Genomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Complete mitochondrial genome of *Saturnia jonasii* (Lepidoptera: Saturniidae): Genomic comparisons and phylogenetic inference among Bombycoidea

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ARTICLE INFO

Keywords:
Saturnia jonasii
Mitochondrial genome
Bombycoidea
Phylogeny

ABSTRACT

The complete mitochondrial genome (mitogenome) of *Saturnia jonasii* (Lepidoptera: Saturniidae) was sequenced and compared to those of 19 other bombycoid species. Furthermore, the mitogenome sequences were used to infer phylogenetic relationships among bombycoid species. The 15,261-bp *Saturnia jonasii* mitogenome contained the typical sets of genes and gene arrangements found in majority of Lepidoptera. All Bombycoidea species, including *Saturnia jonasii*, have a 15–33-bp spacer sequence at the *trnS$_2$-ND1* junction. The phylogenetic reconstruction of bombycoid species consistently and strongly supported monophylies of the families, Saturniidae, Bombycidae, and Sphingidae, based on Bayesian inference (BI) and maximum-likelihood (ML) methods. Among these families, the Bombycidae and Sphingidae species consistently showed a sister relationship, regardless of data partitions; the BI method strongly supported this relationship, whereas it was moderately supported using the ML method.

1. Introduction

In South Korea, two species of *Saturnia* (Lepidoptera: Saturniidae) have been reported. *Saturnia boisduvalii* Jordan, 1911 is distributed in North and South Korea, northern China, the Russian Far East, and southeastern Siberia, whereas *Saturnia japonica* Moore, 1862 is distributed in North and South Korea, central and southern China, Taiwan, Japan, and the Russian Far East [1,2]. A third species, *Saturnia jonasii*, which is distributed in Japan (including Tsushima Island) and Taiwan [2–4], has not been recorded in South Korea. However, a recent morphological and molecular study identified specimens of *Saturnia* from the remote South Korean offshore island of Jejudo as *Saturnia jonasii*, and not *Saturnia boisduvalii* [5]. Consequently, the distribution of *Saturnia boisduvalii* has been reduced to the Korean mainland. The major morphological differences between *Saturnia jonasii* and *Saturnia boisduvalii* included wing pattern elements of the fore- and hindwings and male and female genitalia [5]. DNA barcoding sequence-based phylogeny strongly supported clustering of the specimens from Jejudo with *Saturnia jonasii* collected in Japan [5]. Nonetheless, the minimum sequence divergence between *Saturnia jonasii* and *Saturnia boisduvalii* was slightly lower (4.26–4.71%) than that between most other pairs of *Saturnia* species (approximately 5–7%; [5]). Furthermore, the morphological similarity between the two species has been reported [3], although Kim et al. [5] clarified that the *Saturnia jonasii* specimens collected from Jejudo Island differ morphologically from *Saturnia boisduvalii*. Thus, an extended comparison using mitochondrial genomes of the two species and other members of Bombycoidea would help in establishing the genetic distinction of these two species.

The mitochondrial genome (mitogenome) of the majority of metazoans are circular, double-stranded molecules spanning approximately 16 kb [6], composed of 13 protein-coding genes [subunits 6 and 8 of the F_0 ATPase (ATPase6 and ATPase8), cytochrome oxidase subunits 1–3 (*COI–COIII*), cytochrome B (*CytB*), and NADH dehydrogenase subunits 1–6 and 4L (*ND1–ND6* and *ND4L*)], two rRNA genes [small- and large-subunit rRNAs (lrRNA and srRNA)], and 22 tRNA genes [6]. Additionally, it harbors a control region, known in insect mitochondrial DNA (mtDNA) as the A + T-rich region, which contains the replication origin for both mtDNA strands in *Drosophila* species [7,8]. To date, the mitogenomes of > 1,400 species covering all insect orders have been reported and approximately 350 of these are from Lepidoptera (https://www.ncbi.nlm.nih.gov/; last visited on June 2017). These sequences have greatly contributed to our understanding of population genetics, molecular evolution, and phylogenetics.

In the present study, we determined the complete mitogenome

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https://doi.org/10.1016/j.ygeno.2017.11.004
Received 15 July 2017; Received in revised form 20 November 2017; Accepted 25 November 2017

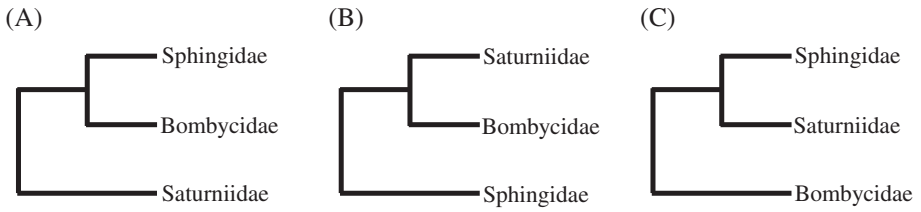

Fig. 1. Representatives of previous phylogenetic hypotheses among Sphingidae, Saturniidae, and Bombycidae. (A) Minet [9], Zwick [11] and Fig. 8A tree from Kim et al. [12]; (B) Regier et al. [10] and Zwick et al. [13]; and (C) Kawahara and Breinholt [14] and Fig. 8B tree from Kim et al. [12].

sequences of *Saturnia jonasii* for the first time. The genomic sequence was compared with that of other species of Bombycoidea in order to determine characteristics such as genome structure, genomic arrangement, nucleotide composition, codon usage, spacing pattern, and A + T-rich region structure. In particular, the sequence divergence of individual genes among three available *Saturnia* species (*Saturnia jonasii*, *Saturnia boisduvalii*, and *Saturnia japonica*) was compared to estimate the genetic divergence between *Saturnia jonasii* and *Saturnia boisduvalii*. Furthermore, a phylogenetic analysis using the available mitogenome sequences of other Bombycoidea species was performed to determine phylogenetic relationships among the families in Bombycoidea. These families (Saturniidae, Bombycidae, and Sphingidae) have previously been extensively examined based on morphology and different molecular markers using different analytical methods [9–12]. Morphological characters supported the sister relationship between Bombycidae and Sphingidae, with the placement of Sphingidae as a sister group to this group [9] (Fig. 1A). However, alternative relationships among the three families have also been observed using various molecular markers and analyses (Fig. 1A, B, C; [10–14]).

2. Materials and methods

2.1. DNA extraction, PCR, and sequencing

Adult *Saturnia jonasii* were collected from Mt. Hallasan, Jejudo Province (33°24′35″N, 126°29′43″E) in 2008, and deposited at Chonnam National University with the accession number CNU SJ4004. A hind leg was used to extract DNA using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Three primer sets that amplify three long overlapping fragments (LFs; LF1, LF2, and LF3) were adapted from Kim et al. [15]: LF1, LF2, and LF3 amplify *COI* and *ND4* (~7.2 kb), *ND5* to *lrRNA* (~6.7 kb), and *lrRNA* to *COI* (~4.8 kb), respectively. Amplification of the LFs was conducted using LA Taq™ (Takara Biomedical, Tokyo, Japan) under the following conditions: 96 °C for 2 min, 30 cycles of 98 °C for 10 s and 48 °C for 15 min, and a final extension step of 72 °C for 10 min. Thereafter, these amplicons were used as templates to amplify 26 overlapping short fragments (SFs) using AccuPower® PCR PreMix (Bioneer, Daejeon, Korea) under the following conditions: initial denaturation for 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 48–52 °C, and 1 min at 72 °C, and a final 7-min extension at 72 °C. The sequences of the primers used, which were also adapted from Kim et al. [15], are shown in Supplementary Table S1. DNA sequencing was conducted using an ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM™ 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). All the products were sequenced from both directions. Individual SF sequences were assembled manually into the complete mitogenome using SeqMan (DNASTAR, Madison, Wisconsin, USA).

2.2. Boundary delimitation and annotation

The tRNA genes were searched using tRNAscan-SE 1.21 [16] with the default search mode. Mito/Chloroplast was set as the source, invertebrate mitogenome set as the genetic code for tRNA isotype prediction, and cove score cut-off set to 1. Twenty-one of the 22 tRNAs were accordingly identified. The remaining tRNA, $trnS_1$, was found by alignments of the potential region with the $trnS_1$ of other lepidopteran species, with particular consideration being given to the anticodons and hand-drawn secondary structures [12]. In order to identify individual protein-coding genes (PCGs), the blastn program in Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used with default settings. Delimitation of genes, along with confirmation of start and stop codons was performed based on the alignment of available *Saturnia* PCGs with those of the other bombycoid species using MAFFT ver. 6 [17] with the gap opening penalty set at 1.53 and the offset value (≈ gap extension penalty) set at 0.5. The two rRNAs and A + T-rich region were identified and delimited using the nucleotide blastn in Blast and further confirmed by alignment against other bombycoid rRNA and A + T-rich region sequences using MAFFT ver. 6 [17].

2.3. Genomic comparison among bombycoid species

Nineteen available mitogenome sequences of Bombycoidea were obtained from GenBank (Table 1) and these sequences, along with the *Saturnia jonasii* mitogenome sequence obtained in the present study, were compared for genome and gene size, A/T nucleotide composition, and the structure of the A + T-rich region. Furthermore, the genetic distance between species and within genera at each gene was calculated using unrooted pairwise distance using PAUP ver. 4.01b10 [33]. These values were plotted using boxplot implemented in JMP software ver. 11.1.1 (SAS Institute, Cary, N.C., USA). The nucleotide sequences of the PCGs were translated based on the invertebrate genetic code for mtDNA. Codon usage and nucleotide composition were determined using MEGA 6 [34]. For each gene, relative synonymous codon usage (RSCU) values were calculated to estimate the degree of nucleotide bias in each codon. Gene overlap and intergenic-spacer sequences were counted manually. The A/T content of each gene, whole genome, and each codon position of the PCGs were calculated using DNASTAR (Madison, USA) [35]. Composition skew analysis, which measures the relative number of As to Ts [AT skew = (A − T)/(A + T)] and Gs to Cs (GC skew = [(G − C)/(G + C)]), was carried out to determine the base composition of nucleotide sequences [36]. The sequence data of *Saturnia jonasii* have been deposited in the GenBank database with the accession no. MF346379.

2.4. Phylogenetic analysis

Each PCG and rRNA was aligned using RevTrans ver. 2.0 [37] for phylogenetic reconstruction of Bombycoidea. The well-aligned blocks were selected using GBlocks 0.91b software [38] and concatenated to generate PCG and rRNA alignment. For phylogenetic analysis, we tested various partition strategies in the final dataset (13,014 bp including gaps): unpartitioned (NP), partitioned into PCGs vs. rRNA genes (2P), four partitions based on PCG codon positions plus RNA genes (4P), or 14 partitions based on each PCG plus RNA genes (14P). The GTR + GAMMA + I model was chosen [39], using Modeltest ver. 3.7 [40], for all analyses. Bayesian inference (BI) and maximum-likelihood (ML) methods were conducted using MrBayes ver. 3.2.6 [41] and RAxML-HPC2 ver. 8.0.24 [42], respectively, which are implemented in the CIPRES Portal ver. 3.1 [43]. For the BI analysis using Markov Chains