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The complete mitochondrial genome of the oriental fruit moth *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae)

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Abstract The oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) currently is one of the economically most destructive pest species of stone and pome fruits worldwide. Here we sequenced the complete mitochondrial genome of this pest. This genome is 15,776 bp long, with an A + T content of 81.24%, containing 37 typical animal mitochondrial genes and an A + T-rich region. All gene are arranged as hypothesized ancestral gene order of insects except for *trnM*, which was shuffled from 3′ downstream of *trnQ* to 5′ upstream of *trnI*. *cox1* gene uses unusual CGA start codon, as that in all other sequenced lepidopteran mitochondrial genome. The secondary structures for the two rRNA genes were predicted. All helices typically present in insect mitochondrial rRNA genes are generated. A microsatellite sequence was inserted into the region of H2347 in *rrnL* in *G. molesta* and two other sequenced tortricid mitochondrial genomes, indicating that the insertion event in this helix might occurred anciently in family Tortricidae. All of the 22 typical animal tRNA genes have a typical cloverleaf structure except for *trnS2*, in which the D-stem pairings in the DHU arm are absent. An intergenic sequence is present between *trnQ* and *nad2* as well as in other sequenced lepidopteran mitochondrial genomes, which was presumed to be a remnant of *trnM* gene and its boundary sequences after the duplication of *trnM* to the upstream of *trnI* in Lepidoptera. The A + T-rich region is 836 bp, containing six repeat sequences of "TTATTATTATTATTAAATA (G)TTT."

Keywords Oriental fruit moth · Mitochondrial DNA · Gene rearrangement · Secondary structure · Intergenic region

Introduction

The oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), originated from East Asia, currently is one of the economically most destructive pest species of stone and pome fruits worldwide [1, 2]. *G. molesta* larvae bore in fruits, causing direct damage, or feed on twigs, causing shoot dieback. Management of this pest is mainly based on the use of the insecticides and pheromone-based mating disruption [3]. In addition to controlling methods, recently, ecological strategies and evolutionary patterns were studied, that might facilitate the managements of this pest. Molecular markers, i.e. amplified fragment length polymorphism (AFLP) and microsatellite (SSR) have been used to investigate the population genetic structure of *G. molesta* [4, 5], however, both are length-based markers from nuclear genome.

Insect mitochondrial genomes are about 16 Kb in size with 37 genes, including 13 protein-coding genes, two ribosomal RNA genes (large and small ribosomal RNAs), and 22 tRNA genes [6]. Additionally, an A + T-rich region is present, functioning on the regulation of transcription and replication [7]. Mitochondrial genomes contain abundant molecular markers, such as sequences, gene arrangement patterns and RNA secondary structures, which

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were frequently used for studies of population genetics, species identification, and phylogeny at different hierarchical levels [8, 9].

Presently, twenty-six complete or nearly complete mitochondrial genomes sequences are available in GenBank for lepidopteran species. However, the number of sequenced lepidopteran mitochondrial genomes is very limited relative to the species-richness of Lepidoptera.

In this study, we describe the complete mitochondrial genome sequence of the oriental fruit moth, *G. molesta*, and compare its features with other available lepidopteran mitochondrial genomes.

Materials and methods

Insects and DNA extraction

Grapholita molesta larvae were collected on the peach trees and kept in absolute alcohol at −80°. Total genomic DNA was extracted from individual larva using a DNeasy tissue kit (Qiagen, Hilden, Germany) following manufacturer protocols.

PCR amplification and sequencing

The *G. molesta* mitochondrial genome was amplified through nine overlapping fragments by PCR amplification using modified universal primers [10, 11] according to the determined lepidopteran mitochondrial genome sequences and specific primers designed in this study.

PCRs were done using Takara LA *Taq* (Takara Biomedical, Japan) under the following conditions: initial denaturation for 2 min at 94° followed by 35 cycles of 10 s at 96°, 15 s at 45–55°, and 1–4 min at 60° and a subsequent final extension for 8 min at 60°. PCR components were added as recommended by Takara LA *Taq*, the manufacturer. PCR products were sequenced directly by primer walking from both directions after purification. Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and run on an ABI 3730 capillary sequencer.

Genome annotation and secondary structure prediction

tRNA genes were initially identified using the tRNAscan-SE search server with default parameters [12]. Sequences longer than 100 bp between the identified tRNA genes were used as queries in Blast searches in GenBank for identification of protein-coding and rRNA genes. Nucleotide sequences of protein-coding gene were translated using the invertebrate mitochondrial genetic code. The exact initiation and termination codons were identified in ClustalX version 2.0 [13] using reference sequences from other insects. The stop codon of these genes was inferred to be the first in-frame stop codon or, when necessary to avoid overlap with the downstream gene, an abbreviated stop codon corresponding well to the stop codon of other insect genes.

The secondary structure of large and small rRNAs (*rrnL* and *rrnS*) were derived from *Drosophila melanogaster* [14] and *Drosophila virilis* [15] with modifications made based

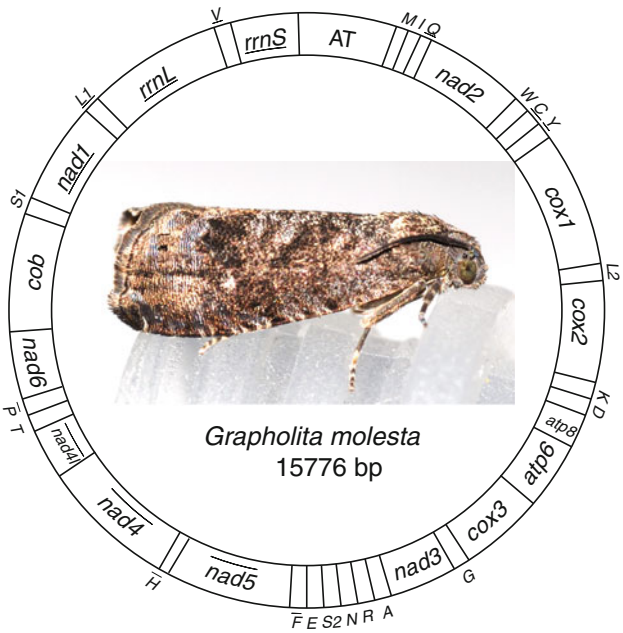

Fig. 1 Structure of *Grapholita molesta* mitochondrial genome. *cox1*, *cox2*, and *cox3* refer to the cytochrome oxidase subunits, *cob* refers to cytochrome b, *nad1–nad6* refer to NADH dehydrogenase components, and *rrnL* and *rrnS* refer to ribosomal RNAs. Transfer RNA genes are denoted by one letter symbol according to the IPUC-IUB single-letter amino acid codes. L1, L2, S1 and S2 denote tRNALeu$^{(CUN)}$, tRNALeu$^{(UUR)}$, tRNASer$^{(AGY)}$ and tRNASer$^{(UCN)}$, respectively. AT indicates A + T-rich region. Gene names with *lines* indicate that the genes are coded on the minority strand while those without *lines* are on the majority strand

on other predicted mitochondrial rRNA secondary structure [16]. Helix numbering follows the convention established at the CRW site [14] and *Apis mellifera* rRNA secondary structure [16] with minor modification. All structures were drawn in XRNA (developed by B. Weiser and available at http://rna.ucsc.edu/rnacenter/xrna/xrna.html).

All tRNA secondary structures were predicted using the tRNAscan-SE search server except for *trnS2*, which was predicted manually according to that predicted in other insects.

Results and discussions

Genome structure and base composition

The complete mitochondrial genome of *G. molesta* is 15,776 bp long, containing 37 typical animal mitochondrial genes and an A + T-rich region (GenBank accession No. HQ116416). All gene are arranged as hypothesized ancestral gene order of insects except for *trnM* [17], which was shuffled from 3′ downstream of *trnQ* to 5′ upstream of *trnI* (Fig. 1). This arrangement pattern of *trnM* was presumed be an synapomorphic character in lepidopteran mitochondrial genomes [18, 19], however, the presently sequenced lepidopteran mitochondrial genomes used for comparative studies are all from the lineage of Ditrysia, representing limited group of Lepidoptera. Thus, more mitochondrial genomes are needed from other groups to prove the universality of *trnM* shuffling in this Order.

The entire mitochondrial genome of *G. molesta* is biased to use A and T, with an A + T content of 81.24%, as that of other insects. The AT skew for the majority strand is −0.064, while GC skew is −0.175, referring to the occurrence of more Ts than As and more Cs than Gs.

Protein-coding genes

All protein-coding genes start with ATN codons (one with ATA, three with ATT, one with ATC, and seven with ATG) except for *cox1* (Table S1). In *G. molesta* mitochondrial genome, *cox1* gene uses unusual CGA start codon, as that in all other sequenced lepidopteran mitochondrial genome. Annotated *cox1* gene in *G. molesta* could be aligned well with its orthologous genes in other lepidopteran mitochondrial genome, confirmed the atypical start site of *cox1* in Lepidoptera.

Seven of 13 protein-coding genes in *G. molesta* harbor the usual termination codon TAA, but *cox1*, *cox2*, *cob*, *nad2*, *nad4* and *nad5* use the incomplete termination codon T (Table S1). The assignment of incomplete stop codon on these genes could avoid overlapping nucleotides between their adjacent genes. These incomplete stop codons are commonly found in metazoan mitochondrial genes [18, 20].

The relative synonymous codon usage was analyzed, indicating a biased usage of A and T nucleotides (Table S2). UUA(Leu), AUU(Ile), UUU(Phe), AUA(Met) were the most frequently used codons as in other insects [18, 21]. All protein-coding genes show more T than A, while genes coded on the majority strand show more C than G and genes coded on the minority strand show less C than G (Table S3). This is congruent with the observation of skew values in insect mitochondrial genomes [22].

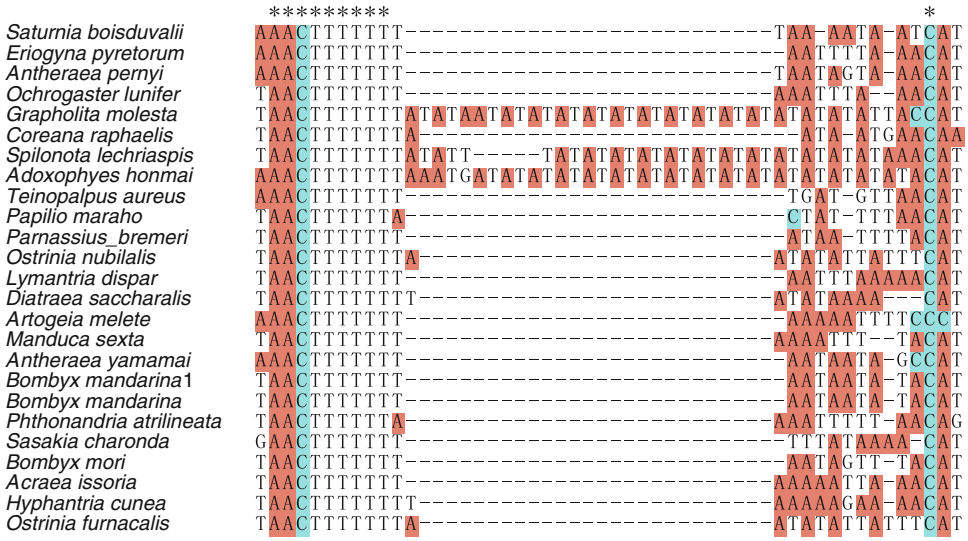

Fig. 2 Alignment of the homologous regions including Helix 2347 of *rrnL* in all sequenced lepidopteran mitochondrial genomes Sequences were downloaded from GenBank, and the accession numbers are as in Table 1

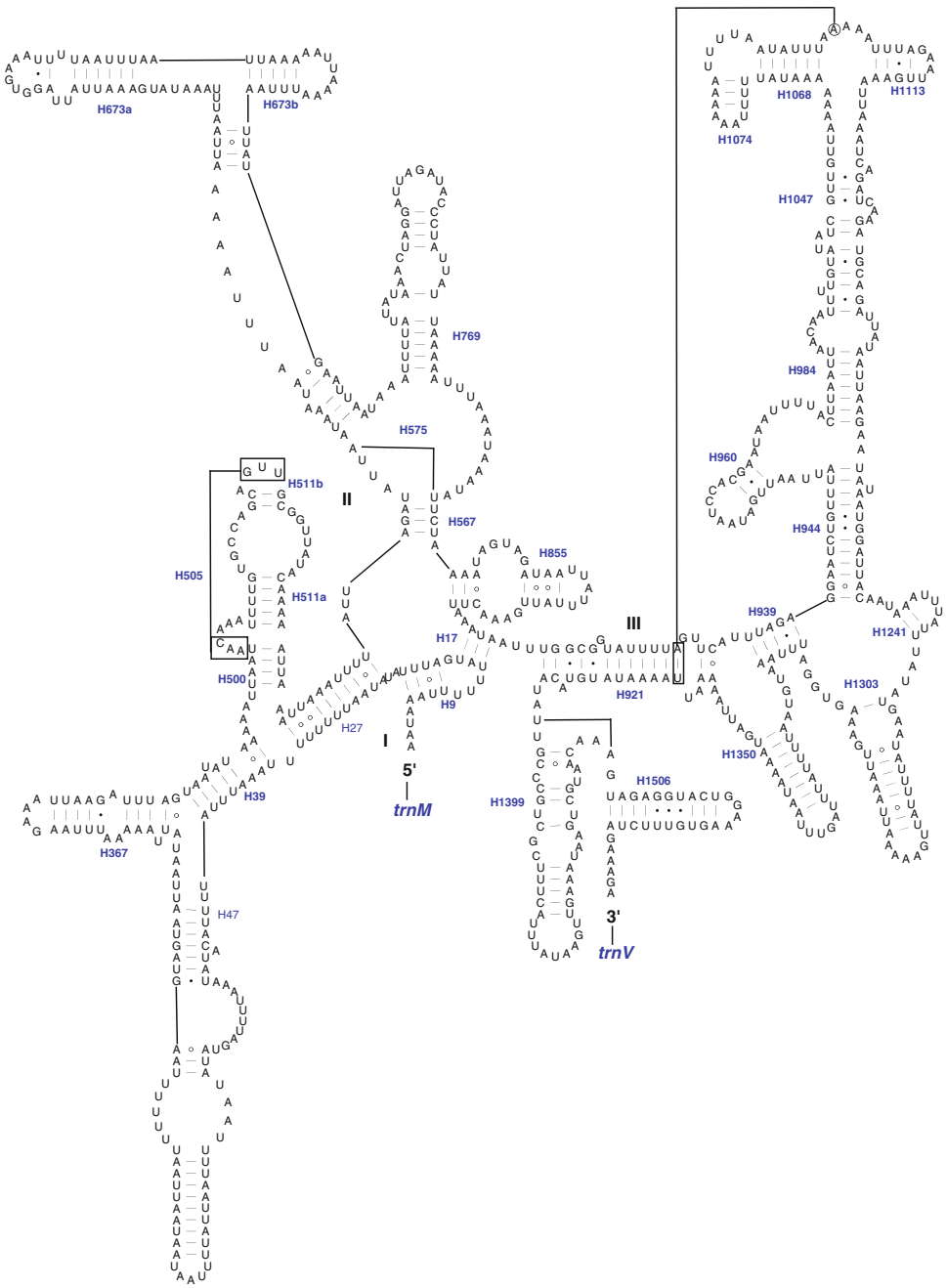

Fig. 3 Predicted *rrnS* secondary structure in *Grapholita molesta* mitochondrial genome. Tertiary interactions and base triples are shown connected by continuous lines. A 5′ half of *rrnL*; B 3′ half of *rrnL*. Base-pairing is indicated as follows: Watson–Crick pairs by *lines*, wobble GU pairs by *dots* and other noncanonical pairs by *circles*

rRNA structure

Both rRNA genes are present in *G. molesta* mitochondrial genome, located between *trnL1* and *trnV* for *rrnL* and between *trnV* and the A + T-rich region for *rrnS*. The length of the *rrnL* is 1382 bp, and the length of *rrnS* is 775 bp.

Both *rrnL* and *rrnS* conform to the secondary structure models proposed for these genes from other insects [20, 23, 24]. Forty-nine helices are present in *G. molesta rrnL* as in *M. sexta* [23], *D. melanogaster* [15] and *A. mellifera* [16], belonging to six domains (Fig. S1). The stem region of H991 was difficult to fold under the criteria of Watson–Crick pairs, and the structure of H991 with a large internal loop among H991, H1057 and H1087 is different from that of *M. sexta*. A 23 bp insertion was present in the loop region between H1664 and H1764 in *M. sexta*. In *G. molesta*, a microsatellite sequence of $(TA)_{12}$ was inserted into the loop region of H2347. Alignment of the homologous regions in other sequenced lepidopteran mitochondrial genomes showed that similar microsatellite sequence of $(TA)_{14}$ was also present in the stem region of H2347 in *Spilonota lechriaspis* [25] and *Adoxophyes honmai* (Lepidoptera: Tortricidae) [26] (Fig. 2), indicating

Table 1 The intergenic sequences between *trnQ* and *nad2* in all presently sequenced lepidopteran mitochondrial genomes

Species	Length (bp)	Sequence	GenBank accession no. and references
Teinopalpus aureus	65	ATATAAATAATGAATTTTTAATTCAAATTTTATTCCCCCTATTTTAAATTTTTTTTAATATTAAT	HM563681
Spilonota lechriaspis	47	ATTAAATGAATTTTCAATTCTAAAAGAAATTATTATTTCTATTTTAT	NC_014294 [25]
Saturnia boisduvalii	53	ATTTTAAATAGAGAATTTCAAATTCTTTTTAATTTATTATTAAATTATTTTAA	EF622227 [31]
Sasakia charonda	87	ATTTTTATAATATGAATTTAATTATATGTACATATATACATATATAATATTCACTTAAGATTTATTTCTTTATTTTAAATTTTATTT	NC_014224
Phthonandria atrilineata	63	ATATTTATATAAAGAATTTATATTTCTATTAAATTATTATAATTATTTTATATATTATACCAA	NC_010522 [32]
Parnassius bremeri	70	AAACTATTGTGCATCTTACACTAAAATATATTTATTGAATTTAAATAACAAAACTAACCCCCTATTTTAG	NC_014053
Papilio maraho	47	TGTAATTTTACACTGAATTTATAATTCAACTTAACCCCCATTTTTAA	NC_014055
Ostrinia nubilalis	62	AATAAATTAAAATATAAATTTAATTTTATATAAAATATTTTTTATTTTAAATTTTTTCCCCT	NC_003367 [33]
Ostrinia furnacalis	62	AATAAATTAAAATATAAATTTAATTTTATATAAAATATTTTTTATTTTAAATTTTTTCCCCT	NC_003368 [33]
Ochrogaster lunifer	72	TTATTAATAAAAATATAAATTAATTATTTAGATTAAATTCTAATAAAAATTATTAAATTATAATTATTTTTA	NC_011128 [34]
Manduca sexta	54	ATTTTATCAAATAGAAATCTTAATTTCTTAAATTACAATAAGTAATTATTTTAT	NC_010266 [23]
Lymantria dispar	47	ATTATTTAATCAATGAATTTATAATTCACCAAATAATTTTATCTTAA	FJ617240
Hyphantria cunea	50	ATTTCTATAATAATGATCTTAAATTCATAATAATTTTTTTTTTATTTTAA	GU592049 [18]
Eriogyna pyretorum	54	ATTTTCTATAAAGAATTTATAATTCTTTCAAATTTATTCATTAAATTATTTTAA	FJ685653 [35]
Diatraea saccharalis	55	AATATTTATATTAATATATTTATAATATACTTATTAATTTAATTTTTTATTTTAT	FJ240227 [36]
Coreana raphaelis	56	TTTTTAATTTAAAAAATAAACTTAAAATTTATTAATGAAATTTATTTCTTTTTTAT	NC_007976 [19]
Bombyx mori	65	ATTTAAATAATTAAAATAAAGAATTTATAATTCTATTAAATATATTTATATTTTTTTTTATTTTA	NC_002355
Bombyx mandarina	48	AATTTAAATAATTAAAATAAAGAATTTATAATTCTATTAAATATATTT	AY301620 [37]
Bombyx mandarina	47	ATTTAAATAATTAAAATAAAGAATTTATAATTCTATTAAATATATTT	NC_003395 [38]

Table 1 continued

Species	Length (bp)	Sequence	GenBank accession no. and references
Artogeia melete	48	TTTAAATAAATAGAACTTAAATTTCTTTTAAATTTTTTTTTATTTTAA	NC_010568 [39]
Antheraea yamamai	53	ATTTTTTAATAAAGAATTGATAATTCTTAGAAATTTATTTATAATTATTTTTG	EU726630 [40]
Antheraea pernyi	56	ATTTTTCTTAATAAAGAATTGATAATTCTTAAAAATTTATTTAATAATTATTTTTA	NC_004622 [41]
Adoxophyes honmai	64	TATTTTTAAAAAGAAATTTATAATTTCTAAAAGAATTTCATTTCTATTTTAAATTTTTTTTTTAT	NC_008141 [26]
Acraea issoria	51	ATTACCAAAATATGAATAAATTTCATTTTAGAATTATAATTCTTATTTTTA	NC_013604 [42]

Note: For unpublished mitochondrial genomes, only GenBank accession numbers are listed

that the insertion event in the region of H2347 in *rrnL* might be an synapomorphic character in family Tortricidae.

The secondary structure of *rrnS* contains 29 helices present in *Manduca sexta* [23] and *A. mellifera* [16], belonging to three domains (Fig. 3). The structures of Helix H47, H673, H1047, H1241 and H1303 are different from those in *M. sexta*. H47 has a small loop in *G. molesta* compared to that in *M. sexta*. This region was variable within species [16, 23, 24], and has been used to predict the phylogenetic relationships among subfamilies of Braconidae (Insecta: Hymenoptera) combined with H39 and H367 [9]. H673 in *G. molesta* was more similar to that in some species of Hymenoptera [20, 24] and Diptera [15] than in species of Lepidoptera [23, 27]. The region of H673 is long, which could yields multiple possible secondary structures. The presently predicted structures of rRNA are mainly based on sequence comparison and mathematical methods, so it is not clear which structures are utilized in situ. The region composed of H1047, H1068, H1074 and H1113 in *G. molesta* was different in length especially in loop regions from that in *M. sexta*, indicating it is another variable region in *rrnS* within species [16, 23].

tRNA structure

All of the 22 typical animal tRNA genes were present in *G. molesta* mitochondrial genome, ranging from 65 to 71 bp. All tRNA genes have a typical cloverleaf structure except for *trnS2* (Fig. S2). The D-stem pairings in the DHU arm are absent in *trnS2*, which has also been reported in other insects [24], and is common in Coleoptera [28]. The structure of *trnS2* could not be identified and folded using conventional tRNA search methods such as tRNAscan-SE. We found the location of *trnS2* by comparisons with those identified in other insects and then determined the exact boundaries according to the secondary structure folded manually. The anticodons for all tRNA genes are identical to their counterparts in most other published insect mitochondrial genomes.

In mitochondrial tRNA genes, noncanonical pairs were common in secondary structures. There are 16 wobble G–U pairs and four U–U pairs present in tRNA secondary structures in *G. molesta*.

Non-coding region

There are 14 non-coding regions ranging from 1 to 62 bp except for the A + T-rich region in *G. molesta* mitochondrial genome. A 62 bp intergenic sequence is present between *trnQ* and *nad2*, from where *trnM* was translocated to the upstream of *trnI*. In all other sequenced lepidopteran mitochondrial genomes, the same *trnM* rearrangement

event occurred with a similar intergenic sequence ranging from 47 to 87 bp left in this region (Table 1). Additionally, the length of this intergenic sequence covers that of typical tRNA genes, thus, we presume that this region might be a remnant of *trnM* gene and its boundary sequences after the duplication of *trnM* to the upstream of *trnI*.

Intergenic spacer region between *trnS1* and *nad1* may correspond to the binding site of mtTERM, a transcription attenuation factor [29], which was evidenced by a 7 bp motif (ATACTAA) conserved across Lepidoptera [23], 5 bp (TACTA) motif conserved across Coleoptera [28] and a 6 bp conserved motif (THACWW) in Hymenoptera [24]. The ATACTA motif is also present in *G. molesta* mitochondrial genome between *trnS1* and *nad1*.

The longest intergenic region in *G. molesta* is the A + T-rich region, between *rrnS* and *trnM*. The length of A + T-rich region is 836 bp, and the A + T content is 95.9%. This region usually contains replication origins in both vertebrates and invertebrates [7, 30]. The sequence of "TTATTATTATTATTAAATA(G)TTT" was repeated six times in the A + T-rich region in *G. molesta*. However, the set of elements that may function in the initiation of genome replication could not be identified [7].

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