Comparative analysis of the mitochondrial genome and genetic relationship between *Philosamia cynthia ricini* and *Philosamia cynthia cynthia*

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ABSTRACT: *Philosamia cynthia cynthia* and *Philosamia cynthia ricini* are silken insects belonging to the family Saturniidae (Lepidoptera). In order to explore the genetic relationships of silken insects from Saturniidae at the molecular level, we used NGS method to assemble complete mitochondrial genome sequences of *P. cynthia ricini* strain B7 and *P. cynthia cynthia* strain C2gx. Assembly results showed that the total lengths of the mitochondrial genome sequences of B7 and C2gx were 15 383 and 15 344 bp, respectively; and both genomes contained 37 genes with an A+T enriched region. A total of 361 transversion or transition sites occurred at 13 PCGs and 1 insertion or deletion site at the *Cox1*. The start codons of the *Cox1* in B7 and C2gx were CGA and ATT, respectively, which were different from the TTAG of other lepidopteran insects. Based on the mitochondrial genome sequences of fifteen lepidopteran insects published in NCBI, we performed phylogenetic analysis and showed that, although the two strains have some genetic differences in the mitochondrial genome, B7 and C2gx were clustered into one branch with close kinship as expected.

KEYWORDS: *Philosamia cynthia ricini*, *Philosamia cynthia cynthia*, mitochondrial genome, genetic relationship

INTRODUCTION

The mitochondrial genome (mt genome) is an important material for studying population genetics, genetics, and evolution due to its conserved gene arrangement, fast nucleotide substitution rate, and small genome size [1, 2]. In most animals, the mitochondrial DNA (mtDNA) has a closed double-stranded circular structure with length between 16–20 kb [3]. It generally contains 37 genes with a conserved gene order, which includes 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), and 2 ribosomes RNA genes (rRNAs) [4].

In recent years, with the continuous development and improvement of the next generation sequencing (NGS) methods, related researches on mt genomes of plants and animals have also gradually expanded. For insects, mt genomes have been sequenced in various insects, such as Hemiptera, Lepidoptera, and Coleoptera, with some basic features compared, such as size, gene content, gene rearrangement, and codon usage, etc [5–7]. Using mt genome sequences, Jiang et al [8]; Hong et al [9]; and Feng et al [10] reconstructed the genetic relationship between different Lepidopteran species, and reached the same conclusions by using traditional classification methods. Song and Liang constructed a phylogenetic tree of Hemiptera based on 29 PCGs from the mt genome of multiple species, and revealed Hemiptera as a monophyletic taxon [11]. However, Cui et al [12] and Li et al [13] found some present systematic errors in the Hemiptera phylogenetic studies, due to effects of compositional heterogeneity. The above-mentioned cases proved that different evolution rates of the same genes between different species could result in the divergence of classification of species based on mt genome. Some highly conserved sequences of mtDNA are commonly used as DNA barcodes, and it has become an important method in species identification and classification. Common PCGs are used as DNA barcodes including the *Cytochrome C Oxidase Subunit 1* (*Cox1*) [14], *Cytochrome C Oxidase Subunit 2* (*Cox2*), and *NADH dehydrogenase*
Philosamia cynthia cynthia (P. cynthia cynthia) and Philosamia cynthia ricini (P. cynthia ricini) are both multivoltine (multiple life cycles a year) insects belonging to the lepidopteran family of Saturniidae. P. cynthia cynthia mainly feeds on Ailanthus altissima (Mill.) Swingle leaf with additionally feeding on Ricinus communis L. leaf, Ilex chinensis Sims leaf, and Firmiana platanifolia (L.f.) Marsili leaf, and diapaus in the form of pupa. Before, P. cynthia cynthia had been considered as a leaf-eating pest, but it has been used as a precious silk and edible insect resource in recent years in lieu of traditional control methods [16]. P. cynthia ricini is native to Assam, India, and was introduced to mainland China in 1940. P. cynthia ricini mainly feeds on Ricinus communis L. leaf, Manihot esculenta Crantz leaf, and Ailanthus altissima (Mill.) Swingle leaf. There is no diapause period under suitable conditions for P. cynthia ricini. Adults of both insects are similar in appearance, but the arrangement of scales on the abdomen is obviously different: horizontal in P. cynthia ricini, while vertical in P. cynthia cynthia. In addition, they can be crossed and the hybrids are fertile [17]. Among lepidopteran insects, Bombyx mori is the first species to have a complete mt genome sequenced. At present, there are many studies on sequencing and structural analysis of mt genomes of B. mori, Antheraea assamensis, etc., and completed mt genomes have been reported for P. cynthia ricini strains [18], but similar researches have been lacking for P. cynthia cynthia. Based on the basic composition structure and bases of P. cynthia ricini mt genome, studies of skewness, codon usage preferences, and kinship with other Saturniidae insects have provided data for genetic relationships of lepidopteran insects [19].

Comparative molecular characteristic studies have been done between the two silk insects of B. mori and Antheraea proylei using PCGs or rRNA [20]. However, comparative analyses of mt genome between P. cynthia cynthia and P. cynthia ricini are lacking. Here, we assembled complete mt genome sequences of P. cynthia cynthia and P. cynthia ricini. In addition, we contrasted the tRNA structural composition and the regions of PCGs in order to identify and analyze the differences of the two insects.

**MATERIALS AND METHODS**

Philosamia cynthia ricini strain B7 was collected from Xuzhou, Jiangsu Province. Philosamia cynthia cynthia strain C2gx was collected from Nanning, Guangxi Province. Both samples were preserved and stored in the Sericultural Research Institute, Chinese Academy of Agricultural Sciences.

Mitochondrial DNA was extracted by the classical “phenol-chloroform-isopentyl alcohol” method [21] from the male pupa of B7, and C2gx. DNA was fragmented using the Covaris machine. The DNA fragments were repaired at protruding ends using a combination of 3′-5′ exonuclease and polymerase, and a single base ‘A’ was introduced at the 3′ of the segment to join the fragments with ligase to selectively enrich DNA fragments with joints at both ends while amplifying the DNA library [22]. Finally, fragment sizes were measured using the Agilent 2100 Bioanalyzer, and library concentration was detected by fluorescence quantification, followed by sequencing on the Illumina sequencing platform [23].

The SPAdes v3.9.0 [24] and A5-miseq v2015-0522 [25] softwares were used to assemble their high quality paired-end reads to construct contigs and scaffolding, respectively. Sequences with high sequencing coverage were extracted and compared with the nt library at NCBI using blastn (BLAST v2.2.31+) to select mitochondrial sequences of each spliced result. The obtained mitochondrial assembly results were first analyzed by mummer v3.1 [26] for collinearity and to determine the positional relationships between contigs, before gap filling between contigs was performed. The results were error-corrected using the Pilon v1.18 software [27] to obtain the final mitochondrial sequences. The assembled complete mt genome sequences were deposited at the MITOS web server for functional annotations [28].

The mt genome circle map was drawn with the cgview visualization software [29]. mt genome, PCGs, and rRNA base composition bias were calculated using formula: AT bias = (A−T)/(A+T); GC bias = (G−C)/(G+C) [30]. Through the calculation of relative synonymous codon usage (RSCU), the codon usages of B7 and C2gx were compared, and the coding protein sequences were analyzed.

ClustalW was used for the multiple sequence alignment of complete mt genome sequences from 15 lepidopteran insects published at NCBI. The phylogenetic tree was constructed using the MEGA 7.0 software by setting up the unrooted neighbor-joining (NJ) method with following parameters: 1000 times bootstrap value, poisson correction, and pairwise deletions [31].
RESULTS

Comparison of mitochondrial genome composition, structure and characteristics

The total lengths of mt genomes of B7 and C2gx were 15383 and 15344 bp, respectively. Both genomes contained 13 PCGs, 2 rRNAs, 22 tRNAs, and an A+T-rich region (OH region) (241 bp in B7 and 250 bp in C2gx). The gene orders of both mt genomes were the same as that of a typical lepidopteran mt genome, with a double-stranded (sense and antisense sequences) closed circular structure. For both genomes, there were 14 genes located in the antisense sequence, including 4 PCGs, 8 tRNAs, and 2 rRNAs. There were 23 genes located in the sense sequence, including 9 PCGs, 14 tRNAs, and 1 OH region (Figs. 1 and 2 and Table 1).

The total lengths of the 13 PCGs were 11164 bp and 11173 bp in B7 and C2gx, respectively. In B7, the Cox1 uses CGA as the start codon, while the other PCGs use the start codon ATT/G/C; whereas in C2gx, all PCGs use the typical start codon ATT/G. The 12 PCGs in B7 and C2gx mt genomes use TAA as the stop codon, and only the NADH dehydrogenase subunit 3 (Nad3) uses TAG as the stop codon. There were 8 and 9 overlapping gene pairs in B7 and C2gx, respectively. Base overlapping may be a way to reduce the sizes of the mt genomes.

The RSCU showed that the mt genomes of B7 and C2gx were both biased toward bases A and T, and the codons that start or stop with A and T were much more frequent than other synonymous codons.

Both mt genomes of B7 and C2gx contained 22 non-coding region sequences with lengths ranging from 1–250 bp. Among them, the OH region was 241 bp long in B7 and 250 bp long in C2gx. The OH region is the main regulatory region for replication and transcription of the mt genome [32]. Previous studies have indicated that there are repetitive sequences in the OH regions of lepidopteran mt genomes. The OH region of Antheraea pernyi contains a 38 bp repetitive unit [33], and (AT)ₙ random repetitive sequences exist in the OH regions of Antheraea yamamai, B. mori and Bombyx mandarina [34]. In current study, the 38 bp repetitive unit was absent in the OH regions of both B7 and C2gx, but a repeat of (AT)₃₀ was found in B7 and a repeat of (AT)₅₀ in C2gx.

Both B7 and C2gx mt genomes contained 22 tRNAs and 2 rRNAs. The tRNA lengths were between 62–72 bp, rrnL lengths were 1295 (B7)/1289 bp (C2gx), and rrnS lengths were 763 (B7)/777 bp (C2gx). The tRNA of both genomes had some mismatched bases. Most of them were UG mismatched bases in typical tRNA secondary structure, and there were a few UU mismatched bases. There was only one pair of AA mismatched bases in tRNAs, which was not a typical secondary structure. In addition, trnA, trnL2, trnN, trnS1, trnS2, trnT, and trnY did not have the typical secondary structure in B7 and trnA, trnL2, trnN, trnS1, trnS2 for C2gx (Figs. 3 and 4).

Mitochondrial protein coding sequences alignment and analysis of mutation sites

Basic local alignment of the entire mt genome sequences of B7 and C2gx showed that the similarity between the two genomes was 96.83%, indicating that the two subspecies are closely related. Insertions or deletions occurred at 23 local sites, and transversion or transition occurred at 454 sites. In order to further explore the compositions within PCGs, we compared 13 PCGs between B7 and C2gx one by one. The results revealed several gene mutations with varying degrees. The insertion or deletion of 13 PCGs only occurred at the start site of the Cox1 in both B7 and C2gx (Fig. 5). A total of 361 transversions or transitions occurred between B7 and C2gx, which mainly concentrated within proteins such as NADH dehydrogenase subunit 2 (Nad2), Cox1, ATP synthase subunit 6 (Atp6), Cytochrome C Oxidase Subunit 3 (Cox3), Nad5, NADH dehydrogenase subunit 4 (Nad4), NADH dehydrogenase subunit 1 (Nad1), and Cytochrome B (Cob). Among these PCGs, Cox1 and Nad5 are the greatest different PCGs between B7 and C2gx (Table 2 and Fig. 5). In addition, we compared the amino acid sequences and their secondary structures to analyze functional differences of the 13 PCGs. We found that except for Nad4 and NADH dehydrogenase subunit 4l (Nad4l), the remaining 11 PCGs amino acid sequences were different at 2–13 sites with overall amino acid sequence similarity over 95%; and the secondary structures were identical. For Nad4 and Nad4l, amino acid sequence similarity between the two subspecies was approximately 90%, and the differences in the secondary structure were also obvious.

Genetic relationship

We constructed phylogenetic tree using NJ method with complete mt genomic sequences of lepidopteran insects that are closely related to B7 and C2gx, including A. yamamai, Actias luna, Actias
Fig. 1 B7 mt genome.

Fig. 2 C2gx mt genome.
Fig. 3 B7 mt genome does not have a typical tRNA secondary structure (trnS2) and mismatched bases (in red circles).

Fig. 4 C2gx mt genome does not have a typical tRNA secondary structure (trnS2) and mismatched bases (in red circles).

Solid lines represent 6 SNP sites, and dashed lines represent 3 SNP sites

Indel region at the start of Cox1 (partial sequences)

Fig. 5 Distribution of SNP and Indel sites in 13 PCGs.

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Table 1 Characteristics of the mt genomes.

| Name     | Gene       | Strand | Start position (bp) | Intergenic nucleotide | Start codon | Stop codon | Anticodon |
|----------|------------|--------|---------------------|-----------------------|-------------|------------|-----------|
| B7/C2gx  | trnM       | N      | 1                   | 65/64                 | 3           | CAT        |
| B7/C2gx  | trnI       | N      | 69/68               | 65                    | -3          | CAT        |
| B7/C2gx  | trnQ       | J      | 131                 | 69                    | 54/56       | TTG        |
| B7/C2gx  | nad2       | N      | 254/255             | 1014                  | 9/3         | ATT        |
| B7/C2gx  | trnW       | N      | 1277/1272           | 68                    | -8          | TCA        |
| B7/C2gx  | trnC       | J      | 1337/1332           | 62                    |             | GCA        |
| B7/C2gx  | trnY       | J      | 1399/1394           | 65/66                 | 9/0         | GTA        |
| B7/C2gx  | cox1       | N      | 1473/1460           | 1536/1545             | -5          | CGA/ATT    |
| B7/C2gx  | trnL2      | N      | 3004/3000           | 68                    | -45         | TAA        |
| B7/C2gx  | cox2       | N      | 3027/3023           | 688                   | 42          | ATG        |
| B7/C2gx  | trnK       | N      | 3757                | 71                    | 33/24       | TTG        |
| B7/C2gx  | trnD       | N      | 3861/3848           | 68/72                 |             | GTC        |
| B7/C2gx  | atp8       | N      | 3929/3920           | 165                   | -7          | ATG/ATT    |
| B7/C2gx  | atp6       | N      | 4087                | 678                   | -1          | ATG        |
| B7/C2gx  | cox3       | N      | 4764/4755           | 789                   | 2           | ATG        |
| B7/C2gx  | trnG       | N      | 5555/5546           | 66                    |             | TCC        |
| B7/C2gx  | nad3       | N      | 5621/5612           | 354                   | -2          | ATT        |
| B7/C2gx  | trnA       | N      | 5973/5964           | 66/67                 | 24/5        | TGC        |
| B7/C2gx  | trnR       | N      | 6063/6036           | 64/68                 | 26/32       | TCG        |
| B7/C2gx  | trnN       | N      | 6153/6136           | 65                    | 14/1        | GTC        |
| B7/C2gx  | trnS1      | N      | 6232/6200           | 66                    | 4           | GCT        |
| B7/C2gx  | trnE       | N      | 6302/6270           | 66                    | -2          | TTC        |
| B7/C2gx  | trnF       | J      | 6366/6334           | 68/67                 |             | GAA        |
| B7/C2gx  | nad5       | J      | 6434/6401           | 1716                  | 30          | ATT        |
| B7/C2gx  | trnH       | J      | 8180/8147           | 66                    | 1           | GTG        |
| B7/C2gx  | nad4       | J      | 8247/8214           | 1341                  | 3           | ATG        |
| B7/C2gx  | nad4L      | J      | 9591/9558           | 291                   | 12/8        | ATG        |
| B7/C2gx  | trnT       | N      | 9894/9857           | 65/64                 |             | TGT        |
| B7/C2gx  | trnP       | J      | 9959/9921           | 65                    | 32          | TGG        |
| B7/C2gx  | nad6       | N      | 10056/10018         | 501                   | 7/6         | ATT        |
| B7/C2gx  | cob        | N      | 10564/10525         | 1152                  | 5           | ATG        |
| B7/C2gx  | trnS2      | N      | 11721/11682         | 66                    | 24/25       | TGA        |
| B7/C2gx  | nad1       | J      | 11811/11773         | 939                   | 2           | ATG        |
| B7/C2gx  | trnL1      | J      | 12752/12714         | 68                    | 33/44       | TAG        |
| B7/C2gx  | rrnL       | J      | 12853/12826         | 1295/1289             | 30          |           |
| B7/C2gx  | trnV       | J      | 14178/14145         | 66                    |             | TAC        |
| B7/C2gx  | rrnS       | J      | 14244/14211         | 763/777               | 59          |           |
| B7/C2gx  | OH         | N      | 15066/15017         | 241/250               | 76/77       |           |

a Strand: J is the antisense strand and N is the sense strand.
b Intergenic nucleotide: the length of the interval between this gene and the previous gene.

artemis aliena, Saturnia jonasii, Neoris haraldi, Ernolatia moorei, Mustilizans hepatica, Rondotia menciana, Triuncina daii, Rotunda rotundapex, B. mandarina, Antheraea assama, Eriogyna pyretorum, A. pernyi, and B. mori. The results indicated that B. cynthia ricini, and B. cynthia cynthis clustered into one branch as expected (Fig. 6). Meanwhile, the 3 Antheraea insects and the 2 Bombyx insects formed their own respective branches, reflecting their close relationships. M. hepatica was the most distant from all the other insects.

DISCUSSION

By comparing PCGs, the biggest difference we found was that the Cox1 of B7 did not use the typical start codon ATT/G. In the lepidoptera mt genome, TTAG is usually used as the start codon for the Cox1, and no canonical is available in tRNA and start region of the Cox1 [35]. Nevertheless, neither B7 nor C2gx had this characteristic. The start codon of Cox1 was
Fig. 6 Genetic relationships of 17 lepidopteran insects.

Table 2 Comparison of 13 coding gene mutation sites in B7 and C2gx.

| Name   | Gene | Bases | Indel | SNP |
|--------|------|-------|-------|-----|
| B7/C2gx| Nad2 | 1014  | 0     | 21  |
| B7/C2gx| Cox1 | 1536/1545 | 9 | 49  |
| B7/C2gx| Cox2 | 688   | 0     | 18  |
| B7/C2gx| Atp8 | 165   | 0     | 3   |
| B7/C2gx| Atp6 | 678   | 0     | 22  |
| B7/C2gx| Cox3 | 789   | 0     | 29  |
| B7/C2gx| Nad3 | 354   | 0     | 10  |
| B7/C2gx| Nad5 | 1716  | 0     | 55  |
| B7/C2gx| Nad4 | 1341  | 0     | 43  |
| B7/C2gx| Nad4l| 291   | 0     | 8   |
| B7/C2gx| Nad6 | 501   | 0     | 20  |
| B7/C2gx| Cob  | 1152  | 0     | 48  |
| B7/C2gx| Nad1 | 939   | 0     | 35  |

was that the OH region in C2gx was 9 bp longer than in B7. The two respectively contained (AT)$_{59}$ and (AT)$_{61}$ repeats and had a poly T structure with an ATAGA guide length of 19 bp. Based on previous researches, the origin of lagging strand replication in *Drosophila melanogaster* was related to (AT) repeats region [36]. In human mt genome, the change in the structure of tRNA was mapped to the origin of some lagging strands [37]. Thus, these sequences may play an important role in the transcription of their mitochondrial genes. And the start sequence of the OH region of C2gx was a 13 bp poly T structure, which was considered as the signal recognition site of the mt genome light chain replication initiation [38].

For the comparison of tRNAs between B7 and C2gx, most of them had UG mismatched bases in tRNAs and the remaining are U-U mismatched. Both B7 and C2gx had a pair of AA mismatched bases in trnN. Base mismatches may affect tRNAs function; however, tRNAs may retain their normal functions through some modifications or sharing codons with neighboring downstream genes [37].

We also comparatively analyzed the mutation
sites in the PCGs. We found that there were 9 bases insertions or deletions in the Cox1. In addition, the Cox1 could be used as a genetic marker among species in tapeworms, which was used to distinguish different strains of the same species [39] and applicable to B7 and C2gx. At the protein level, the differential expression of Cox1 could be used to further differentiate the genetic relationship between B7 and C2gx. Besides, there were a few mutation sites in Atp8 and Nad4l; while Nad4/5, Cox1, and Cob were also quite different due to the gene of transversion or transition. The Nad is relatively conserved in different taxa, but with high mutation rates among species, it was considered suitable as a genetic marker for species identification and diagnosis. The Atp played an important role in animal and plant energy metabolism and cell development. The Cox was crucial to growth, development, and energy utilization; while the Cob was greatly conserved and could be used as a potential genetic differentiation marker. In this study, we also analyzed the amino acid sequences and the secondary structures of the 13 PCGs. Whether the marked differences of Nad4 and Nad4l in the composition and structure may be another important reason for the divergence in related functions and genetic evolution of B7 and C2gx needs further experiments.

CONCLUSION

In this study, we used NGS method to assemble the mt genomes of B7 and C2gx to analyze the whole mt genome, OH regions, tRNAs, and PCGs. The results indicated that there were indeed some mutation sites and different aspects in B7 and C2gx, and these could be related to genetic relationship. Finally, by constructing a phylogenetic tree using the NJ method and based on complete mt genomic sequences of 17 lepidopteran insects, we found that B7 and C2gx shared a high degree of genetic relationship as expected.

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