Description of Nearly Completed Mitochondrial Genome Sequences of the Garden Chafer *Polyphylla laticollis manchurica*, Endangered in Korea (Insecta: Coleoptera)

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Abstract

In this study, we present the nearly complete mitogenome sequences of the garden chafer, *Polyphylla laticollis manchurica*, which is listed as an endangered species in Korea. The *P. l. manchurica* mitogenome, which includes unfinished whole A+T-rich region and a partial srRNA was 14,473-bp long, possessing typical sets of genes (13 PCGs, 22 tRNA genes, and 2 rRNA genes). Gene arrangement of the *P. l. manchurica* mitogenome was identical to the common one found in the majority of insects. The 5 bp-long motif sequence (TAGTA) that has been suggested to be the possible binding site for the transcription termination peptide for the major-strand was also found in the *P. l. manchurica* mitogenome between tRNA^{Ser}(UCN) and ND1. The start codon for COI gene and ATPase8 was designated as a typical TTG. All tRNAs of the *P. l. manchurica* showed a stable canonical clover-leaf structure of other mt tRNAs, except for tRNA^{Ser}(AGN), DHU arm of which could not form stable stem-loop structure. As has been previously determined, the high A/T content was unanimously observed in *P. l. manchurica* in terms of A/T bias in the third codon position (73.5%) compared with the first (66.4%) and second codon position (66.2%). The PCGs encoded in major-strands are slightly T-skewed, whereas those of the minor-strand are A-skewed, indicating strand asymmetry in nucleotide composition in the Coleoptera including *P. l. manchurica*.

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Int. J. Indust. Entomol. 27(1), 185-202 (2013)

Received : 00 A 2013
Accepted : 00 A 2013

Keywords:

Mitochondrial genome,
Polyphylla laticollis manchurica,
Garden chafer,
A/T bias

Introduction

Animal mitochondrial genomes (mitogenomes) are approximately 16~20 kbp, and are encoded with a remarkably

conserved set of 37 genes: 13 protein-coding genes (PCGs), two ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes, and one major non-coding sequence, which is termed the control region (Boore, 1999). This control region in insect instead is called

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as the A+T-rich region due to the high adenine and thymine (A/T) content, and in fact, this region contains the highest A/T content of any region of the mitogenomes in insects (Kim *et al.*, 2010).

The mitogenome information has greatly been devoted to our understanding of several fields of biology (*i.e.*, comparative and evolutionary genomics, molecular evolution, and phylogenetics). However, still newly sequenced insect mitogenome information provides us with new insights into genomic structures (Wan *et al.*, 2012) and the evolutionary relationships of several levels of taxonomic groups (Kim *et al.*, 2011; Cameron *et al.*, 2009), and gene arrangement (Wang *et al.*, 2013).

Up to now, more than 250 mitogenome sequences have been determined from a variety of insects, but this list includes only ~34 coleopteran species (<http://www.ncbi.nlm.nih.gov/genomes/ORGANELLES>). Considering that the suborder Polyphaga contains the vast majority of beetle diversity, with at least 300,000 described species (90% of the beetles) belonging to more than 100 families in four infraorders (Hammond, 1992), genomic information is extremely limited. In particular, the complete mitogenome sequence of the infraorder Scarabaeiformia in Polyphaga is available only for two species (Cameron *et al.*, 2009; Sheffield *et al.*, 2009). Recently, Kim *et al.* (2013a, 2013b) additionally reported two complete mitogenome sequences of Scarabaeiformia, the whiter-spotted flower chafer, *Protaetia brevitaris* (Scarabaeidae) and the two-spotted stag beetle, *M. blanchardi* (Lucanidae).

In this paper, we report the mitogenome sequence of *Polyphylla laticollis manchurica* to describe the genome via comparison to those of pre-existing coleopteran insects in terms of whole genome organization, arrangement, and the major characteristics of individual *genes*. *P. l. manchurica* is distributed throughout Korea, including Jeju Island, and also in Mongolia and Japan, as well as Eastern China (Won *et al.*, 1998). Due to the rarity the species is listed as an endangered species in Korea (Won *et al.*, 1998).

Materials and Methods

Specimen collection and genomic DNA extraction

The garden chafer, *Polyphylla laticollis manchurica* (Scarabaeiformia: Scarabaeidae), was collected in Yeongwol,

Gwangwondo-Province in Korea. *P. l. manchurica* is listed as a first degree endangered species in Korea, and thus, proper permission was obtained from relevant supervisory office before collection. Total genomic DNA was extracted using the WizardTM Genomic DNA Purification kit (Promega, USA) following the manufacturer's instruction.

PCR amplification, cloning, and sequencing

Three short fragments, corresponding each about 500~700 bp were amplified from three genes, such as COI (SF1), CytB (SF2), and srRNA (SF3) (Fig. 1). Primers for these

Fig. 1. Circular map of the mitochondrial genomes of *Polyphylla laticollis manchurica*. The abbreviations for the genes are as follows: COI, COII, and COIII refer to the cytochrome oxidase subunits, CytB refers to cytochrome B, ATP6 and ATP8 refer to subunits 6 and 8 of F₀ ATPase, and ND1 ~ 6 refer to components of NADH dehydrogenase. tRNAs are denoted as one-letter symbols consistent with the IUPAC-IUB single letter amino acid codes. The one-letter symbols L, L*, S and S* denote tRNA^{Leu}(CUN), tRNA^{Leu}(UUR), tRNA^{Ser}(AGN), and tRNA^{Ser}(UCN), respectively. Gene names that are not underlined indicate a clockwise transcriptional direction, whereas underlined genes indicate a counter-clockwise transcriptional direction. The *P. l. manchurica* mitogenome was amplified each from three short (SF1, SF2, and SF3) and three long (LF1, LF2, and LF3) overlapping fragments, shown as single lines within a circle. The unknown region that possibly contains partial srRNA and the A+T-rich region is shaded.

Table 1. List of primers used to amplify and sequence the mitochondrial genomes of *Polyphylla laticollis manchurica*

Fragment name	Primer name	Direction	Position	Sequence (5'-3')
Short fragments				
SF1	LCO1490	F	1423-1447	GGTCAACAAATCATAAAGATATTGG
	HCO2198	R	2375-2401	TAAACTTCAGGGTGACCAAAAAATCA
SF2	PBSF3F0720	F	6980-6999	CCTATATAACGAATATCTTG
	PBSF3R0720	R	7409-7428	AATGCAGGAATATTAAGTGC
SF3	PBSF5F	F	13946-13968	GACGGGCAATATGTGCATATTTT
	PBSF5R	R	14476-14497	TGCCAGCAATTGCGGTTATACA
Long fragments				
LF1*	PLCOIF3	F	1836-1861	TATTTTITAGCCTCCATCTTGCAGGAA
	PL8KbR1	R	10503-10526	AGCAATCAGCCATAGTTTACATCA
LF2*	PL8KbF3	F	10779-10801	ATTATGGGGCGGATTTCGAGTTG
	SF5R	R	14474-14495	TGCCAGCAATTGCGGTTATACA
LF3	SF5F3	F	14309-14330	ACATTTCATAATAAGGGTATC
	PLCOIR4	R	1615-1634	CCGAATCCCCGATTATAAT
Internal primers				
LF3	PLSF1F	F	8-25	TGTCTGAAAAAGAGTCAC
	PLSF1R	R	714-736	ATATGATTAATTGAGGAGTAAGC
	PLSF2F	F	561-582	TCCTTAATTATACTGACCTGGC
	PLSF2R	R	1109-1128	ATTTTAAAGGTGGTCTTGAG
	PLSF3F	F	995-1012	CAAAATATTCACCATAGC
	PLSF4R	R	1640-1660	CTAGTATTAGGGGTACTAGTC
	PLSRRNAF	F	14085-14108	ATCTATGCGCTGTACCTTGATCTG

*Sequencing was completed by shotgun method.

short fragments were designed via the alignment of several coleopteran mitogenomes sequenced in their entirety. These short fragments were amplified with AccuPower® PCR PreMix (Bioneer, Korea) using an initial denaturation at 94°C for 4 min, followed by 35 cycles of 30 s denaturation at 94°C, 40 sec annealing at 50-55°C, and a 60 sec extension at 72°C. The final extension step was continued for 8 min.

Using the sequence information obtained from the short fragments, three primer sets specific to each species were designed to amplify three long fragments (LF1 ~ LF3) which overlapped with the short fragments (SF1 ~ SF3). PCR cycles were as follows: denaturation for 2 min at 96°C, followed by 30 cycles of 10 sec at 98°C and 15 min at 58-65°C, and a

final 10 min extension at 72°C. In order to sequence the long fragments, both primer walking and shotgun approaches were used, because the success of sequencing varied depending on fragments. Nevertheless, both approaches were unsuccessful for LF3, and, thus, the partial LF3 that encompasses the 5'-end of the srRNA and the whole A+T-rich region was unfinished. We believe this may have happened because this region is exceptionally long, containing unexpectedly long repeat regions and a high A/T content, considering previous sequence results of other coleopteran species, such as *P. brevitarsis* and *M. blanchardi* (Kim *et al.*, 2013a, 2013b). Primer information for each short fragment, long fragment, and internal primers for primer walking are provided (Table 1).

For the primer walking method, internal primers were directly used to complete the sequences of the long fragment subsequent to purification with OIAquick PCR Purification Kit (Qiagen, USA). For the shotgun approach the long PCR fragments were subjected to shearing into 1~5 kb fragments (Gene Machine, USA) and were cloned into the pUC118 vector (Takara Biomedical, Japan). Each resultant plasmid DNA was isolated using a Wizard Plus SV Minipreps DNA Purification System (Promega, USA). DNA sequencing was conducted using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI PRISM® 3100 Genetic Analyzer (PE Applied Biosystems, USA).

Gene identification and structure

The boundary of individual thirteen mitochondrial protein-coding genes (PCGs) and individual two rRNAs were determined through the alignment of the homologous sequences of known full-length coleopteran mitochondrial genome sequences using the CLUSTAL X program (Thompson *et al.*, 1997). The nucleotide sequences of the PCGs were translated on the basis of the invertebrate mtDNA genetic code (<http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi?chapter=cgencodes>). The translated amino acid sequence was further utilized to identify and delimitate the 13 PCGs. The tRNA genes were identified by their predicted cloverleaf secondary structure and anticodon sequences using the tRNAscan-SE version 1.1 with the option of invertebrate codon predictors and a cove score cut off of 1 (Lowe and Eddy 1997). The tRNA^{Ser}(AGN) and tRNA^{Asn} were initially identified through alignment of the nucleotide sequences of the tRNA genes of known full-length coleopteran mtDNA sequences, and were further confirmed by hand-drawing their secondary cloverleaf structure by inspection of respective anticodon sequences. The sequence data determined in this study have been deposited to GenBank under the accession number KF544959.

Comparative mitochondrial gene analyses

We compared 35 species of coleopteran insects including the currently sequenced *P. l. manchurica*. Nucleotide composition

of each gene, whole genome, and each codon position of PCGs were calculated using DNASTAR (Burland, 2000). Frequencies of both codon and amino acid were calculated using SWAAP ver. 1.0.3 (<http://www.bacteriamuseum.org/SWAAP/SwaapPage.htm>). Gene overlap and intergenic-space sequences were hand-counted. Nucleotide composition, termed “compositional skew” was calculated for the PCGs between two strands, and the whole genome with the EditSeq program included in the Lasergene software package (www.dnastar.com) using the following formula proposed by Perna and Kocher (1995): GC-skew = (G-C)/(G+C) and AT-skew = (A-T)/(A+T), where C, G, A, and T are the frequencies of the four bases.

Results and discussion

General features

The *Polyphylla laticollis manchurica* mitogenome contains typical sets of genes, such as 13 PCGs, 22 tRNA genes, and 2 rRNA genes (Table 2). Typically, coleopteran insects contain one large non-coding region, named the A+T-rich region in insects, but we were unsuccessful in sequencing this region and the neighboring partial 5'-region of srRNA. In GenBank, a substantial number of incomplete mitogenome sequences lacking the A+T-rich region and neighboring sequences are registered and published as incomplete mitogenomes (Sheffield *et al.*, 2008). Possible reasons for such failure may be the presence of secondary structural motifs and the excessive length of the A+T region. Several coleopteran species have been reported to have an extraordinary long A+T-rich region and an extra non-coding sequences neighboring to the region. For example, the seven-spotted lady beetle has been reported to have a 4,469 bp-long A+T-rich region which is composed of a 2,214 bp of non-repeat region and a 2,255 bp repeat region (Kim *et al.*, 2012). Furthermore, the whiter-spotted flower chafer, *P. brevitarsis*, has a 5,646-bp long A+T-rich region which is composed of a 1,804 bp non-repeat region and a 3,841 bp repeat region (Kim *et al.*, 2013a). In addition, insect mitogenomes infrequently contain substantially longer intergenic spacer sequences which are composed of tandem repeat unit located neighboring to the A+T-rich region. For example, the Korean firefly, *Pyrocoelia rufa*, has a 1724-bp long intergenic spacer (Bae *et al.*, 2004). More extreme case is the two-spotted stag beetle,

Table 2. Summary of the mitochondrial genome of *Polyphylla laticollis manchurica*

Gene	D	Nucleotide number	Size (bp)	Anticodon	Codon		O/S
					Start	Stop	
tRNA ^{Ile}	F	1-63	63	GAT (29-31)			
tRNA ^{Gln}	R	61-129	69	TTG (97-99)			+3
tRNA ^{Met}	F	129-197	69	CAT (159-161)			+1
ND2	F	198-1205	1008		ATA	TAA	
tRNA ^{Trp}	F	1220-1286	67	TCA (1251-1253)			-15
tRNA ^{Cys}	R	1279-1339	61	GCA (1307-1309)			+8
tRNA ^{Tyr}	R	1341-1405	65	GTA (1372-1374)			-1
COI	F	1405-2943	1539		TTG	TAA	
tRNA ^{Leu(UUR)}	F	2939-3003	65	TAA (2968-2970)			+5
COII	F	3004-3688	685		ATA	T-tRNA	
tRNA ^{Lys}	F	3689-3759	71	CTT (3719-3721)			
tRNA ^{Asp}	F	3768-3834	67	GTC (3800-3802)			-0
ATPase8	F	3835-3990	156		TTG	TAG	
ATPase6	F	3987-4658	672		ATA	TAA	+4
COIII	F	4658-5445	788		ATG	TA-tRNA	+1
tRNA ^{Gly}	F	5445-5507	63	TCC (5475-5477)			
ND3	F	5508-5861	354		ATA	TAG	
tRNA ^{Ala}	F	5860-5923	64	TGC (5889-5891)			+2
tRNA ^{Arg}	F	5924-5989	66	TCG (5953-5955)			
tRNA ^{Asn}	F	5987-6051	65	GTT (6018-6020)			+3
tRNA ^{Ser(AGN)}	F	6052-6118	67	TCT (6088-6090)			
tRNA ^{Glu}	F	6119-6181	63	TTC (6149-6151)			
tRNA ^{Phe}	R	6180-6245	66	GAA (6211-6213)			+2
ND5	R	6246-7964	1719		ATT	TAA	
tRNA ^{His}	R	7965-8028	64	GTG (7996-7998)			
ND4	R	8028-9362	1335		ATG	TAA	+1
ND4L	R	9356-9646	291		ATG	TAA	+7
tRNA ^{Thr}	F	9655-9718	64	TGT (9686-9688)			
tRNA ^{Pro}	R	9719-9783	65	TGG (9750-9752)			
ND6	F	9785-10288	504		ATC	TAA	-1
CytB	F	10288-11430	1143		ATG	TAG	+1
tRNA ^{Ser(UCN)}	F	11429-11493	65	TGA (11458-11460)			+2
ND1	R	11514-12467	954		ATA	TAA	
tRNA ^{Leu(CUN)}	R	12528-12465	64	TAG (12499-12497)			
16S rRNA	R	12466-13814	1286				
tRNA ^{Val}	R	13815-13884	70	TAC (13851-13853)			
srRNA (incomplete)	R	13885-14473	589				
A+T-rich region		unfinished					

Direction (D) of gene is presented as F for forward and R for reverse direction.

tRNA abbreviations follow the IUPAC-IUB three-letter code.

O/S, number of overlapping(+)/intergenic space sequence(-).

Table 3 . Characteristics of the coleopteran mitochondrial genomes sequenced in their entirety and near entirety

Taxon	Size (bp)	A+T %	No. codons	PCGb		rRNA		srRNA		A+T-rich region		GenBank accession no.	References
				A/T %	Size (bp)	A/T %	Size (bp)	A/T %	Size (bp)	A/T %	Size (bp)		
Polyphaga													
<i>Apatides fortis</i>	16,171	67.2	3,684	66.1	1,276	70.8	736	69.2	1,626 d	65.9		FJ613421	Sheffield <i>et al.</i> (2009)
<i>Chrysochroa fulgidissima</i>	15,592	70.0	3,718	68.8	1,302	73.6	797	71.8	875	76.7		EU826485	Hong <i>et al.</i> (2009)
<i>Acmaeodera</i> sp.	16,217	68.4	3,706	66.2	1,294	74.3	774	69.6	1,533d	75.9		FJ613420	Sheffield <i>et al.</i> (2009)
<i>Cyphon</i> sp.	15,919	75.2	3,709	72.8	1,297	80.8	781	82.1	1,043	85.2		EU877949	Sheffield <i>et al.</i> (2008)
<i>Pyrophorus divergens</i>	16,120	69.4	3,707	67.5	1,269	72.8	805	72.4	1,470	75.0		EF398270	Arnoldi <i>et al.</i> (2007)
<i>Rhagophthalmus lufengensis</i>	15,982	79.6	3,697	78.1	1,270	82.2	786	83.3	1,367	86.8		DQ888607	Li <i>et al.</i> (2007)
<i>Rhagophthalmus ohbai</i>	15,704	79.1	3,692	77.5	1,277	82.4	785	83.5	1,044	85.3		AB267275	Li <i>et al.</i> (2007)
<i>Pyrocoelia rufa</i>	17,739	77.4	3,691	76.3	1,242	81.6	765	81.8	1,522	87.6		AF452048	Bae <i>et al.</i> (2004)
<i>Chauliognathus opacus</i>	14,893	76.9	3,667	76.1	1,265	80.8	858	79.6	313d	64.9		FJ613418	Sheffield <i>et al.</i> (2009)
<i>Protaetia brevitarsis</i>	20,319	74.5	3,702	73.4	1,238	77.9	785	76.4	5,654	75.3		KC775706	Kim <i>et al.</i> (2013a)
<i>Polyphylla laticollis manchurica</i>	14,473	69.8	3,704	68.7	1,286	75.5	589 c	70.2	- c	- c		KF544959	This study
<i>Rhopaea magnicornis</i>	17,522	75.5	3,698	74.4	1,279	78.7	800	75.6	2,827	78.2		FJ859903	Cameron <i>et al.</i> (2009)
<i>Metopodontus blanchardi</i>	21,628	67.0	3,711	65.6	1,268	71.4	747	69.2	3100	71.5		KF364622	Kim <i>et al.</i> (2013b)
<i>Lucanus mazama</i>	15,261	67.1	3,650	65.6	1,258	72.3	745	72.1	410d	63.2		FJ613419	Sheffield <i>et al.</i> (2009)
<i>Priasilpha obscura</i>	16,887	76.5	3,698	73.6	1,283	81.4	788	81.3	1,744c	87.0		EU877952	Sheffield <i>et al.</i> (2008)
<i>Cucujus clavipes</i>	15,642	76.3	3,691	74.8	1,279	81.2	781	78.6	1,005	82.1		GU176341	Song <i>et al.</i> (2010)
<i>Anoplophora glabripennis</i>	15,774	78.3	3,700	77.0	236	71.2	806	78.0	1,115	88.0		DQ768215	An <i>et al.</i> (Unpublished)
<i>Psacothea hilaris</i>	15,856	76.7	3,720	75.9	1,273	80.0	808	80.6	1,190	78.6		FJ424074	Kim <i>et al.</i> (2009)
<i>Crioceris duodecimpunctata</i>	15,880	76.9	3,694	75.2	1,277	82.5	804	79.6	1,220	83.3		AF467886	Stewart and Beckenbach (2003)
<i>Tribolium castaneum</i>	15,881	71.7	3,698	69.2	1,280	76.0	774	76.2	1,239	82.5		AJ312413	Friedrich and Muquim (2003)
<i>Adellum</i> sp.	16,449	72.7	3,692	70.4	1,280	78.8	759	75.9	1,157d	79.3		FJ613422	Sheffield <i>et al.</i> (2009)
<i>Mordella atrata</i>	15,540	71.9	3,681	70.6	1,280	75.1	758	73.2	989	77.0		FJ859904	Cameron <i>et al.</i> (2009)
<i>Chaetosoma scaritides</i>	15,511	79.1	3,685	77.3	1,282	82.4	779	82.2	862d	91.0		EU877951	Sheffield <i>et al.</i> (2008)

Table 3 . Continued

Taxon	Size (bp)	A+T %	No. codons ^a	PCGb	IrRNA		srRNA		A+T-rich region		GenBank accession no.	References
					Size (bp)	A/T %	Size (bp)	A/T %	Size (bp)	A/T %		
<i>Naupactus xanthographus</i>	15,026	76.9	3,704	75.7	1,298	81.1	894	78.5	216	80.1	GU176345	Song et al. (2010)
<i>Sphenophorus</i> sp.	15,052	76.3	3,705	74.9	1,294	81.2	787	78.4	374	85.3	GU176342	Song et al. (2010)
<i>Euspilotus scissus</i>	14,763	73.1	3,631	71.7	1,273	78.4	763	76.4	276	78.3	GU176344	Song et al. (2010)
<i>Necrophila americana</i>	16,902	74.7	3,698	73.2	1,293	80.4	792	74.4	2,196	79.0	GU176343	Song et al. (2010)
<i>Tropisternus</i> sp.	16,372	74.4	3,698	72.5	1,289	79.8	780	75.3	1,705	81.1	GU176339	Song et al. (2010)
Myxophaga												
<i>Sphaerius</i> sp.	15,735	80.8	3,709	79.8	1,315	84.1	784	84.1	953c	89.6	EU877950	Sheffield et al. (2008)
<i>Hydroscapha granulum</i>	15,975	77.2	3,732	75.5	1,339	83.5	789	79.2	1,144	86.3	AM493667	Pons et al. (2010)
Adephaga												
<i>Trachypachus holmbergi</i>	15,991	79.5	3,726	78.4	1,321	81.9	788	82.5	851c	84.9	EU877954	Sheffield et al. (2008)
<i>Calosoma</i> sp.	16,462	78.5	3,725	76.7	1,332	81.3	798	80.5	1,482	87.0	GU176340	Song et al. (2010)
<i>Aspidytes niobe</i>	14,257	79.0	3,729	78.4	1,317	82.5	444	77.7	- c	- c	AM493668	Pons et al. (2010)
<i>Macrogyrus oblongus</i>	16,643	78.0	3,713	75.26	1,312	83.08	791	81.67	1,808	88.83	FJ859901	Cameron et al. (2009)
Archostemata												
<i>Tetraphalerus bruchi</i>	15,689	70.0	3,705	65.8	1,322	68.1	791	64.2	1,000 d	78.4	EU877953	Sheffield et al. (2008)

^aTermination codons excluded in total codon count. ^bProtein coding genes. ^cIncomplete sequence. ^dNo annotation available from GenBank.

	ND1 →	tRNA ^{Ser} (UCN) →
Polyphaga		
<i>Apatides fortis</i>	<u>CTTATTTAGTTA</u> CAAAATTT	TAGTATTAAGT
<i>Chrysochroa fulgidissima</i>	<u>TTGTGTTAGGCA</u> ATAAAATTT	TAGTATAAGTT
<i>Acmaeodera</i> sp.	<u>TACAGTTAACTA</u> ACAAAATTT	TAGTACTTAAG
<i>Cyphon</i> sp.	<u>ATTATTTAGTTG</u> ATTAAATTT	TAGTAATAAGT
<i>Pyrophorus divergens</i>	<u>TTTGTTTAGTTA</u> ATTAAATTT	TAGTATAAGTT
<i>Rhagophthalmus lufengensis</i>	<u>ATTATTTAGTTA</u> ATAAAATTT	TAGTATAAAG
<i>Rhagophthalmus ohbai</i>	<u>ATTATTTAGTTA</u> ATAAAATTT	TAGTATTAAGT
<i>Pyrocoelia rufa</i>	<u>ATAATTTAGTTA</u> ATTAAATTT	TAGTAAAGTTA
<i>Chauliognathus opacus</i>	<u>TTAATTTAGTCA</u> ATAAAATTT	TAGTAAAGTTA
<i>Protaetia brevitarsis</i>	<u>TTTATTTAATGG</u> ATTAAATTT	TAGTATATTTA
<i>Polyphylla laticollis manchurica</i>	<u>TTGATTTAATGC</u>ATGAATTTT	<u>TAGTAGTCTAA</u>
<i>Rhopaea magnicornis</i>	<u>ATAATATAATGT</u> ATAAAATTT	TAGTAAAAAAA
<i>Metopodontus blanchardi</i>	<u>CTAATTTAGTGA</u> ATAAAATTT	TAGTAGTAAGC
<i>Lucanus mazama</i>	<u>TCTATTTAGTGA</u> ATAAAAAAT	TAGTAAACAGG
<i>Priasilpha obscura</i>	<u>TTTATTTAGTCA</u> ATAAAATTT	TAGTACAAGTT
<i>Cucujus clavipes</i>	<u>ATGGTTTAGTTA</u> ATTAAATTT	TAGTATAAGTC
<i>Anoplophora glabripennis</i>	<u>TTACTTTAGTTA</u> ATAAAATTT	TAGTATAAGTT
<i>Psacotheta hilaris</i>	<u>TTGCTTTAGTTA</u> ATAAAATTT	TAGTATAAGTT
<i>Crioceris duodecimpunctata</i>	<u>TTTATTTAGTTA</u> ATAAAATTT	TAGTACAAATT
<i>Tribolium castaneum</i>	<u>TTAATTTAGTTA</u> ATAAAATTT	TAGTATAAAGT
<i>Adelium</i> sp.	<u>TTATTGTAGTTA</u> ATAAAATTT	TAGTATAAAGT
<i>Mordella atrata</i>	<u>TTATTTTAGTTA</u> AGTTTTTTT	TAGTAAAAACT
<i>Chaetosoma scaritides</i>	<u>TTAAATTAGTTA</u> ATAAAATTT	TAGTATAAGCC
<i>Naupactus xanthographus</i>	<u>AATATTTAGTTA</u> ATTATTTT	TAGTACGTTTA
<i>Sphenophorus</i> sp.	<u>ATTTTATACTTA</u> ATTATTTT	TAGTACTAAAG
<i>Euspilotus scissus</i>	<u>TTGGCTTAGTTA</u> ATAAAATTT	TAGTATAAGTT
<i>Necrophila americana</i>	<u>TTGATTTAGTTA</u> ATAAAATTT	TAGTAGTAAGT
<i>Tropisternus</i> sp.	<u>ATTATTTAGTTA</u> ATAAAATTT	TAGTATAAGTT
Myxophaga		
<i>Sphaerius</i> sp.	<u>ATTATATAATGA</u> AATATTTT	TAGTAATAAAT
<i>Hydroscapha granulum</i>	<u>TTAATTTAGTGA</u> ATTATTTT	TAGTAATTAAA
Adephaga		
<i>Trachypachus holmbergi</i>	<u>TTAATTTAGTGA</u> ATTATTTT	TAGTAAAAAGT
<i>Calosoma</i> sp.	<u>TTAATTTAGTGA</u> ATTATTTT	TAGTAAAGTTA
<i>Aspidytes niobe</i>	<u>TTAATTTAGTCA</u> ATTATTTT	TAGTAAAAAAT
<i>Macrogyrus oblongus</i>	<u>TTGATTTAGTGA</u> ATTATTTT	TAGTAAAGTTA
Archostemata		
<i>Tetraphalerus bruchi</i>	<u>CGCTTTAAGTTA</u> ATTATCTA	TAGTATAGGTT

Fig. 2. Alignment of the internal spacer region located between ND1 and tRNA^{Ser}(UCN) from coleopteran species, including *Polyphylla laticollis manchurica*. The boxed nucleotides indicate the conserved pentanucleotide region (TAGTA) detected in coleopteran insects. Underlined and dotted nucleotides, respectively, indicate the adjacent partial sequences of the ND1 gene and tRNA^{Ser}(UCN) gene. The arrows indicate the transcriptional direction.

which contains a 4,051-bp long, large non-coding sequence located between tRNA^{Ile} and tRNA^{Gln}, along with a 3,100-bp long A+T-rich region, resulting in a genome size of 21,628 bp, which is at least 5 kb larger than typical animal mitogenomes (Kim *et al.*, 2013b). Considering these, it would be a reasonable speculation that the unfinished region is unusually large. The secondary structural motifs in the *P. l. manchurica* mitogenome might be another source that prevented the polymerases correctly passing across this region, resulting in casual amplification and making subsequent sequencing impossible (Carapelli *et al.*, 2006). Several coleopteran mitogenomes which have extraordinary long A+T-rich region also have several long repeat sequences (Kim *et al.*, 2012; 2013a, 2013b). These repeat sequences can easily form stem-and-loop structures (Zhang and Hewitt, 1997; Kim *et al.*, 2007), and thus, the unfinished A+T-rich region, along with the neighboring region of the *P. l. manchurica* mitogenome is highly likely to have such motifs. Nevertheless, this failure was not fully understood, considering our previous success in sequencing this region from several coleopteran insects (Kim *et al.*, 2012, 2013a, 2013b).

The *P. l. manchurica* mitogenome has an identical gene order that has been found in the majority of insect species (Boore, 1998). Among 35 complete or nearly complete coleopteran mitogenomes (Table 3), only *Tribolium castaneum* differs from the most common type, by the movement of the tRNA^{Glu} to a position 3'-downstream of tRNA^{Phe}, thus resulting in an order of tRNA^{Phe} and tRNA^{Glu}, rather than the order tRNA^{Glu} and tRNA^{Phe} (Friedrich and Muqim 2003).

Overlapping and intergenic spacer sequences

The *P. l. manchurica* mitochondrial genes harbor a total of 47 bp of intergenic spacer sequences, which are spread over six regions, ranging in size from 1 to 20 bp, with the longest being located between tRNA^{Ser}(UCN) and ND1 (Table 2). Similar sized intergenic spacer sequences are found in the majority of sequenced coleopteran insects (16 ~ 22 bp), except for those of *Rhagophthalmus lufengensis* and *R. ohbai*, which are only 5-bp long (Li *et al.*, 2007). Previously, it has been shown that the region contains a 5 bp-long motif sequence (TAGTA) in the coleopteran insects (Sheffield *et al.*, 2008). Our additional analysis, including all available coleopteran species and *P. l. manchurica*, consistently revealed a motif sequence in the region (Fig. 2). This 5-bp

consensus sequence was suggested to be the possible binding site for mtTERM, the transcription termination peptide, with the consideration that the intergenic spacer sequence is detected at the end site of the major-strand coding region in the circular mtDNA (Taanman, 1999).

Next longer intergenic spacer sequence is located between ND2 and tRNA^{Tyr} as 14 bp (Table 2). A search on this region from other coleopteran insects has shown fluctuating length: only a few spacer sequences in most species (e.g., 4 bp in *Pyrophorus divergens*; Arnoldi *et al.*, 2007), abuttal between the two genes (e.g., *Psacotha hilaris*; Kim *et al.*, 2009), and overlapping between the two genes (2 bp in *Tribolium castaneum*; Friedrich and Muqim, 2003). An exceptional case is that of *P. rufa*, which has a 1,724-bp long tandem repeat unit between the two genes (Bae *et al.*, 2004). The *P. l. manchurica* mitochondrial genes overlap in a total of 33 bp at 12 locations, with the longest overlap measuring 8 bp, and located between tRNA^{Tyr} and tRNA^{Cys}. Similarly-sized overlapping sequences are also detected in several coleopteran insects (e.g., *P. brevitarsis*; Kim *et al.*, 2013a; *Metopodotus blanchardi*; Kim *et al.*, 2013b), but abuttal (*P. rufa*; Bae *et al.*, 2004) and overlapping regions (8 bp in *Pyrophorus divergens*; Arnoldi *et al.*, 2007) are also found between the two tRNAs.

Start and stop codon

The *P. l. manchurica* mitogenomes harbors 3,704 codons, excluding termination codons, and this number is identical to those of *Naupactus xanthographus* (Song *et al.*, 2010) and most similar to those of *Sphenophorus* sp. (3,705 codons; Song *et al.*, 2010) and *Tetraphalerus bruch* (3705 codons; Sheffield *et al.*, 2008).

All PCGs, with the exception of COI and ATPase8 of the *P. l. manchurica* mitogenomes have the typical ATN codon (Table 2). The initiation site for the COI gene and the precedent tRNA^{Tyr} does not harbor the typical start codon, except for the infrequent, but typical invertebrate start codon TTG that is located at the beginning region of the COI, overlapping one nucleotide with the 5'-end of the tRNA^{Tyr} (Fig. 3). Thus, we designated the TTA as the COI start codon. Only a few other sequenced coleopteran insects have a typical ATN start codon, but these are all located inside neighboring tRNA^{Tyr} (Fig. 3). Thus, Sheffield *et al.* (2008) previously suggested that start codon for COI genes should be chosen to minimize

	← tRNA ^{Tyr}	COI →							
Polyphaga									
<i>Apatides fortis</i>	ataaatttacagtttaccgcttaaaactcagccaccttaacg	<u>AAT</u>	AAA	TGG	CTA	NKWL			
<i>Chrysocroa fulgidissima</i>	gtgagaaaatttacaatttaccgcctacctcagccgcccc	<u>ACG</u>	AAT	AAA	TGA	CTT	TNKWL		
<i>Acmaeodera</i> sp.	tagatttacaatctaccgcctaaaactcggccatctcaacg	<u>AAT</u>	AAA	TGG	CTA	NKWL			
<i>Cyphon</i> sp.	ataaatttacagtttattgctctatactcagccatctcgccg	<u>AAT</u>	AAG	TGG	CTC	NKWL			
<i>Pyrophorus divergens</i>	gaataaaatttacaatttaccgctataaactcagcc <u>ATC</u> TCA	CTG	AAC	AAA	TGA	TTA	MSLNKWL		
<i>Rhagophthalmus lufengensis</i>	taaataaaatttacaatttattgcttaaaactcagccacttta	<u>CTA</u>	AAT	AAA	TGA	TTA	LNKWL		
<i>Rhagophthalmus ohbai</i>	taaataaaatttacaatttattgcttaaaactcagccacttta	<u>CTA</u>	AAT	AAA	TGA	TTA	LNKWL		
<i>Pyrocoelia rufa</i>	ataaataaaatttacaatttattgcttaaaactcagccacttta	<u>CTA</u>	AAT	AAA	TGA	TTT	MLLNKWF		
<i>Chauiognathus opacus</i>	taaattttacaatttattgcttaaaactcagccacttta	<u>AAC</u>	AAA	TGA	TTA	NKWL			
<i>Protaetia brevitarsis</i>	gtaaatttacagtttaccgcttaagctcagccactttttcg	<u>AAC</u>	AAA	TGA	TTA	NKWL			
<i>Polyphylla laticollis manchurica</i>	<u>tgtaaaatttacaatttaccgcctaaaactcggccattttac</u>	<u>TTG</u>	AAG	AAA	TGA	TTA	LKKWL		
<i>Rhopaea magnicornis</i>	gtaaatttacagtttaccgcttaaatctcgccactttcactg	<u>AAC</u>	AAA	TGA	TTA	NKWL			
<i>Metopodontus blanchardi</i>	ataagttttacagcttaccgcttctctcagccattttaccg	<u>AAC</u>	AAG	TGA	TTA	NKWL			
<i>Lucanus mazama</i>	aataagttttacagcttaccgcttaaatctcagccactttgccc	<u>AAT</u>	AAA	TGA	TTG	NKWL			
<i>Priasilpha obscura</i>	aaaatttaaaatccctaaatttttaatttttaaccaattttccg	<u>AAC</u>	AAG	TGA	CTA	NKWL			
<i>Cucujus clavipes</i>	taaattttacaatttattgcttaaaactcagccattttaccg	<u>AAC</u>	AAA	TGA	TTA	NKWL			
<i>Anoplophora glabripennis</i>	tacgtaaatttacaatttactacctaattctcggtc <u>ATT</u> TTA	CCG	AAT	AAG	TGA	TTA	MLPNKWL		
<i>Psacotheca hilaris</i>	taggttaaaatttaccgcttactcctaaactcagcc <u>ATT</u> TTA	CCG	AAC	AAA	TGA	TTA	MLPNKWL		
<i>Crioceris duodecimpunctata</i>	atgcataaaatttaccgcttattgctttttatcagcc <u>ATT</u> TCA	CCG	AAT	AAA	TGG	CTG	MSPNKWF		
<i>*Tribolium castaneum</i>	tctgtaaatttaccgcttaaaTCTCAGGCCACTTCAACTAAA	CTG	AAT	AAA	TGA	TTA	KLNKWL		
<i>Adelium</i> sp.	ataaatttacaatttattgcttgaatctcgccatttataccg	<u>AAC</u>	AAA	TGA	TTA	NKWL			
<i>Mordella atrata</i>	ataaatttacaatttaccgcttaaatctcgccactctgccc	<u>AAC</u>	AAA	TGA	CTT	NKWL			
<i>Chaetosoma scaritides</i>	ataaatttacaatttattgcttatactcagccattttaccg	<u>AAC</u>	AAA	TGA	TTA	NKWL			
<i>Naupactus xanthographus</i>	ataaatttaccgcttattacattttttcagccattttaccg	AAT	AAA	TGA	TTT	NKWF			
<i>Sphenophorus</i> sp.	acaattttatcgcttaattctcagccactttacc <u>ATA</u> CATTTA	CCG	AAT	AAA	TGA	TTA	MHLPNKWL		
<i>Euspilotes scissus</i>	taaattttacaatttattgcttgaatctcagccattttaccg	AAT	AAA	TGA	CTT	NKWL			
<i>Necrophila americana</i>	taaattttacaatttattgcttgaatctcagccactttaccg	AAT	AAA	TGA	TTA	NKWL			
<i>Tropisternus</i> sp.	ataaatttaccgcttattgcttgaatctcagccactttaccg	AAT	AAA	TGA	TTA	NKWL			
Myxophaga									
<i>Sphaerius</i> sp.	ttcagtttacagtttattgcttaaaatttaccgctttttaccg	<u>CAA</u>	AAA	TGA	TTA	QKWL			
<i>*Hydroscapha granulum</i>	ataaatttaccgcttattacgcttagctcggccattTTACCG	CGA	CGA	TGG	ATA	TTC	LPRRW		
Adephaga									
<i>Trachypachus holmbergi</i>	aattttacagtttattgcttaaatattctcagccattttaccg	<u>CGA</u>	CAA	TGA	TTA	RQWL			
<i>Calosoma</i> sp.	aaattttacagtttattacattattctcagccattttaccg	<u>CGA</u>	TGG	ATA	TTC	RRWM			
<i>Aspidytes niobe</i>	aaataaaatttaccgcttattgcttaaatctcagccattttta	<u>CCG</u>	<u>CGA</u>	CAA	TGA	TTA	PRQWL		
<i>Macrogyrus oblongus</i>	agttttactgcttaaatctcagccattttaccct <u>TTT</u> CTGTAC	CCG	CAA	CAA	TGA	TTA	FLYPQQWL		
Archostemata									
<i>Tetraphalerus bruchi</i>	atagaatttacagttctatcacctaaactcagccattttaccg	<u>CAA</u>	CGA	TGA	TTG	QRWL			

Fig. 3. Alignment of the initiation context of COI genes of coleopteran insects including that of *Polyphylla laticollis manchurica*. The first four to seven codons are shown in uppercase letters on the right-hand side of the figure. Underlined nucleotides indicate the adjacent partial sequence of tRNA^{Tyr}. Arrows indicate the direction of transcription. Boxed nucleotides indicate currently known translation initiators for the COI of coleopteran. The start codon for *P. l. manchurica* was designated respectively as TTG.

intergenic spacer sequences and gene overlaps. In this regard, they proposed asparagines (AAT or AAC) as the start codon for COI gene, because those are the first non-overlapping in-frame codons, and found at the corresponding position in all sequenced Polyphaga in Coleoptera (Fig. 3; Sheffield *et al.*, 2008). Furthermore, they hypothesized that asparagines may function as a molecular synapomorphy for Polyphaga (Sheffield *et al.*, 2008). However, our newly sequenced *P. l. manchurica*, which belongs to Polyphaga, instead have AAG (Lysine) at the corresponding position and have the typical TTG precedent to AAG. Thus, our *P. l. manchurica* is the only example that does not follow the unanimous COI start codon in Polyphaga. The *P. l. manchurica* ATPase8 also has the TTG start codon (Fig. 4). Previously, the TTG codon was found in ND1 gene for coleopteran *P. rufa* (Bae *et al.*, 2004) and dipteran *Anopheles quadrimaculatus* (Mitchell *et al.*, 1993),

and COI gene for lepidopteran *Caligula boisduvalii* (Hong *et al.*, 2008), but has never been found for the start codon for ATPase8 in Coleoptera (data not shown).

Eleven of the 13 PCGs have a complete termination codon of TAA or TAG, but the COII and COIII genes harbor the incomplete termination codon, T (Table 2). The most common interpretation of this phenomenon is that TAA termini are created via post-transcriptional polyadenylation (Ojala *et al.*, 1981).

tRNA and rRNA genes

The *P. l. manchurica* mitogenomes harbors 22 tRNA genes that are interspersed thorough genomes (Fig. 5). Except for tRNA^{Ser}(AGN), the dihydrouridine (DHU) arm of which forms a simple loop, all tRNAs were folded into the typical clover-leaf structure. The aberrant tRNA^{Ser}(AGN) has been reported in many

	tRNA ^{Asp} →	ATPase8 →							
Polyphaga									
<i>Apatides fortis</i>	<u>tagattgtcatttttaaaataattacttagtaataaaccccg</u>	ATT	CCT	CAA	ATA	GCC	IPQMA		
<i>Chrysochroa fulgidissima</i>	ttaaggaataataaaaattatttgagcgccagctattccta	ATA	CCA	CAA	ATA	GCA	MPQMA		
<i>Acmaeodera</i> sp.	<u>ttagcttgtcgagctaaaaattttaactactaataattttt</u>	ATT	CCA	CAA	GCG	CTA	IPQAL		
<i>Cyphon</i> sp.	<u>aggttgtcaatctaaaatcatttttaaaataaatatttttt</u>	ATT	CCT	CAA	ATA	GCT	IPQMA		
<i>Pyrophorus divergens</i>	<u>tagcttgtcagctaaaaattttaatacattaataattttta</u>	ATT	CCG	CAA	ATA	GCT	IPQMA		
<i>Rhagophthalmus lufengensis</i>	<u>acttgtcatgttttaataattattataaataataattttta</u>	ATT	CCA	CAA	ATA	GCA	IPQMA		
<i>Rhagophthalmus ohbai</i>	<u>acttgtcatgttttaataattattataaataataattttta</u>	ATT	CCA	CAA	ATA	GCA	IPQMA		
<i>Pyrocoelia rufa</i>	<u>ttaatttgtcaaatataaaataattttcaaaaataattttta</u>	ATT	CCA	CAA	ATA	AGC	IPQMS		
<i>Chauiognathus opacus</i>	<u>cgtagtttgtcaaaactaaaatcattgaaaaataattttta</u>	ATT	CCA	CAA	ATA	TCA	IPQMS		
<i>Protaetia brevitarsis</i>	<u>ttaatttgtcaagttaaaaataatcattattgataactttta</u>	ATC	CCA	CAA	ATA	TCA	IPQMS		
<i>Polyphylla laticollis manchurica</i>	<u>taacttgtcaagttagaattacctcgaaagcggtagttttta</u>	TTG	CCT	CAA	ATA	GCA	LPQMA		
<i>Rhopaea magnicornis</i>	<u>atttagtttgtcaaaactaaaataacotttttaggtagttttta</u>	ATT	CCA	CAA	ATA	ATA	IPQMM		
<i>Metopodontus blanchardi</i>	<u>cgtagtttgtcaaaactaaaattacttataggtatcctttta</u>	ATT	CCA	CAA	ATA	GCT	IPQMA		
<i>Lucanus mazama</i>	<u>cattaaactgtcatatttaaaattatcagctgtagtctttta</u>	ATT	CCT	CAA	ATA	GCC	IPQMA		
<i>Priasilpha obscura</i>	<u>tttgtcaaaactaaaattattaacccattagataattttct</u>	ATT	CCT	CAA	ATA	ATA	IPQMM		
<i>Cucujus clavipes</i>	<u>ttaatttgtcaaatataaaattatttaaaataataattttct</u>	ATT	CCT	CAA	ATA	GCC	IPQMA		
<i>Anoplophora glabripennis</i>	<u>tagcttgtcagctaaaattattttaaaataataattttta</u>	ATT	CCT	CAA	ATA	GCA	IPQMA		
<i>Psacotheta hilaris</i>	<u>tagcttgtcagctaaaattattttaaaataataattttta</u>	ATT	CCA	CAA	ATA	GCA	IPQMA		
<i>Crioceris duodecimpunctata</i>	<u>ttagtttgtcaaaactaaaattatttggtaaaataattttta</u>	ATT	CCT	CAA	ATA	GCA	IPQMA		
<i>Tribolium castaneum</i>	<u>caaaactaaagtcaacgtaaacctgttagtttttaattcctc</u>	ATT	CCT	CAA	ATA	GCC	IPQMA		
<i>Adelium</i> sp.	<u>tatatataaattacctttaaggataatagtcaaaataatttta</u>	ATA	CCA	CAA	ATA	GCT	IPQMA		
<i>Mordella atrata</i>	<u>attaaacttgtcaagttaaaataattcattattagttttta</u>	ATT	CCT	CAA	ATA	GCT	IPQMA		
<i>Chaetosoma scaritides</i>	<u>atatatttctgcaaggtcaagtaactttaagtgatttttt</u>	ATA	CCA	CAA	ATA	GCT	IPQMA		
<i>Naupactus xanthographus</i>	<u>ttagtttgtcaaaactaaaattattaactataataattttta</u>	ATT	CCA	CAA	ATA	GCT	IPQMA		
<i>Sphenophorus</i> sp.	<u>tcttaaacattttaatagtaaaattagtaacttacttctaag</u>	ATC	CCA	CAA	ATA	TCT	IPQMS		
<i>Euspilotes scissus</i>	<u>tcttaaacatttttatagtaaaactaaagattacttctaag</u>	ATT	CCT	CAA	ATA	GCA	IPQMA		
<i>Necrophila americana</i>	<u>cttaaacatcccatagtatataacgaataacttctaag</u>	ATT	CCT	CAA	ATA	GCA	IPQMA		
<i>Tropisternus</i> sp.	<u>tcttaaacacatttatagtaaaattagcacttacttctaag</u>	ATT	CCA	CAA	ATA	GCC	IPQMA		
Myxophaga									
<i>Sphaerius</i> sp.	<u>gtatatataacgaataacttctaatagaataacctaattttata</u>	ATT	CCA	CAA	ATA	GCA	IPQMA		
<i>Hydroscapha granulum</i>	<u>gcattgtcaaaactaaaattattaattttttaatttctttta</u>	ATA	CCA	CAA	ATA	GCT	MPQMA		
Adephaga									
<i>Trachypachus holmbergi</i>	<u>agaatgtcaaaactaaaattatttaaaattattatttctttta</u>	ATT	CCA	CAA	ATA	GCC	IPQMA		
<i>Calosoma</i> sp.	<u>agaatgtcaaaactaaaattatttaataattatttcttttg</u>	ATT	CCT	CAA	ATA	GCT	IPQMA		
<i>Aspidytes niobe</i>	<u>tagaatgtcaaaactaaaattatttaataattatttctttta</u>	ATT	CCT	CAA	ATA	GCA	IPQMA		
<i>Macrogyrus oblongus</i>	<u>tagaatgtcaaaactgaattatttaaaataattatttctttta</u>	ATC	CCT	CAA	ATA	GCC	IPQMA		
Archostemata									
<i>Tetraphalerus bruchi</i>	<u>ttagattgtcaaaactgaagtcattataataattatttcttct</u>	ATG	CCT	CAA	ATA	TCA	MPQMS		

Fig. 4. Alignment of the initiation context of ATPase8 gene of coleopteran insects, including that of *Polyphylla laticollis manchurica*. The first five codons are shown in uppercase letters on the right-hand side of the figure. Underlined nucleotides indicate the adjacent partial sequence of tRNA^{Asp}. Arrows indicate the direction of transcription. Boxed nucleotides indicate currently known translation initiators for the ATPase8 gene of coleopteran insects. The start codon for *P. l. manchurica* was designated respectively as TTG.

metazoan species, including insects (Wolstenholme 1992; Garey and Wolstenholme, 1989). For the proper function of a tRNA the DHU arm, which is involved in tertiary interaction requires proper folding (Rich and RajBhandary, 1976). The nuclear magnetic resonance analysis from nematodes has shown that the aberrant tRNA^{ser}(AGN) also functions in a similar way to that of usual tRNAs by structural adjustment to fit in the ribosome (Ohtsuki *et al.*, 2002).

The size of tRNAs ranged from 61 (tRNA^{Cys}) to 71 bp (tRNA^{Lys}) in *P. l. manchurica* (Table 2). Interestingly, tRNA^{Lys} is often slightly larger than tRNAs in many other coleopteran mitogenomes. In fact, 29 among 35 coleopteran species analyzed in this study ranked the tRNA^{Lys} as the longest tRNA at 71 bp (data not shown). In the varying tRNA size, the size of aminocycle stem as 7 bp, anticodon loops as 7 bp, and anticodon stem as 5 bp were all well conserved in all *P. l. manchurica* tRNAs. Most of size variation among tRNAs stemmed from the length variation in DHU and TΨC arms, within

which loop sizes (3 ~ 13 bp) are more variable than stem sizes (3 ~ 7 bp). With the numerous number of G-U base pairs, which form a weak bond in the tRNAs the *P. l. manchurica* tRNAs contained 11 mismatches (three U-C, and each two A-C, U-U, G-A, and A-A mismatches) (Fig. 5). As with all other insect mitogenome sequences, two rRNA genes were detected in *P. l. manchurica*. The 18S rRNA is located between tRNA^{Leu}(CUN) and tRNA^{Val} and the 16S rRNA is located between tRNA^{Val} and the presumable A+T-rich region, respectively (Fig. 1).

Nucleotide composition, codon usage, and skewness

The nucleotide composition of the mitogenome of *P. l. manchurica* is also biased toward A/T content at 69.9% (Table 3). This value is well within the range found in the sequenced

Fig. 5. Predicted secondary clover-leaf structures for the 22 tRNA genes of *Polyphylla laticollis manchurica*. The tRNAs are labeled with the abbreviations of their corresponding amino acids. The one-letter symbols L, L*, S and S* denote tRNA^{Leu}(CUN), tRNA^{Leu}(UUR), tRNA^{Ser}(AGN), and tRNA^{Ser}(UCN), respectively. Arms of tRNAs (clockwise from top) are the amino acid acceptor (AA) arm, TΨC (T) arm, the anticodon (AC) arm, and the dihydrouridine (DHU) arm. Nucleotide sequences from 5' to 3' are indicated from the left side of the amino acid stem. Dashes (-) indicate Watson-Crick base-pairing, and centered asterisks (*) indicate G-U base-pairing.

coleopteran insects, where these values range from 67.2% in *Apatides fortis* to 80.8% in *Sphaerius* sp. (Table 3). To evaluate the degree of the base bias, the AT-skew and GC-skew each in the whole genome and whole PCGs from the major strand and each major- and minor-strand PCGs was measured from coleopteran insects, including *P. I. manchurica* (Table 4). Overall, whole

genomes of all coleopteran species including the *P. I. manchurica* are obviously A- and C-skewed, whereas whole PCGs are T- and C-skewed, indicating that Ts are clearly more favored over As in the PCGs, and the evolutionary pattern of PCGs differ from the remaining genes. In the majority of coleopteran species, including *P. I. manchurica*, the major-strand, in which nine PCGs (ND2,

Table 4. Composition and skewness in the coleoptera mitochondrial genomes

Order	Major-strand PCGs				Minor-strand PCGs				Whole PCGs*				Whole genome*			
	A/T%	AT skew	GC skew	A/T%	AT skew	GC skew	A/T%	AT skew	GC skew	A/T%	AT skew	GC skew	A/T%	AT skew	GC skew	
Polyphaga																
<i>Apatides fortis</i>	63.8	0.057	-0.315	69.8	0.377	-0.270	66.1	-0.120	-0.113	67.1	0.170	-0.295				
<i>Chrysochroa fulgidissima</i>	66.9	0.021	-0.232	71.4	0.388	-0.274	68.6	-0.143	-0.054	69.9	0.153	-0.237				
<i>Acmaeodera</i> sp.	63.8	-0.026	-0.275	70.0	0.344	-0.192	66.2	-0.156	-0.114	68.4	0.114	-0.249				
<i>Cyphon</i> sp.	70.5	-0.062	-0.190	76.3	0.269	-0.294	72.8	-0.146	-0.028	75.1	0.072	0.228				
<i>Pyrophorus divergens</i>	65.6	0.042	-0.282	70.5	0.399	-0.327	67.5	-0.135	-0.068	69.4	0.165	0.291				
<i>Rhagophthalmus lufengensis</i>	77.0	-0.036	-0.122	79.8	0.298	-0.266	78.1	-0.139	0.016	79.6	0.104	-0.196				
<i>Rhagophthalmus ohbai</i>	76.3	-0.023	-0.162	79.3	0.302	-0.268	77.4	-0.133	-0.010	79.1	0.119	-0.226				
<i>Pyrocoelia rufa</i>	75.1	-0.015	-0.161	78.1	0.300	-0.231	76.2	-0.128	-0.022	77.4	0.105	0.158				
<i>Chauliognathus opacus</i>	74.7	0.004	-0.130	78.4	0.277	-0.158	76.1	-0.108	-0.029	76.8	0.112	-0.153				
<i>Protaetia brevitarsis</i>	71.3	-0.077	-0.197	76.6	0.228	-0.293	73.3	-0.138	-0.031	74.5	0.065	-0.319				
<i>Polyphyla laticollis manchurica</i>	67.2	-0.080	-0.250	71.2	0.228	-0.285	68.7	-0.139	-0.061	69.8	0.043	-0.270				
<i>Rhopaea magnicornis</i>	72.5	-0.108	-0.182	77.6	0.198	-0.299	74.4	-0.144	-0.019	75.5	0.005	0.238				
<i>Metopodotus blanchardi</i>	63.6	-0.023	-0.278	68.8	0.311	-0.335	65.6	-0.139	-0.064	67.0	0.107	-0.266				
<i>Lucanus mazama</i>	63.3	-0.067	-0.255	69.0	0.283	-0.284	65.5	-0.155	-0.068	67.1	0.074	-0.272				
<i>Priasilpha obscura</i>	71.5	-0.064	-0.190	77.1	0.227	-0.292	73.6	-0.130	-0.029	76.5	0.052	0.263				
<i>Cucujus clavipes</i>	72.9	-0.102	-0.211	77.7	0.212	-0.374	74.7	-0.146	-0.012	76.3	0.026	-0.282				
<i>Anoplophora glabripennis</i>	75.1	-0.114	-0.131	79.8	0.180	-0.263	76.9	-0.140	0.001	78.3	0.011	0.205				
<i>Psacotha hilaris</i>	74.0	-0.129	-0.127	78.6	0.199	-0.276	75.8	-0.153	0.010	76.6	0.011	-0.213				
<i>Crioceris duodecimpunctata</i>	73.0	-0.076	-0.114	78.7	0.233	-0.235	75.2	-0.139	0.001	76.8	0.044	0.162				
<i>Tribolium castaneum</i>	66.9	-0.005	-0.286	72.6	0.301	-0.347	69.1	-0.125	-0.070	71.6	0.109	0.305				
<i>Adellum</i> sp.	68.1	0.025	-0.200	73.9	0.330	-0.243	70.3	-0.119	-0.050	72.7	0.137	-0.222				
<i>Mordella atrata</i>	68.8	-0.081	-0.219	73.4	0.268	-0.282	70.6	-0.156	-0.045	71.9	0.069	0.255				
<i>Chaetosoma scaritides</i>	75.8	-0.083	-0.129	79.8	0.228	-0.265	77.3	-0.141	0.007	79.0	0.042	0.200				

Table 4. Continued

Order	Major-strand PCGs				Minor-strand PCGs				Whole PCGs*				Whole genome*			
	A/T%	AT skew	GC skew	A/T%	AT skew	GC skew	A/T%	AT skew	GC skew	A/T%	AT skew	GC skew	A/T%	AT skew	GC skew	GC skew
Polyphaga																
<i>Naupactus xanthographus</i>	73.9	-0.093	-0.170	78.4	0.242	-0.254	75.6	-0.152	-0.025	76.8	0.042	-0.220				
<i>Sphenophorus</i> sp.	73.1	-0.102	-0.203	77.8	0.239	-0.260	74.9	-0.157	-0.045	76.3	0.030	-0.238				
<i>Euspilotes scissus</i>	70.1	-0.111	-0.191	74.1	0.218	-0.328	71.7	-0.155	-0.006	73.1	0.023	-0.246				
<i>Necrophila americana</i>	70.9	-0.088	-0.138	76.8	0.216	-0.237	73.2	-0.140	-0.013	74.6	0.029	-0.193				
<i>Tropisternus</i> sp.	70.4	-0.006	-0.175	75.7	0.305	-0.274	72.4	-0.126	-0.023	74.4	0.120	-0.226				
Myxophaga																
<i>Sphaerius</i> sp.	78.3	-0.086	-0.058	82.0	0.163	-0.257	79.7	-0.116	0.050	80.6	0.010	0.147				
<i>Hydrosapha granulum</i>	73.6	-0.101	-0.073	78.5	0.227	-0.267	75.5	-0.147	0.041	77.2	0.030	-0.152				
Adephaga																
<i>Trachypachus holmbergi</i>	77.0	-0.093	-0.060	80.6	0.190	-0.225	78.4	-0.131	0.039	79.4	0.020	0.153				
<i>Calosoma</i> sp.	74.8	-0.085	-0.127	79.5	0.216	-0.291	76.6	-0.138	0.014	78.4	0.038	-0.197				
<i>Aspidytes niobe</i>	76.9	-0.078	-0.069	80.5	0.218	-0.219	78.4	-0.138	0.040	79.0	0.052	-0.145				
<i>Macrogyrus oblongus</i>	73.2	-0.073	-0.161	78.5	0.246	-0.266	75.2	-0.142	-0.017	78.0	0.042	0.208				
Archostemata																
<i>Tetraphalerus bruchi</i>	65.4	0.153	-0.278	66.3	0.460	-0.221	65.8	-0.085	-0.089	66.9	0.247	0.251				

Stop codon was excluded in the count.

*The skewness of whole PCGs and whole genome was calculated from major strand.

Table 5. Frequency of four most frequent codons of coleopteran insects

Species	codon				Total
	TTA (L)	ATT (I)	TTT (F)	ATA (I)	
Polyphaga					
<i>Apatides fortis</i>	5.62	5.40	5.43	4.18	20.63
<i>Chrysochroa fulgidissima</i>	5.90	7.39	4.61	7.31	25.21
<i>Acmaeodera</i> sp.	5.29	7.07	6.66	4.26	23.28
<i>Cyphon</i> sp.	9.81	8.84	6.09	7.90	32.64
<i>Pyrophorus divergens</i>	4.48	6.43	4.97	6.26	22.14
<i>Rhagophthalmus lufengensis</i>	10.60	10.12	7.63	9.12	37.47
<i>Rhagophthalmus ohbai</i>	10.46	9.89	7.23	9.10	36.68
<i>Pyrocoelia rufa</i>	9.60	10.27	7.51	8.59	35.97
<i>Chauiognathus opacus</i>	9.87	9.14	7.12	7.83	33.96
<i>Protaetia brevitaris</i>	10.10	8.70	6.08	7.56	32.44
<i>Polyphylla laticollis manchurica</i>	8.29	7.32	5.56	7.05	28.22
<i>Rhopaea magnicornis</i>	11.49	9.41	6.06	7.36	34.32
<i>Metopodontus blanchardi</i>	4.93	6.63	5.01	5.90	22.47
<i>Lucanus mazama</i>	5.51	7.02	4.41	6.06	23.00
<i>Priasilpha obscura</i>	9.82	9.60	6.87	8.36	34.65
<i>Cucujus clavipes</i>	10.51	9.35	6.56	8.16	34.58
<i>Anoplophora glabripennis</i>	12.45	10.52	5.86	8.57	37.40
<i>Psacotheta hilaris</i>	11.92	10.06	5.23	8.50	35.71
<i>Crioceris duodecimpunctata</i>	11.69	8.99	5.93	8.69	35.30
<i>Tribolium castaneum</i>	6.62	8.32	4.95	6.54	26.43
<i>Adelium</i> sp.	7.10	7.32	5.72	6.61	26.75
<i>Mordella atrata</i>	8.10	8.45	4.97	6.74	28.26
<i>Chaetosoma scaritides</i>	11.80	10.39	7.19	8.98	38.36
<i>Naupactus xanthographus</i>	11.88	9.48	5.78	9.02	36.16
<i>Sphenophorus</i> sp.	10.58	9.91	5.24	8.88	34.61
<i>Euspilotus scissus</i>	8.57	8.62	5.48	7.49	30.16
<i>Necrophila americana</i>	9.82	9.17	5.95	7.57	32.51
<i>Tropisternus</i> sp.	8.82	7.84	5.17	6.25	28.08
Myxophaga					
<i>Sphaerius</i> sp.	13.56	10.95	7.68	8.90	41.09
<i>Hydroscapha granulum</i>	12.36	9.03	6.41	8.74	36.54
Adephaga					
<i>Trachypachus holmbergi</i>	13.29	9.77	7.43	9.21	39.70
<i>Calosoma</i> sp.	12.19	9.21	7.09	8.56	37.05
<i>Aspidytes niobe</i>	12.55	10.65	8.21	9.41	40.82
<i>Macrogysus oblongus</i>	10.72	9.24	6.89	9.26	36.11
Archostemata					
<i>Tetraphalerus bruchi</i>	4.37	5.26	4.97	6.02	20.62
Average	9.45	8.74	6.11	7.68	31.98

ND3, ND6, COI, COII, COIII, ATPase6, ATPase8, and CytB) are encoded is slightly T-skewed (AT skew = -0.129 ~ 0.153), whereas the minor-strand, in which four PCGs (ND1, ND4, ND4L, and ND5) are encoded is obviously A-skewed (AT skew = 0.163 ~ 0.460), although both strands are C-skewed (Table 4). Thus, the two strands are sharply distinct in A/T-skewness, indicating that mutational pressures that favor Ts or As are starkly different between the two strands. It has been suggested that the lagging strand, equivalent to the major strand, should be more prone to chemical conversion of As to Gs and Cs to Ts, by the mechanism called deamination than the leading strand, and this may have resulted in the enriched Ts and Gs in the lagging strand, and As and Cs in the leading strand (Reyes *et al.*, 1998). Nevertheless, current coleopteran mitogenomes pretty strongly display C-skewness rather than G-skewness in the major strand (Table 4). Thus, the strand-based inequality has yet to be clearly understood.

The genome-wise A/T bias is also reflected in the codon usage of *P. l. manchurica* mitogenomes (Table 5). The codons TTA (Leu), ATT (Ile), TTT (Phe), and ATA (Met) are the four most frequently used codons in the *P. l. manchurica* PCGs, accounting for 28.22% and on average these four codons accounted for 31.98% in Coleoptera (Table 5). Considering 60 codons are available, excluding each two start and stop codons (ATA, ATG, TAA, and TAG) the overuse of these four codons is obvious. These four codons are all comprised of A or T nucleotides, thus indicating the biased usage of A and T nucleotides in the coleopteran insects including *P. l. manchurica* PCGs.

The analysis of the base composition at each codon position of the concatenated 13 PCGs of *P. l. manchurica* showed 68.6% of the A/T content in the third codon position. This value is higher than that of the first (66.4%) and second (66.2%) codon positions, and on average, the A/T content in the third codon position was 74.9%, ranging from 65.4% to 82.1% in Coleoptera (Table 6). In a comparison among species-diverse insect orders, Hymenoptera is the highest on average at 93.1% (Hong *et al.*, 2008), Lepidoptera is next at 92.1% (Kim *et al.*, 2010), and Diptera rank third at 92.0% (Cameron *et al.*, 2009), indicating that the Coleoptera is the least A/T-biased. Probably, this aspect has implications for phylogenetic analysis because of the different degrees of A/T bias, resulting in compositional heterogeneity among insect orders as a major source of systematic bias in phylogeny (Jermini *et al.*, 2004; Sheffield *et al.*, 2009).

Table 6. Summary of base composition at each codon position of the concatenated 13 PCGs in coleopteran mitochondrial genomes

Species	1st codon position				2nd codon position				3rd codon position				Overall			
	A	T	C	G	A	T	C	G	A	T	C	G	A	T	C	G
<i>Apatides fortis</i>	34.8	37.3	10.8	16.2	27.0	49.6	14.8	11.1	40.0	48.6	4.5	6.9	29.1	37.0	18.9	15.0
<i>Chrysoschoa fulgidissima</i>	31.0	31.4	16.7	20.9	19.0	46.0	20.1	15.0	37.7	40.4	13.2	8.8	29.2	39.3	16.7	14.9
<i>Acmaeodera</i> sp.	31.3	32.8	16.0	20.8	19.1	46.6	20.0	15.2	37.9	39.4	14.3	8.9	28.9	39.6	16.9	14.6
<i>Cyphon</i> sp.	38.0	30.8	16.6	14.3	30.5	40.2	18.1	11.2	44.1	32.7	16.2	7.1	37.5	34.6	17.0	11.0
<i>Pyrophorus divergens</i>	32.2	29.8	17.3	20.7	20.3	45.3	20.3	14.0	37.9	35.1	17.7	9.3	30.1	36.7	18.4	14.7
<i>Rhagophthalmus lufengensis</i>	35.6	35.5	11.2	17.7	19.9	47.7	19.0	13.4	45.8	48.6	3.6	2.1	33.8	43.9	11.3	11.1
<i>Rhagophthalmus ohbai</i>	36.1	35.8	11.5	16.7	20.1	47.5	19.0	13.4	44.9	46.6	5.4	3.1	33.7	43.3	12.0	11.1
<i>Pyrocoelia rufa</i>	36.1	34.6	12.2	17.2	19.7	47.7	19.1	13.5	45.0	44.3	6.7	3.9	33.6	42.2	12.7	11.5
<i>Chauliognathus opacus</i>	37.1	39.2	11.6	18.1	19.3	47.6	18.9	14.1	45.1	45.7	3.3	6.0	35.3	43.6	11.9	12.1
<i>Protaetia brevitarsis</i>	34.0	39.2	12.9	14.0	27.5	42.0	16.0	14.5	33.3	44.2	12.2	10.3	31.6	41.8	13.7	12.9
<i>Polyphylla laticollis manchurica</i>	33.5	32.9	14.5	18.8	20.0	46.2	19.2	14.4	35.2	38.3	15.7	10.6	29.5	39.1	16.5	14.6
<i>Rhopaea magnicornis</i>	35.9	37.9	10.2	16.0	22.1	48.7	16.1	13.2	41.0	51.7	4.2	3.1	33.0	46.1	10.1	10.8
<i>Metopodotus blanchardi</i>	31.7	29.5	17.1	21.4	19.0	46.1	19.6	15.1	33.8	36.3	18.0	11.7	28.2	37.3	18.2	16.0
<i>Lucanus mazama</i>	36.5	36.4	10.3	16.6	22.0	48.1	16.2	13.5	40.8	49.2	6.2	3.6	33.1	44.6	10.9	11.3
<i>Priasilpha obscura</i>	37.7	31.8	16.1	14.3	29.9	41.5	17.9	10.7	45.3	32.1	15.4	7.2	37.6	35.1	16.5	10.7
<i>Cucujus clavipes</i>	32.6	35.9	12.9	18.2	22.6	48.1	16.9	15.5	42.9	49.6	4.5	2.1	33.5	48.6	11.9	12.9
<i>Anoplophora glabripennis</i>	33.4	35.5	13.0	18.1	20.5	47.2	18.4	13.9	44.2	48.1	4.9	2.7	32.7	43.6	12.1	11.6
<i>Psacotha hilaris</i>	31.7	35.1	13.7	19.5	20.2	46.7	19.0	14.1	43.7	48.0	5.3	3.0	31.9	43.3	12.7	12.2
<i>Crioceris duodecimpunctata</i>	33.9	35.2	12.9	18.0	20.3	47.1	18.3	14.3	42.1	45.5	7.5	5.0	32.1	42.6	12.9	12.4
<i>Tribolium castaneum</i>	32.3	31.4	16.4	20.0	19.3	46.3	20.2	14.2	38.7	37.4	15.7	8.2	30.1	38.4	17.4	14.1
<i>Adelium</i> sp.	35.3	29.1	13.2	15.0	19.9	48.3	17.6	14.3	39.9	46.9	5.6	4.0	33.8	42.3	11.1	12.2
<i>Mordella atrata</i>	36.3	35.6	11.3	16.8	21.7	48.1	16.5	13.6	38.8	46.2	9.0	5.9	32.3	43.3	12.3	12.1
<i>Chaetosoma scaritides</i>	39.6	34.1	13.4	13.0	30.0	42.5	16.8	10.7	47.0	37.6	10.2	5.2	38.9	38.1	13.5	9.6
<i>Naupactus xanthographus</i>	35.9	34.1	13.4	13.0	30.0	42.5	16.8	10.7	47.0	37.6	10.2	5.2	32.1	43.6	12.5	11.9
<i>Sphenophorus</i> sp.	36.5	36.4	10.3	16.6	21.4	48.5	16.3	13.6	38.8	46.2	9.0	5.9	31.6	43.3	13.1	12.0
<i>Euspilotus scissus</i>	35.8	38.3	10.8	15.1	26.0	48.6	14.2	11.2	41.0	47.6	5.1	6.3	30.3	41.4	14.2	14.1
<i>Necrophila americana</i>	35.4	37.5	10.5	16.6	21.7	48.5	16.6	13.3	40.9	51.4	4.9	2.8	31.5	41.7	13.6	13.2
<i>Tropisternus</i> sp.	31.6	39.3	10.5	16.6	23.1	49.3	17.2	10.2	41.9	49.3	4.9	2.8	31.7	40.8	14.1	13.5
<i>Sphaerius</i> sp.	40.1	37.2	10.3	12.5	30.2	41.3	16.6	11.9	47.9	41.3	7.8	3.0	39.4	39.9	11.6	9.1
<i>Hydroscaapha granulum</i>	33.4	37.1	11.0	18.3	20.4	47.0	18.1	14.3	42.7	45.8	5.9	5.4	32.2	43.3	11.7	12.7
<i>Trachypachus holmbergi</i>	38.2	34.6	12.8	14.4	29.9	43.9	15.8	10.4	48.4	38.8	7.8	5.0	38.8	39.1	12.1	9.9
<i>Calosoma</i> sp.	35.3	29.1	13.2	15.0	19.9	48.3	17.6	14.3	39.9	46.9	5.6	4.0	33.1	43.6	11.5	11.8
<i>Aspidytes niobe</i>	35.9	37.1	10.4	16.4	20.4	48.2	17.5	13.7	45.1	48.5	3.0	3.3	33.8	44.6	10.3	11.2
<i>Macrogyrus oblongus</i>	35.4	32.5	12.4	18.6	21.6	46.2	17.9	12.8	42.9	47.6	4.5	2.1	32.9	42.6	11.1	12.1
<i>Tetraphalerus bruchi</i>	39.4	22.9	21.0	16.6	29.7	34.3	23.1	12.8	52.5	17.5	21.6	8.5	40.5	24.9	21.9	12.6

Stop codon was excluded in the count.

Acknowledgements

This work was supported by research grants from the National Institute of Biological Resources-“The Genetic Evaluation of Important Biological Resources” awarded to Iksoo Kim.

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