *L. sculptilimacula*, and *L. entomophila*). Five concatenated datasets were used in the phylogenetic analysis: 1) 12 protein-coding genes (*atp6*,



**Fig. 1.** Arrangement of mitochondrial genomes of six *Liposcelis* species and the typical mitochondrial genome of insect. The circular mt genomes have been linearized for ease of comparison. Gene names are the standard abbreviations used in this study. Different PCGs and rRNA genes are with different colours; tRNA genes are in white; pseudogenes are in gray; non-coding regions are in black.

*atp8*, *cox1*, *cox2*, *cox3*, *cob*, *nad1*, *nad2*, *nad3*, *nad4*, *nad5*, *nad6*, PCG123rRNA hereafter) and two rRNA genes (*rrnL* and *rrnS*); 2) 12 protein-coding genes (PCG123 hereafter); 3) the first and second codon positions of the 12 protein-coding genes and 2 rRNA genes (PCG12rRNA hereafter); 4) the first and second codon positions of the 12 protein-coding genes (PCG12 hereafter); and 5) the amino acid sequences of the 12 protein-coding genes (AA hereafter). The barklouse *Lepinotus reticulatus* was selected as the outgroup [47]. For *L. bostrychophila*, the BJ, HLM, and BB strains were selected as representative sequences of Group 1, Group 2, and Group 3, respectively [25]. All mt genome sequences used in this study are shown in Table S2. The *nad4L* gene was not found in *L. entomophila*, and thus, it was excluded from the phylogenetic analysis. Every protein-coding gene was aligned using the Translation Align function (Genetic code: Invertebrate Mitochondrial, Protein alignment options: Muscle Alignment) implemented in Geneious 10.1.3 with the default parameters. All stop codons were removed. The rRNA genes were aligned using the MAFFT v7.0 online server [48] with the G-INS-I strategy. Ambiguous positions in the alignment of protein-coding genes and rRNA genes were removed using Gblocks v0.91b [49]. Sequences were concatenated using SequenMatrix 1.7.8 [50].

The five concatenated alignment datasets were used in Bayesian (BI) and maximum likelihood (ML) analyses with MrBayes 3.2.6 [51] and the IQ-TREE web server [52]. For BI analysis, PartitionFinder [53] was used to evaluate the best nucleotide substitution models. The best sequence partitions and corresponding models selected are shown in Table S3. Two independent sets of Markov chains (one cold and three heated chains) were run for 10 million generations. The trees were sampled every 1000 generations with the first 25% discarded as burn-in. For ML analyses, optimal evolutionary models were selected with the “Auto” option, and the model for each partition was chosen based on Bayesian Information Criterion. ML phylogenetic trees were constructed using an ultrafast bootstrap method with 1000 replicates.

### 3. Results

#### 3.1. The mt genome of the booklouse *L. brunnea*

In total, we obtained 19,057,662 sequencing reads, 250 bp in size, from the genomic DNA of *L. brunnea*, and 143,276, 132,587, and

132,600 reads were mapped to chromosomes I, II, and III, respectively (Table 1, Fig. 2). Each chromosome was verified by two overlapping fragments obtained from long-PCR amplification (amplicons are shown in Fig. 3). Two amplicons, A1 and A2, overlapped by 2863 bp and 3314 bp in chromosome I; B1 and B2 overlapped by 3330 bp and 1463 bp in chromosome II; and C1 and C2 overlapped by 2510 bp and 1681 bp in chromosome III.

All 37 typical mt genes (Table S4) were annotated in the mt genome of *L. brunnea*, including 13 PCGs, 2 rRNA genes, and 22 tRNA genes (Fig. S2). Chromosome I was 7333 bp in size, consisting of 6 PCGs (the *cox1*, *cox2*, *nad1*, *nad2*, *nad4*, and *nad5* genes) and 3 tRNA genes (the *trnM*, *trnI*, and *trnS2* genes); chromosome II was 5513 bp in size, with 2 PCGs (the *atp6* and *atp8* genes), 16 tRNA genes (the *trnA*, *trnV*, *trnL1*, *trnL2*, *trnM*, *trnP*, *trnF*, *trnW*, *trnT*, *trnN*, *trnQ*, *trnY*, *trnC*, *trnK*, *trnR*, and *trnE* genes), and the *rrnS* gene; chromosome III was 5317 bp in size, containing 5 PCGs (the *cox3*, *cob*, *nad3*, *nad4L*, and *nad6* genes), 9 tRNA genes (the *trnG*, *trnI*, *trnM*, *trnW*, *trnS1*, *trnS2*, *trnK*, *trnH*, and *trnD* genes), and the *rrnL* gene. The *trnM* gene was present in all three chromosomes, the *trnI* and *trnS2* genes were present in chromosomes I and III, and the *trnW* and *trnK* genes were present in chromosomes II and III. Moreover, the *trnC* gene lacks a TΨC arm, and tRNA-G only has a diminutive TΨC arm.

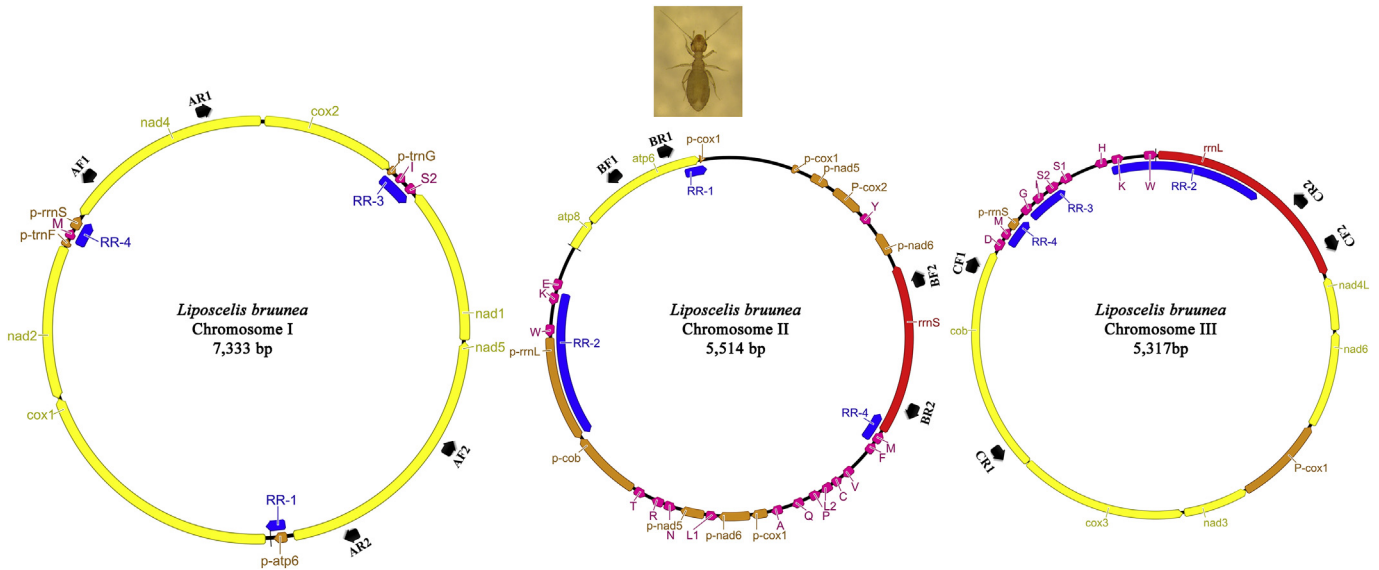
#### 3.2. Gene clusters, pseudogenes, and RRs in the mt genome of *L. brunnea*

Only clusters including no more than two genes were detected among booklice: *atp8-atp6*, *atp6-cox3*, *cox3-nad3*, *nad4-cox2*, *cox3-cox1*, and *nad4-nad1* (Table S5). Surprisingly, only the cluster *atp8-atp6* is very common across many lineages in insects [54], and it is shared by almost all booklice, which is also evidence of the extraordinary gene rearrangement that has occurred in booklice mt genomes.

Sixteen pseudogenes ranging from 32 bp to 546 bp were found to exist across the three chromosomes (Table S4): four of them in chromosome I, ten in chromosome II, and two in chromosome III. Twelve of the pseudogenes originated from PCGs, while three and one stemmed from rRNA and tRNA genes, respectively. Four RRs were found across the three chromosomes. RRI was found on chromosomes I and II; it is 119 bp in size and contains pseudo-*atp6* and 37 bp non-coding sequences. RRII was found on chromosomes II and III; it is 760 bp in

**Table 1**  
Mitochondrial minichromosomes of the booklouse, *Liposcelis brunnea*, identified by Illumina Sequencing.

Chromosome	Size(bp)	Size of coding region (bp)	Size of non-coding region (bp)	Number of illumina sequence reads	Mean coverage	GenBank accession number
I	7333	7009	324	143,276	4885	MG820271
II	5513	2685	2828	132,587	6012	MG820272
III	5317	4520	797	132,600	6235	MG820273



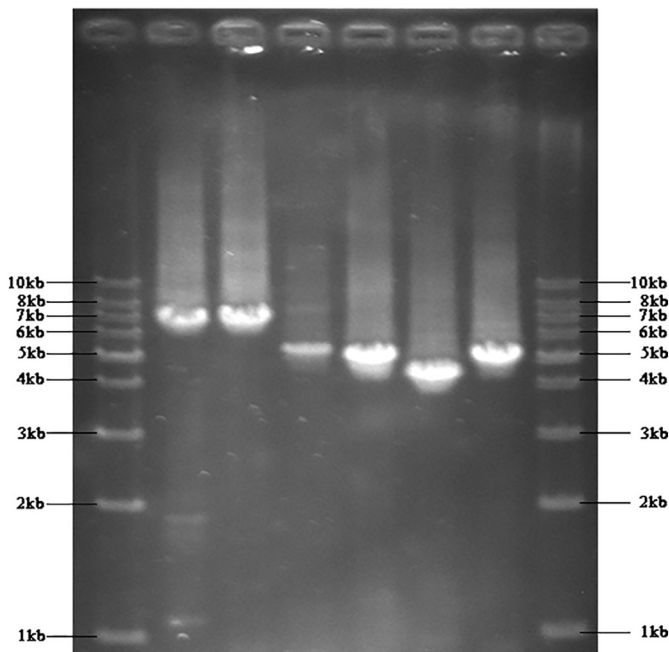
**Fig. 2.** The mitochondrial genome organizations of three chromosomes of *Liposcelis brunnea*. Circular maps were drawn with Geneious (Kearse et al. 2012). The transcriptional direction was indicated with arrows. PCGs were shown in yellow, rRNA genes in red, tRNA genes in purple, pseudogenes in orange, repeat regions in blue. The Long PCR primers used to verify the facticity of three chromosomes were indicated by black arrows. Abbreviations of gene names were: *cox1–3* for cytochrome oxidase subunits 1–3, *cob* for cytochrome *b*, *nad1–6* and *nad4L* for NADH dehydrogenase subunits 1–6 and 4 L, *rnl* and *rns* for large and small rRNA subunits, *atp6* and *atp8* for ATP synthase subunits 6 and 8. The tRNA genes were indicated with their one-letter corresponding amino acids. Pseudogenes were labeled with P- and original gene name.

size, with *trnK*, *trnW*, pseudo-*rnl*, and 98 bp non-coding sequences. RRIII was found on chromosomes I and III; it is 214 bp in size and contains *trnI*, *trnS2* (TGA), pseudo-*trnG*, and 29 bp non-coding sequences. RRIII was found on all three chromosomes; it is 153 bp in size and contains *trnM* and pseudo-*rns* sequences. As a result of the RRs, the number of *trnK*, *trnW*, *trnI*, and *trnS2* genes doubled; that of the *trnM* gene tripled; and pseudogenes (pseudo-*atp6*, pseudo-*rnl*, pseudo-*trnG*, pseudo-*rns*-1, pseudo-*rns*-2) emerged.

### 3.3. Phylogenetic relationships in the genus *Liposcelis*

We generated 10 phylogenetic trees (Fig. 4, Fig. S3) with the same topology. In the genus *Liposcelis*, *L. brunnea* and *L. decolor* formed a clade with a posterior probability (PP) of 1 and bootstrap support value (BSV) of 100. They were placed as the sister group to all other booklice with strong support (PP 1). The *L. bostrychophila* strains *L. paeta* and *L. sculptilimacula* clustered together (PP 1, BSV 100) and were placed as the sister group of *L. entomophila* (PP 1, BSV 1). The phylogeny obtained in this study was in accord with that reported in previous studies on booklice [25,28].

**Chromosome I Chromosome II Chromosome III**  
A1 A2 B1 B2 C1 C2



**Fig. 3.** Long-PCR amplicons of three circular chromosomes of *Liposcelis brunnea*. A1A2, B1B2 and C1C2 had overlap parts in both side to confirm the reality of Chromosome I, II and III, respectively. DNA marker: 1 kb DNA Ladder (Tiangen).

## 4. Discussion

It is normal for an insect genus to possess one mt genome arrangement [7,55]. *Liposcelis* species together with parasitic lice do not follow this pattern and have been found to have a high degree of inheritance divergence [21,23]. Based on the present knowledge, the genus *Liposcelis* is to date the only insect genus with five different types of mt genome fragmentation patterns. The mt genomes of *L. decolor* and *L. sculptilimacula* have a regular one-chromosome structure [26,27]; the mt genomes of *L. paeta*, *L. entomophila*, and the asexual *L. bostrychophila* are fragmented into two chromosomes [23,24]; a *Liposcelis* species from the USA (demonstrated to be cryptic species of *L. bostrychophila*) has a five- or seven-chromosome mt genome [28]. We identified a fifth pattern after sequencing the mt genome of *L. brunnea* and verifying its three-chromosome structure.

### 4.1. Ancestral gene arrangement characters in *Liposcelis*

Shared gene clusters, to some extent, are important available ancestral characters and useful tools for exploring relationships among species. A matrix showing shared gene clusters in booklice was built (Table 2). *Drosophila yakuba* [56] and *L. brunnea* share gene clusters with all booklice. *L. bostrychophila*, *L. paeta*, and *L. sculptilimacula* have at least two shared clusters with each other, while *L. entomophila* only shares the gene cluster *nad4-cox2* with

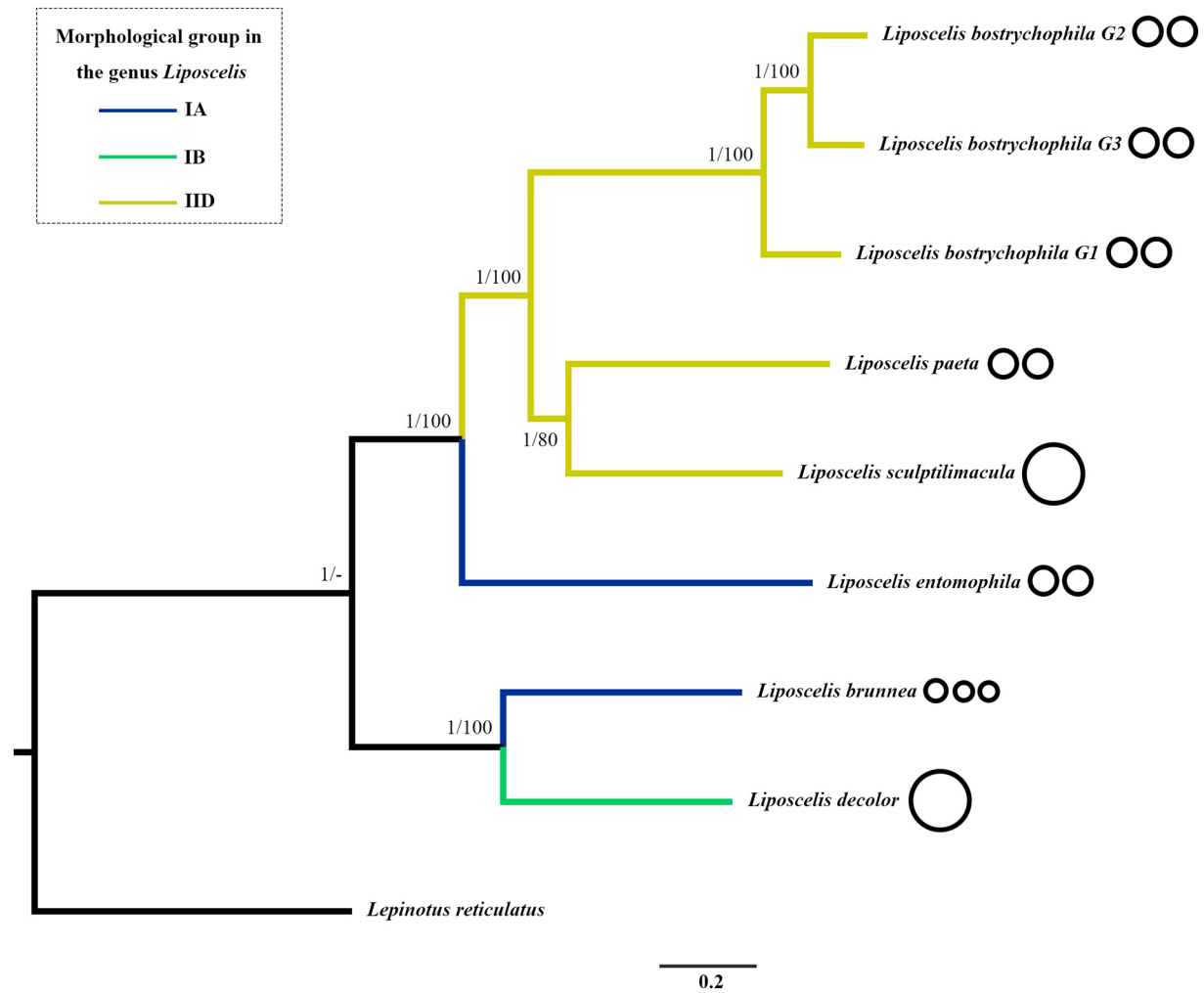


Fig. 4. The phylogenetic tree in genus *Liposcelis*. Consensus tree were produced by PCG123rRNA dataset by BI and ML methods. Different morphological groups were labeled.

*L. brunnea*. At the genus level, there were too few shared gene clusters in *Liposcelis* for comparison to the mt genomes of other genera [54]. Moreover, the gene rearrangements in the *Liposcelis* species differed substantially from those in the ancestor insects, other psocids, or even with each other [27]. Thus, the gene clusters we obtained were not sufficient for the construction of the ancestral mt genome of *Liposcelis*. Even though we tried to simulate the process of gene rearrangement in this genus, the large number of rearrangement events made the results of each attempt implausible. With the increasing number of mt genomes being sequenced in this genus, the number of ancestral characters that can be extracted for ancestral genome sequence reconstruction is also increasing.

#### 4.2. Pseudogenes, repetitive genes, and RRs in *Liposcelis*

In *Liposcelis* studies [23,24], protein-coding pseudogenes have been found in all fragmented mt genomes, rRNA pseudogenes have been annotated in *L. brunnea* and *L. paeta*, and tRNA pseudogenes have been identified in *L. brunnea*. To avoid annotation errors, we conducted three steps in the mt pseudogene searches. First, we applied the identical pseudogene identification methods used in the present study to all booklice species. Second, ARWEN [40] and tRNAscan [41] were used to identify tRNA secondary structures while those structures with low scores were considered as well. Third, the borders of RRs between fragmented chromosomes were carefully checked. As a result of the

Table 2  
Shared gene clusters between every two booklice in matrix.

	<i>Drosophila yakuba</i>	<i>Liposcelis brunnea</i>	<i>Liposcelis bostrychophila</i>	<i>Liposcelis entomophila</i>	<i>Liposcelis paeta</i>	<i>Liposcelis sculptilimacula</i>	<i>Liposcelis decolor</i>
<i>Drosophila yakuba</i>							
<i>Liposcelis brunnea</i>	2						
<i>Liposcelis bostrychophila</i>	1	1					
<i>Liposcelis entomophila</i>	1	1	0				
<i>Liposcelis paeta</i>	1	1	3	0			
<i>Liposcelis sculptilimacula</i>	1	1	2	0	2		
<i>Liposcelis decolor</i>	1	1	1	0	1	1	

searches, four tRNA pseudogenes were discovered in three groups of *L. bostrychophila* [24], and they were attributed to RRs between the fragmented chromosomes. Two pseudo-*trnE* were found in both chromosomes of *L. bostrychophila* Group 1; a pseudo-*trnK* was found in the first chromosome of *L. bostrychophila* Group 2; and a pseudo-*trnK* was found in the second chromosome of *L. bostrychophila* Group 3.

Pseudogenes are considered to be redundant sequences and in the process of being eliminated. Several studies on parasitic lice and booklice have provided reasonable explanations of this process [16,24]. Multiple mt chromosomes are generated by certain molecular events. In each event, a fragment containing the control region and other genes is excised from the typical mt genome chromosome and forms a circular chromosome by the joining of the two free ends. If the new chromosome has selection advantages, the counterpart in the typical chromosome becomes redundant and is eliminated, and pseudogenes are subsequently generated. Our findings for booklice are in accord with this process (Table 3): there is no pseudogene in *L. decolor* and *L. sculptilimacula*, which have regular one-chromosome mt genomes; pseudogenes exist in all other booklice with fragmented mt genomes. In many studies, only pseudo-tRNA genes were found, indicating that tRNA genes might be the tail of mt genome evolution [57–61]. Pseudo-tRNA genes, instead, were not found in *L. paeta* and *L. entomophila*, possibly because of their incomplete mt genomes. More work is needed to obtain the complete mt genome information for these two booklice. On the other hand, pseudogenes that originated from PCGs were present in all booklice with fragmented mt genomes. One possible explanation is that it takes a long time for pseudogenes from PCGs to be eliminated because they are longer than pseudogenes from tRNA genes.

Four RRs were found in the mt genome of *L. brunnea* ranging from 119 bp to 759 bp, which is good evidence for tracing the fragmentation process. These RRs included the *trnK*, *trnW*, *trnI*, *trnS2*, *trnG*, and *trnM* genes, resulting in these genes having multiple copies in the mt genome of *L. brunnea*. Two pseudogenes (pseudo-*atp6* and pseudo-*rnl*) were also found in two RRs. In addition, there are repetitive tRNA genes in *L. bostrychophila* and *L. brunnea*, all of which are attributed to RRs being shared between chromosomes. This leads to several questions. Will RRs have separate functions? Will they work cooperatively? If only one RR will be ultimately retained, which one will be selected?

### 4.3. Relationships among traditional taxonomy, shared gene clusters, phylogeny, and mt genome fragmentation

The accordance between morphological taxonomy and molecular phylogeny has become a popular research topic since the emergence of molecular phylogeny. Some studies have supported the accordance between molecular phylogeny and morphological traits [62–65], while others have reached the opposite conclusion [66–68]. Morphology-based taxonomy remains one of the most important cornerstones of zoological classification. In the genus *Liposcelis*, all booklice are divided into four groups of species (A, B, C, D) belonging to two sections (I and II); they are IA, IB, IIC, and IID. The booklice *L. brunnea* and *L. entomophila* belong to IA; *L. decolor* is a part of IB; and *L. bostrychophila*, *L. sculptilimacula*, and *L. paeta* belong to IID. The group and section divisions are based on easily visible characters of tergite fusions and chaetotaxy. This standard was proposed by Badonnel [69–71] and confirmed by Lienhard [33] and Mockford [72], but not tested by phylogenetic analysis [73].

Shared gene clusters, as discussed above, are important characters to infer species relationships. Thus, it is of value to determine the relationship between shared gene clusters and morphological classifications. Three booklice from IID (*L. bostrychophila*, *L. sculptilimacula*, and *L. paeta*) had more shared gene clusters with each other (2 or 3) than with other booklice (0 or 1). In IA, *L. entomophila* had an exclusive shared gene cluster (*nad4-cox2*) with *L. brunnea*. More shared gene clusters existed at the intra-group level, meaning that the trait of a shared

**Table 3**  
Pseudogenes in *Liposcelis* species.

Species of booklice	Mt genome chromosome number	Total pseudogene number	The number of the pseudogene														
			pseudo- <i>atp6</i>	pseudo- <i>nad1</i>	pseudo- <i>nad2</i>	pseudo- <i>nad3</i>	pseudo- <i>nad4</i>	pseudo- <i>nad5</i>	pseudo- <i>nad6</i>	pseudo- <i>cob</i>	pseudo- <i>cox1</i>	pseudo- <i>cox2</i>	pseudo- <i>cox3</i>	pseudo- <i>rns</i>	pseudo- <i>rml</i>	pseudo-tRNA gene	
<i>Liposcelis brunnea</i>	3	16	1	-	-	-	-	2	2	1	4	1	1	-	2	1	2
<i>Liposcelis bostrychophila</i> Group1	2	5	1	2	-	-	1	1	-	-	-	-	-	-	-	-	2*
<i>Liposcelis bostrychophila</i> Group2	2	3	-	-	-	-	1	2	-	-	-	-	-	-	-	-	1*
<i>Liposcelis bostrychophila</i> Group3	2	4	-	-	-	-	1	2	-	-	-	-	1	-	-	-	1*
<i>Liposcelis entomophila</i>	2	15	2	-	1	-	1	1	-	4	3	2	1	-	-	-	-
<i>Liposcelis paeta</i>	2	8	1	1	-	1	-	-	-	-	-	-	1	-	-	2	-
<i>Liposcelis decolor</i>	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Liposcelis sculptilimacula</i>	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

\* Newly found pseudogenes

gene cluster is completely in accord with the morphological standard based on all available data. Hence, shared gene clusters as well as morphological standards have the same relationship with other traits. Based on the inferred phylogeny, three booklice from IID (*L. bostrychophila*, *L. sculptilimacula*, and *L. paeta*) formed a clade that was consistent with the morphological standard and shared gene cluster characters. This accordance was not found in IA, possibly because *L. brunnea* had a closer phylogenetic relationship with *L. decolor* (IB) but not *L. entomophila*. No conclusion could be drawn for IB and IIC because of the lack of data. More mt genome data are necessary to obtain a comprehensive understanding of their relationships.

Finally, we explored the relationships among mt genome fragmentation, morphological standards, shared gene clusters, and phylogeny. Three booklice, *L. bostrychophila*, *L. sculptilimacula*, and *L. paeta*, belonging to IID with more shared gene clusters had both fragmented and regular mt genomes. IA, containing *L. brunnea* and *L. entomophila*, only had fragmented mt genomes but different types (two-chromosome or three-chromosome mt genome); the mt genome of *L. decolor* representing IB was a regular one-chromosome form. Based on the inferred phylogeny, the fragmented and regular one-chromosome mt genome structure coexisted in almost every clade in the phylogenetic tree (Fig. 4). The correlation between molecular phylogeny and mt genome fragmentation, as has been reported in parasitic lice [21], was nonexistent in booklice.

In this study, the mt genome of *Liposcelis brunnea*, the type species of the genus *Liposcelis*, was sequenced and found to have an atypical three-chromosome structure. This is the only three-chromosome mt genome reported in insects and the fifth mt genome fragmentation type found in *Liposcelis*. Our analysis revealed close relationships among variable gene rearrangements, phylogeny, and morphological identification traits present in *Liposcelis*. Our results provide valuable data for further exploring mt genome fragmentation, gene rearrangement, and evolution.

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## Conflicts of interest

The authors declare that they have no competing interests.

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