Journal of Insect Science (*2017*) 17(2): 63; 1–9
doi: 10.1093/jisesa/iex041
Research

New Mitogenomes of Two Chinese Stag Beetles (Coleoptera, Lucanidae) and Their Implications for Systematics

Zi-Qi Lin,[1] Fan Song,[2] Teng Li,[3] Yuan-Yuan Wu,[1] and Xia Wan[1,4]

[1]Department of Ecology, School of Resources and Engineering, Anhui University, 111 Jiulong Rd., Hefei 230601, P. R. China (1056955062@qq.com; 1406971729@qq.com; wanxia@ahu.edu.cn), [2]Department of Entomology, China Agricultural University, West Campus, 2 Yuanmingyuan West Rd., Beijing 100193, P. R. China (812532519@qq.com), [3]Department of Zoology and Developmental Biology, School of Life Sciences, Lanzhou University, 222 Tianshui South Rd., Lanzhou 730000, P. R. China (58032831@qq.com), and [4]Corresponding author, e-mail: wanxia@ahu.edu.cn

Subject Editor: Yoonseong Park

Received 22 October 2016; Editorial decision 0 Month 0000

Abstract

Although conspicuous and well-studied, stag beetles have been slow to join the genomic era. In this study, mitochondrial genomes of two stag beetles, *Sinodendron yunnanense* and *Prosopocoilus confucius*, are sequenced for the first time. Both of their genomes consisted of 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), 2 ribosomal RNAs (rRNAs), and a control region. The mitogenome of *S. yunnanense* was 16,921 bp in length, and *P. confucius* was 16,951 bp. The location of the gene *trnL*(*UUR*), between the A + T–rich and control region in *S. yunnanense*, is the first observed in Lucanidae. In *P. confucius*, an unexpected noncoding region of 580 bp was discovered. Maximum likelihood and Bayesian inference on the 13 mitochondrial PCGs were used to infer the phylogenetic relationships among 12 representative stag beetles and three scarab beetles. The topology of the two phylogenetic trees was almost identical: *S. yunnanense* was recovered as the most basal Lucanid, and the genus *Prosopocoilus* was polyphyletic due to *P. gracilis* being recovered sister to the genera *Dorcus* and *Hemisodorcus*. The phylogenetic results, genetic distances and mitogenomic characteristics call into question the cohesion of the genus *Prosopocoilus*. The genetic resources and findings herein attempts to redress understudied systematics and mitogenomics of the stag beetles.

Key words: mitogenomes, gene rearrangement, noncoding region, Chinese stag beetles, phylogenomics

Lucanidae (stag beetles) is a small family in Scarabaeoidea, containing about 1,800 described species and subspecies worldwide (Krajcik 2001, Bartolozzi and Sprecher-Uebersax 2006, Fujita 2010). Most studies support Lucanidae as a monophyletic group and an early diverging lineage in scarab beetles (Howden 1982, Scholtz et al. 1994, Ahrens et al. 2014) although a recent study has questioned Lucanid monophyly (Mckenna et al. 2015). There is a robust body of literature on taxonomy of this family (Arrow 1950, Didier and Séguy 1953, Benesh 1960, Lawrence and Newton 1995, Krajcik 2001, Holloway 2007), with particular interest in sexual selection behavior and male polymorphism. As typical, molecular markers have more recently been used to enrich stag beetle systematics. Hosoya and Araya (2005) reconstructed the first molecular phylogeny of stag beetles (based on the molecular markers *cox1* and *rrnL*) although using a small data set of only Japanese species. There has been particular attention to species delimitation and conservation of lucanids from Japan and Taiwan Island, partly because these beetles are popular as pets in these places (Huang and Lin 2010, Kubota et al. 2011, Lin et al. 2011, Tsai et al. 2014, Tsai and Yeh 2016). Kim and Farrell (2015) performed the first comprehensive phylogenetic study, and revealed a Gondwanan origin of Darwin's stag beetle.

Undoubtedly these studies have shed light on the evolution of Lucanidae although using short gene fragments. Further, although widely used in phylogenetics, phylogeography and population genetics in insects (Timmermans et al. 2010, Cameron 2014a, Yuan et al. 2016), only three complete mitogenomes have been reported for lucanids; *Lucanus mazuma* (Sheffield et al. 2009), *Prosopocoilus blanchardi* (Kim et al. 2015), and *Prosopocoilus gracilis* (Wu et al. 2016, our recent work).

To address these insufficiencies, we herein report complete mitogenomes of two representatives from the Chinese fauna, *Sinodendron yunnanense* and *Prosopocoilus confucius*. In *Sinodendron*, four species are recognized worldwide; three are distributed in the Palearctic and Nearctic, whereas *S. yunnanense* is oriental, with a restricted habitat in Southwest China and quite

Table 1 Details on primers used in this study

Gene	Primer name	Sequence (5′–3′)	Reference
cox1	COI–F1	CAACATTTATTTTGATTTTTTGG	Simon et al. 1994
	COI–R1	TCCAATGCACTAATCTGCCATATTA	Simon et al. 1994
cytb	Cytb–F2	GAGGAGCAACTGTAATTACTAA	Balke et al. 2004
	Cytb–R2	AAAAGAAARTATCATTCAGGTTGAAT	Balke et al. 2004
rrnL	16S–F1	CCGGTTTGAACTCAGATCATG	Hosoya et al. 2001
	16S–R1	TAATTTATTGTACCTTGTGTATCAG	Hosoya et al. 2001
nad4	PcND4F1	AATCATTGACCCTGAAACAG	This study
	PcND4R1	AGGGAGATTTCTTTGAGGTG	This study

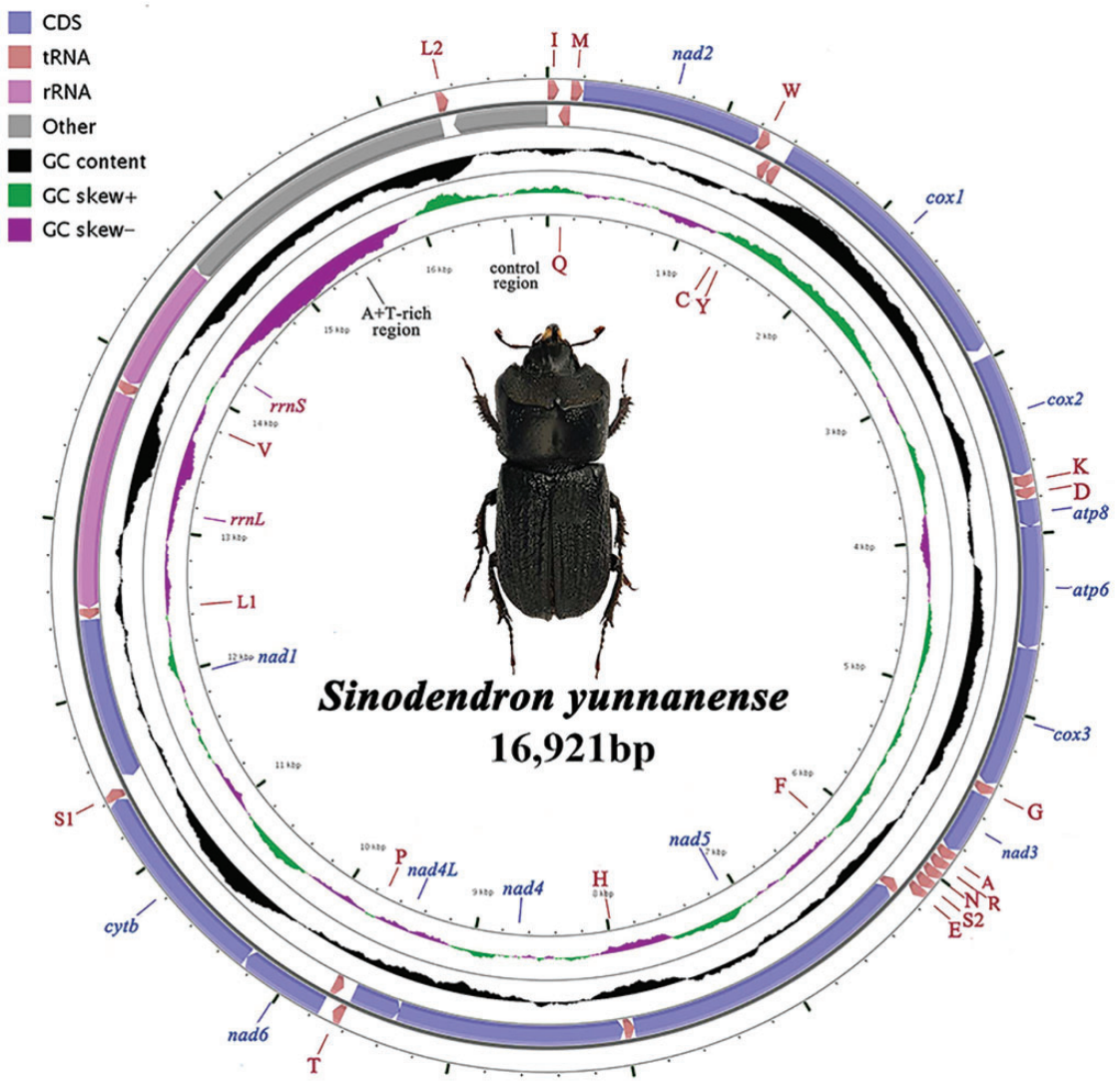

Fig. 1 Mitochondrial genome maps of *Sinodendron yunnanense*. The abbreviations for the genes are as follows: *cox1–3* refer to the cytochrome C oxidase subunits; *cytb* refers to cytochrome B; and *nad1–6* refers to NADH dehydrogenase subunits; *atp6* and *atp8* refer to subunits 6 and 8 of ATPase; *rrnL* and *rrnS* refer to ribosomal RNA of 12S and 16S. From outer to innermost, the first circle shows the gene map, the second circle shows GC content, and the third shows GC skew calculated as (G − C)/(G + C). There is a unique gene rearrangement: trnL2 (UUR) located between the A + T–rich and control region.

rare in the field. The species *P. confucius* is a large but otherwise typical member of the genus, also with limited distribution in South China. We aim to create a more robust understanding of the evolution of lucanid beetles by developing mitochondrial phylogenomics, with a particular focus on *Prosopocoilus*, including a comparison among *P. confucius*, *P. blanchardi* and *P. gracilis*.

Materials and Methods

Sample Collection and DNA Extraction

The specimen of *S. yunnanense* was collected by Dr. Hong-liang Shi from Lanping, Yunnan, China, in July 2012, and the specimen of *P. confucius* was collected from Wuming, Guangxi, China, by Ying-Hua Wu in July 2011. Total genomic DNA was extracted from the

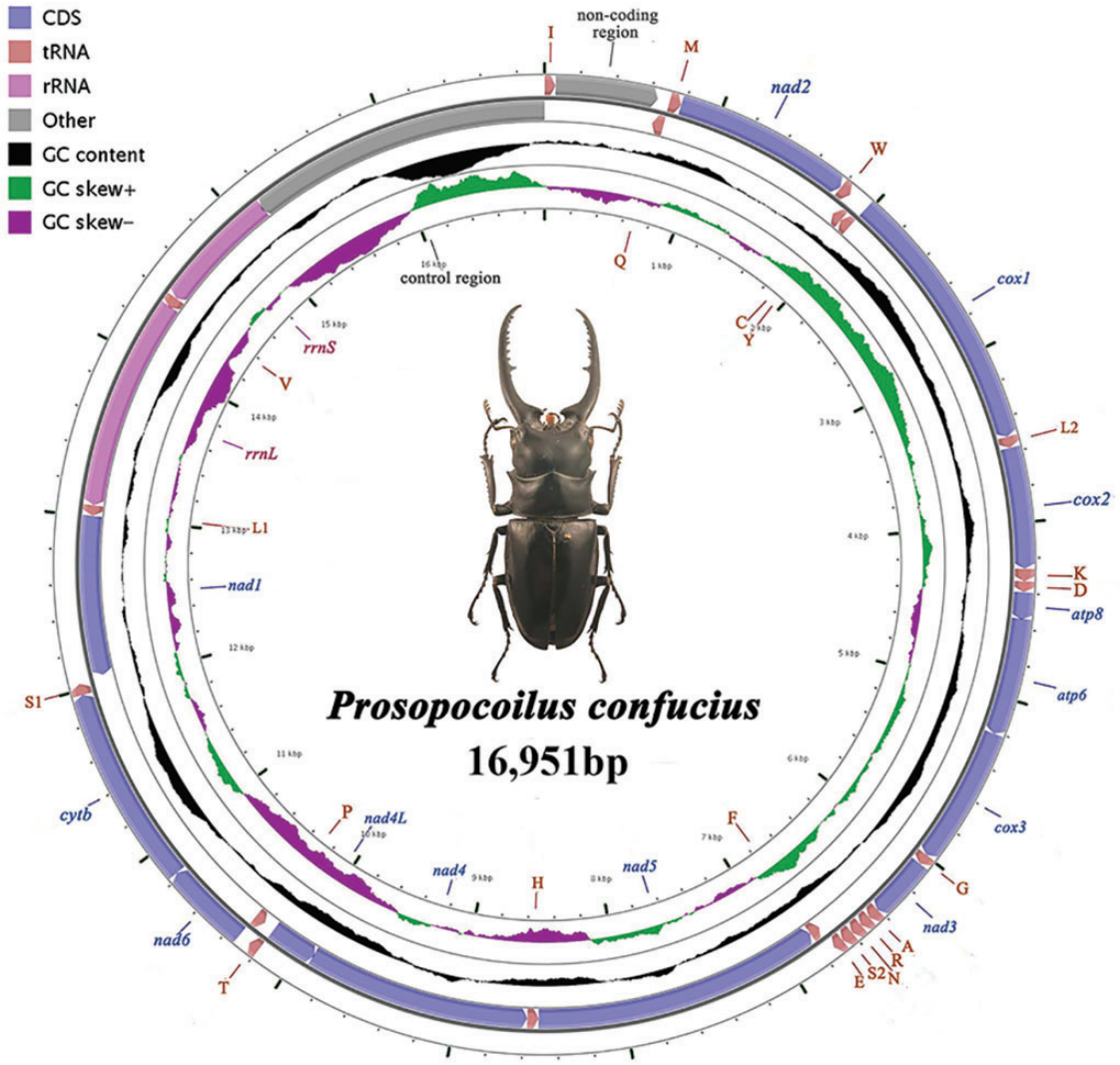

Fig. 2 The mitochondrial genome map of *Prosopocoilus confucius*. There is a noncoding region of 580 bp between *trnI* and *trnQ*.

muscle of a single *S. yunnanense* using the Qiagen DNAeasy Kit. For *P. confucius*, a small portion of the muscle from a specimen preserved in 100% ethanol was used for total DNA extraction with the Blood & Tissue Kit (Qiagen, Germany). The voucher specimens (*S. yunnanense*: MAHUSi012; *P. confucius*: MAHU00102) were deposited at the Museum of Anhui University, China, and the sequences were submitted to GenBank with the accession numbers KP735804 (*S. yunnanense*) and KP552119 (*P. confucius*).

Primer Design, Polymerase Chain Reaction Amplification, and Sequencing

Complete mitogenomes were assembled from amplified fragments, with all primers used for amplification listed in Table 1. PCR amplification reactions were carried out in 25 µl volumes containing 10 µM of each primer (forward and reverse) 1 µl, 2 µl template DNA, 12.5 µl 2 × EasyTaq SuperMix (+dye), and 8.5 µl sterile double-distilled water to make up a final volume of 25 µl. The polymerase chain reaction amplifications were performed under the following conditions: an initial denaturation at 94 °C for 2 min, followed by 35–37 cycles of denaturation at 94 °C for 40 seconds, annealing at 52–58 °C for 50 seconds, and elongation at 70 °C for 1 min, and then a final extension step at 72 °C for 7 min. The temperature of annealing was determined by the length of fragments. Sequencing was conducted with the Illumina HiSeq 2000 platform. Cluster strands created by bridge amplification were primed and all four fluorescently labeled, and 3-OH blocked nucleotides were added to the flow cell with DNA polymerase. The cluster strands were extended in single nucleotides. Following the incorporation step, the unused nucleotides and DNA polymerase molecules were washed away, a scan buffer added to the flow cell, then the optics system scanned each lane of the flow cell in imaging units (tiles). Once imaging was completed, chemicals that effect cleavage of the fluorescent labels and the 3-OH blocking groups were added to the flow cell, which prepares the cluster strands for another round of fluorescent nucleotide incorporation.

Mitogenome Assembly, Annotation, and Analysis

The mitogenomes were assembled using SOAP denovo (BGI company, Shenzhen, China) and preliminary annotations made with the MITOS WebServer (http://mitos.bioinf.uni-leipzig.de/index.py). tRNA genes and their secondary structures were inferred using tRNAscan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/). Those not identified by tRNAscanoSE, in addition to 16S ribosomal RNA (*rrnL, lrRNA*), and 12S ribosomal RNA (*rrnS, srRNA*), were determined according to sequence similarity with related species. The

Table 2 Mitogenome organization of *Sinodendron yunnanense*

Gene	Strand	Region	Length (bp)	Start codon	Stop codon	Anticodon	Intergenic nucleotides (bp)
trnI	J	1–65	65	–	–	GAT	–3
trnQ	N	63–131	69	–	–	TTG	–1
trnM	J	131–199	69	–	–	CAT	0
nad2	J	200–1213	1014	–	TAA	–	2
trnW	J	1,216–1,281	66	–	–	TCA	–8
trnC	N	1,274–1,335	62	–	–	GCA	0
trnY	N	1,336–1,401	66	–	–	GTA	1
cox1	J	1,403–2,938	1536	AAT	TAA	–	46
cox2	J	2,985–3,672	688	ATG	T	–	0
trnK	J	3,673–3,743	71	–	–	CTT	–1
trnD	J	3,743–3,806	64	–	–	GTC	0
atp8	J	3,807–3,962	156	ATT	TAA	–	–7
atp6	J	3,956–4,636	681	ATG	TAA	–	–1
cox3	J	4,636–5,424	789	ATG	TAA	–	2
trnG	J	5,427–5,489	63	–	–	TCC	0
nad3	J	5,490–5,843	354	ATT	TAG	–	–2
trnA	J	5,842–5,906	65	–	–	TGC	–1
trnR	J	5,906–5,971	66	–	–	TCG	–1
trnN	J	5,971–6,035	65	–	–	GTT	–1
trnS(AGN)	J	6,035–6,103	69	–	–	ACT	–1
trnE	J	6,103–6,165	63	–	–	TTC	–2
trnF	N	6,164–6,228	65	–	–	GAA	0
nad5	N	6,229–7,951	1723	ATA	T	–	–3
trnH	N	7,949–8,011	63	–	–	GTG	0
nad4	N	8,012–9,347	1336	ATG	T	–	–7
nad4L	N	9,341–9,631	291	ATT	TAA	–	2
trnT	J	9,634–9,697	64	–	–	TGT	0
trnP	N	9,698–9,760	63	–	–	TGG	1
nad6	J	9,762–10,259	498	ATC	TAA	–	–1
cytb	J	10,259–11,401	1143	ATG	TAG	–	–2
trnS(UCN)	J	11,400–11,464	65	–	–	TGA	19
nad1	N	11,484–12,434	951	ATA	TAG	–	1
trnL(CUN)	N	12,436–12,499	64	–	–	TAG	0
rrnL	N	12,500–13,777	1278	–	–	–	0
trnV	N	13,778–13,847	70	–	–	TAC	0
rrnS	N	13,848–14,614	767	–	–	–	0
A+T–rich region	–	14,615–16,301	1687	–	–	–	0
trnL(UUR)	J	16,302–16,367	66	–	–	TAA	0
Control region	–	16,368–16,921	554	–	–	–	0

protein-coding genes (PCGs) were determined by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) under the invertebrate mitochondrial genetic code. Nucleotide compositions, codon usage, and relative synonymous codon usage (RSCU) values of PCGs were calculated with MEGA version 6.05 (Tamura et al. 2013). PCGs were translated with DNAMAN v7.0.2.176 (Lynnon Biosoft, Vaudreuil-Dorion, Canada). Mitogenomes were mapped with CGView (Grant and Stothard 2008, available at http://stothard.afns.ualberta.ca/cgviewserver/). Composition skew analysis was conducted according to formulas AT skew = [A − T]/[A + T] and GC skew = [G − C]/[G + C] (Perna and Kocher 1995).

Phylogenetic Analyses

Phylogenetic analyses of stag beetles, including the newly sequenced *S. yunnanense* and *P. confucius* and 13 species retrieved from GenBank, were performed using a concatenated dataset of the 13 PCGs. Models of nucleotide substitution were selected according to the Akaike Information Criterion (AIC) with jModelTest v2.1.4 (Posada 2008). Phylogenetic trees were generated from ML analysis using RAxML (Stamatakis 2014) and Bayesian inference (BI) with MrBayes v3.2.5 (Huelsenbeck and Ronquist 2001), both under the GTR + I + G model. Node support in the ML tree was estimated through bootstrap analysis with 1,000 replicates. The BI was conducted with two simultaneous Markov chain Monte Carlo runs of 2 million generations, sampled every 1,000 steps, with the first 25% discarded as burn-in. Phylogenetic trees were viewed and edited in Figtree v1.4.3 Rambaut 2016.

Results and Discussion

Genome Composition and Base Content

The complete mitogenomes of *S. yunnanense* and *P. confucius* were 16,921 bp (Fig. 1), and 16,951 bp (Fig. 2), respectively. Details of the 13 PCGs, 22 tRNAs, 2 rRNAs, and control region are given in Tables 2 and 3. Their compositions were typical of the Coleoptera (Sheffield et al. 2009, Kim et al. 2015, Wu et al. 2016, Du et al. 2016, Yang et al. 2016). Of the 37 genes, 23 (9 PCGs and 14 tRNAs) genes were located on the J-strand, with the remaining 4 PCGs, 2 rRNAs, and 8 tRNAs on the N-strand. Typical of metazoan mitogenomes (Cameron 2014a), the single tRNA gene *trnS* (*AGN*) lacked the cloverleaf secondary structure, due to the structure of the dihydrouridine arm. The lengths of the tRNAs ranged from 61 to 71

Table 3 Mitogenome organization of *Prosopocoilus confucius*

Gene	Strand	Region	Length (bp)	Start codon	Stop codon	Anticodon	Intergenic nucleotides (bp)
trnI	J	1–62	62	–	–	GAT	0
noncoding region	–	63–642	580	–	–	–	0
trnQ	N	643–711	69	–	–	TTG	–1
trnM	J	711–778	68	–	–	CAT	0
nad2	J	779–1,792	1014	ATA	TAG	–	2
trnW	J	1,795–1,860	66	–	–	TCA	–8
trnC	N	1,853–1,913	61	–	–	GCA	–1
trnY	N	1,913–1,977	65	–	–	GTA	1
cox1	J	1,979–3,509	1531	AAT	T	–	0
trnL(UUR)	J	3,510–3,573	64	–	–	TAA	0
cox2	J	3,574–4,261	688	ATA	T	–	0
trnK	J	4,262–4,332	71	–	–	CTT	–1
trnD	J	4,332–4,393	62	–	–	GTC	0
atp8	J	4,394–4,549	156	ATT	TAG	–	–7
atp6	J	4,543–5,211	669	ATG	TAA	–	–1
cox3	J	5,211–5,995	785	ATG	TA	–	–1
trnG	J	5,995–6,056	62	–	–	TCC	0
nad3	J	6,057–6,410	354	ATA	TAG	–	–2
trnA	J	6,409–6,473	65	–	–	TGC	–1
trnR	J	6,473–6,536	64	–	–	TCG	–1
trnN	J	6,536–6,602	67	–	–	GTT	–1
trnS(AGN)	J	6,602–6,670	69	–	–	TCT	–1
trnE	J	6,670–6,732	63	–	–	TTC	–2
trnF	N	6,731–6,794	64	–	–	GAA	0
nad5	N	6,795–8,511	1717	ATT	T	–	0
trnH	N	8,512–8,578	67	–	–	GTG	0
nad4	N	8,579–9,914	1336	ATG	T	–	–7
nad4L	N	9,908–10,195	288	ATG	TAA	–	2
trnT	J	10,198–10,261	64	–	–	TGT	0
trnP	N	10,262–10,327	66	–	–	TGG	5
nad6	J	10,333–10,830	498	ATG	TAA	–	–1
cytb	J	10,830–11,972	1143	ATG	TAA	–	–2
trnS(UCN)	J	11,971–12,035	65	–	–	TGA	18
nad1	N	12,054–13,004	951	ATA	TAG	–	0
trnL(CUN)	J	13,005–13,067	63	–	–	TAG	0
rrnL	N	13,068–14,342	1275	–	–	–	–8
trnV	N	14,335–14,403	69	–	–	TAC	0
rrnS	N	14,404–15,150	747	–	–	–	–14
Control region	–	15,137–16,951	1815	—	–	–	0

Table 4 AT-content, AT-skew, and GC-skew of five Lucanidae mitochondrial genomes

		Genome			PCGs			tRNAs			rRNAs		
Genus	Species	A+T%	AT-skew	GC-skew	A+T%	AT-skew	GC-skew	A+T%	AT-skew	GC-skew	A+T%	AT-skew	GC-skew
Lucanus	*mazama*	67.125	0.074	−0.272	65.646	−0.154	−0.068	72.426	0.020	0.141	72.192	−0.093	0.350
Prosopocoilus	*confucius*	67.851	0.077	−0.362	66.092	−0.154	−0.072	71.309	0.070	0.146	70.277	−0.065	0.431
Prosopocoilus	*gracilis*	66.091	0.106	−0.332	64.469	−0.137	−0.077	69.729	0.042	0.113	69.797	−0.102	0.417
Prosopocoilus	*blanchardi*	67.015	0.107	−0.265	65.220	−0.149	−0.074	70.146	0.050	0.096	70.620	−0.127	0.368
Sinodendron	*yunnanense*	75.061	0.035	−0.271	73.109	−0.140	−0.057	76.326	0.023	0.115	78.582	−0.035	0.370

nucleotides in both species. In both species *lrRNA* was located between *trnL* (*CUN*) and *trnV*, and was 1,278 bp in *S. yunnanense*, and 1,275 bp in *P. confucius*. *srRNA* was 767 bp in length and located upstream of the A + T–rich region in *S. yunnanense*, whereas 747 bp and upstream of the control region in *P. confucius*.

The mitogenome nucleotide composition was A (38.8%), C (15.8%), T (36.2%), and G (9.1%) in *S. yunnanense*, and A (36.5%), C (21.9%), T (31.3%), and G (10.3%) in *P. confucius*, with an AT content of 75% and 67.8%, respectively. The higher AT content of *S. yunnanense* is relative to the A + T–rich region. In five lucanid species, the heavy strand of the mitogenome has a positive AT-skew (0.035 to 0.107) and negative GC-skew (–0.362 to –0.271). In contrast, *S. yunnanense* rRNA genes have negative AT-skew and positive GC-skew. Interestingly, the AT-skew and GC-skew of PCGs were negative, and positive in the tRNA genes (Table 4). It has been proposed that nucleotide composition strand bias might be related to replication and transcription mechanisms (Hassanin et al. 2005, Song et al. 2016a).

PCGs and Codon Usage

All PCGs of *S. yunnanense* and *P. confucius* had the conventional start codon for invertebrate mitochondrial PCGs (ATN) with the

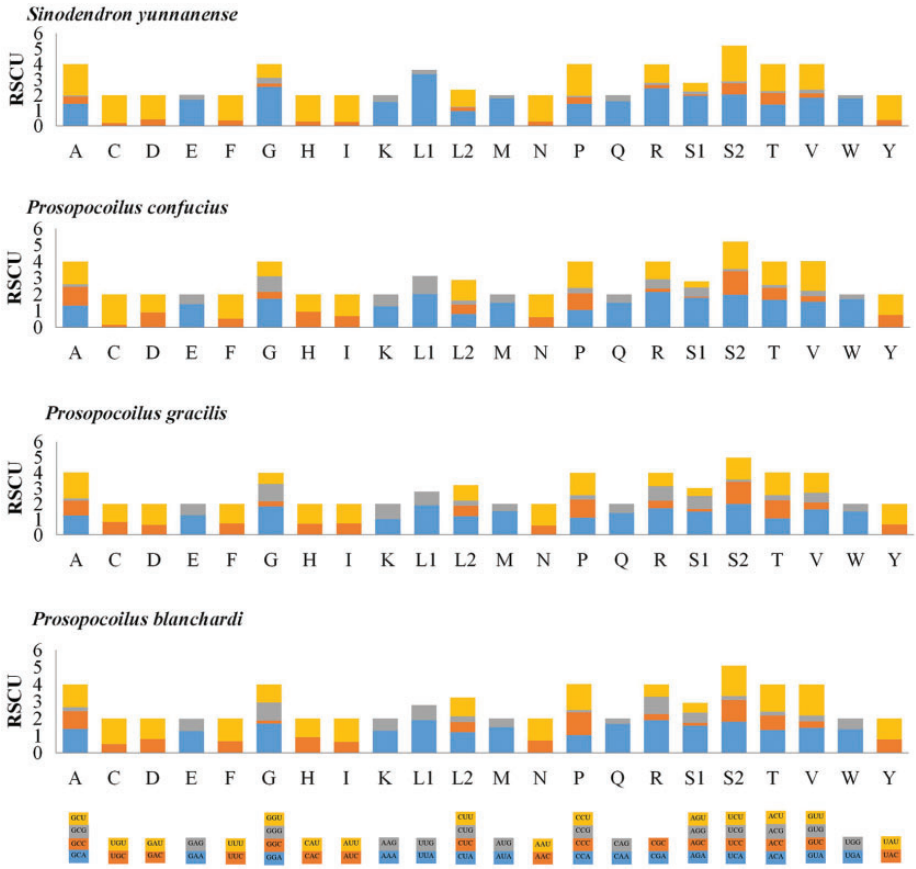

Fig. 3 RSCU in the *S. yunnanense, P. confucius, P. gracilis* and *P. blanchardi*. Lower bars give color codes. Y axis gives RSCU.

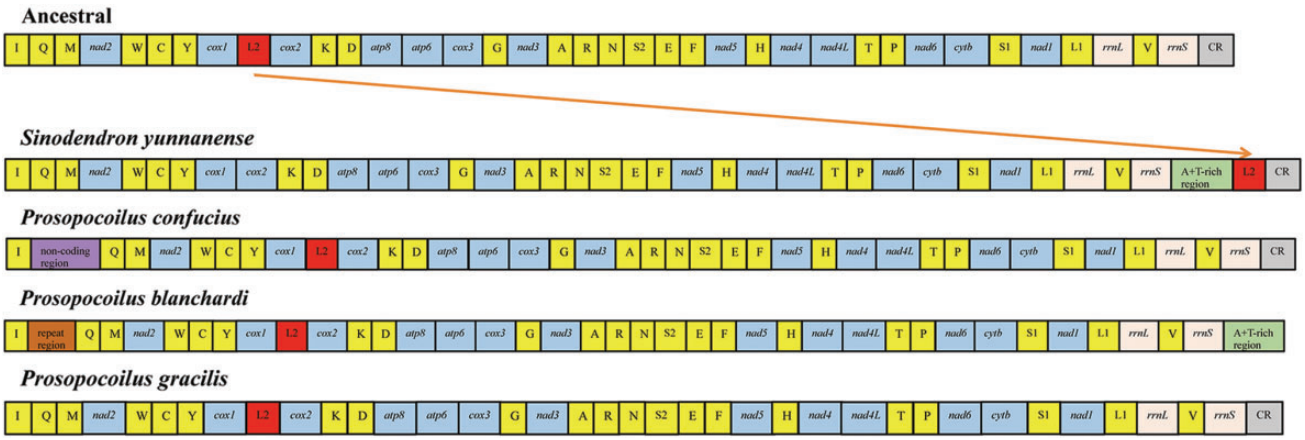

Fig. 4 Mitochondrial gene order of putative ancestor, *S. yunnanense, P. confucius, P. gracilis* and *P. blanchardi*. There is a unique gene rearrangement: *trnL*(*UUR*) located between the A + T–rich and control region in *S. yunnanense*. A non–coding region is in the same position of *P. confucius* and *P. blanchardi*. The mitochondrial gene order of *P. gracilis* is as per the ancestor.

exception of *cox1* (AAT) as found previously (Sheffield et al. 2008). In *S. yunnanense*, 10 (eight in *P. confucius*) of the 13 PCGs shared the typical termination codons TAA and TAG, whereas in the remaining genes an incomplete stop codon T or TA was inferred. It is not uncommon in insects that incomplete codon structures signal a halt of protein translation (Wu et al. 2014, Li 2014, Cheng et al. 2016). The RSCU analysis indicated that codons including A or T at the third position were overrepresented in comparison to other synonymous codons (Fig. 3), which could reflect nucleotide bias.

Intergenic Spacers and Noncoding Region

There was frequent occurrence of intergenic spacers in the two new mitogenomes, interspersed throughout the PCGs and RNA genes, and ranging from 1 to 580 bp in length (Tables 2 and 3). Among them, a 7-bp conserved motif (TACTAAA) upstream of *nad1* was proposed as a binding site for the mitochondrial transcription termination factor, and noted in various beetles and other insects (Cameron and Whiting 2008, Sheffield et al. 2009, Cameron 2014b, Kim et al. 2015, Wu et al. 2016, Yang et al. 2016).

A notable result in *P. confucius* was a 580 bp noncoding region located between *trnI* and *trnQ*, with three structural sections: $(C)_{14}$ in the initial third; $(A)_{14}$ in the center, and a $(C)_9$ in the latter third. This noncoding region is also present and at the same position in *P. blanchardi*, albeit longer (4,051 bp) and more complex (with 17 tandem repetitive sequence, each of which is composed of two subunits; Kim et al. 2015). These features appear to reflect both congeneric and phylogenetic distances (Fig. 6). Moreover, this noncoding region did not occur in *P. gracilis*, despite being a longstanding member of *Prosopocoilus*. Indeed, the mitogenome of this species was structured more typical of insects. Absence of this noncoding region in *P. gracilis* and paraphyly of *Prosopocoilus* in relation to this species (Fig. 6) raises a question on its taxonomic status.

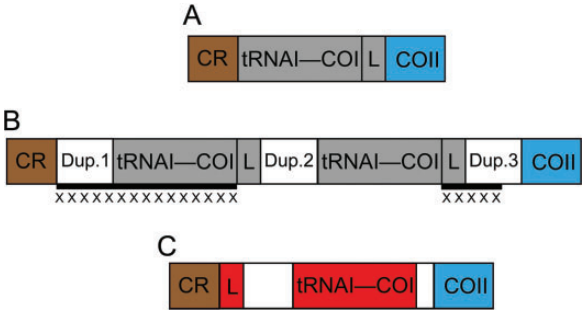

Fig. 5 The TDRL model was introduced to interpret the gene rearrangement in *S. yunnanense*. (A) The putative ancestral arrangement. (B) Genes in gray were duplicated to form the intermediate. (C) Some regions were lost to form the current arrangement in *S. yunnanense*. The X in B indicates the randomly lost regions.

New Gene Rearrangement

Gene arrangements of most insect mitogenomes are of the type exemplified by *Drosophila yakuba*, considered to be the ancestral state (Cameron 2014a). However, gene rearrangement events have been reported in many lineages (Timmermans and Vogler 2012, Song et al. 2016b), with a moderate frequency in Coleoptera (Wei and Chen 2011, Li et al. 2013). Although as the most diverse animal order (with >390,000 described species; Zhang 2013), there are merely ~80 reported mitogenomes of Coleopterans, belonging to 32 families and representing all four suborders (as of February 2016). Gene rearrangement events have been reported in 10 of these, in the form of reversal or absence of tRNAs; in Anthicidae (1), Byrrhoidea (3), Chrysomelidae (3), Curculionidae (1), Lycidae (1), and Mordellidae (1) (Timmermans and Vogler 2012, Nie and Yang 2014).

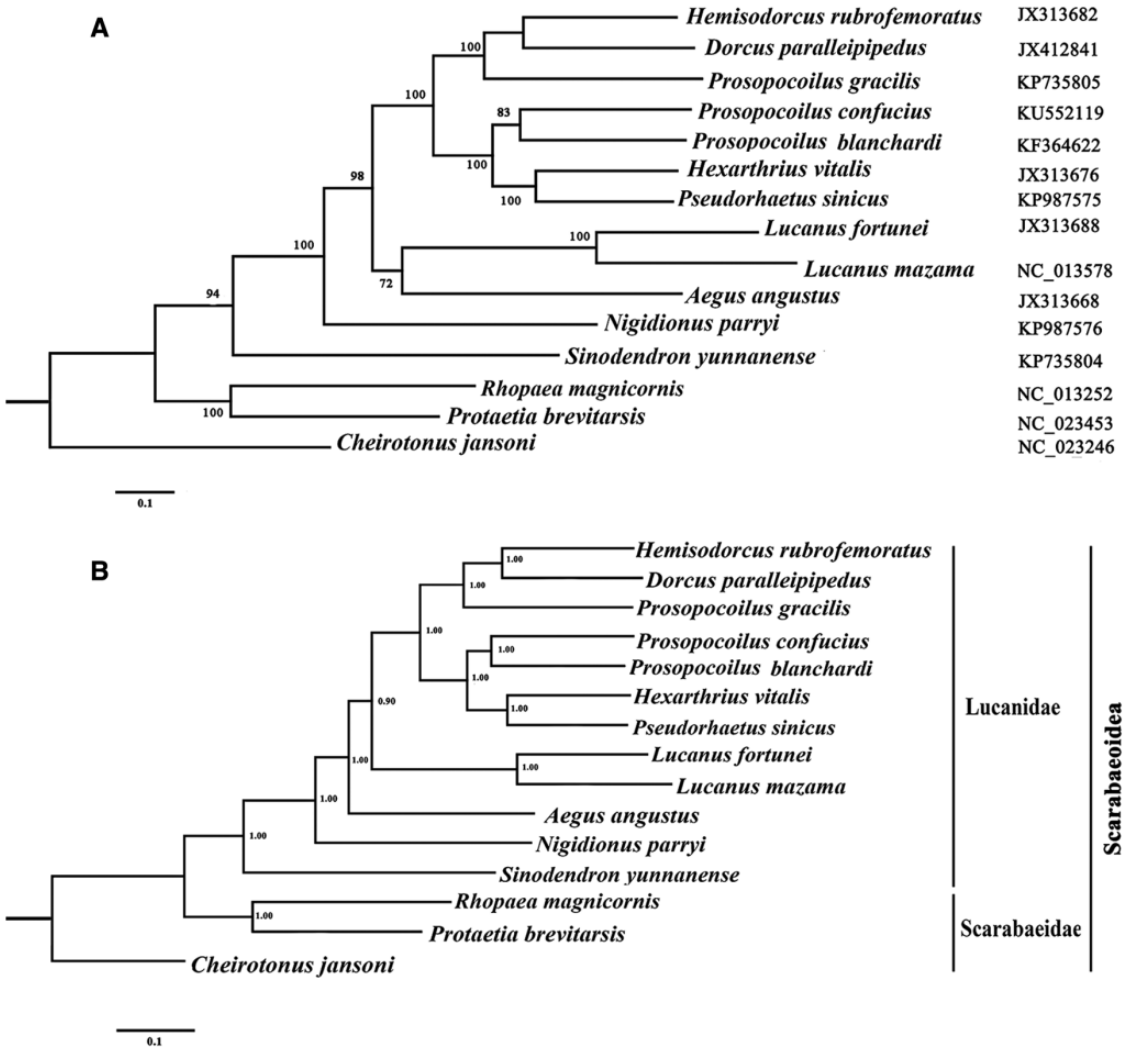

Fig. 6 Phylogenetic reconstruction of 15 beetles using a concatenated dataset of mitochondrial PCGs. (A) ML and (B) BI.

In this study, we report a novel gene rearrangement of *S. yunnanense*, a first for Lucanidae. *trnL* (*UUR*), ancestrally located between *cox1* and *cox2*, was translocated to a position between A + T–rich region and control region (Fig. 4). Moreover, intergenic spacers were observed between *trnL (UUR)* and *trnI*, and *cox1* and *cox2*, which is thought to be evidence of random gene losses (San et al. 2006). The TDRL model (tandem duplication followed by random loss; Moritz et al. 1987) was introduced to interpret the transposition of *trnL* (*UUR*) (Fig. 5). Additionally, recombination is another possible mechanism since illicit priming is unlikely due to the lack of a replication origin in this region (Wei et al. 2009). Data for more representatives are needed to determine the universality of the rearrangement in Lucanidae and to better understand the causal mechanisms.

Phylogenetic Analysis

ML and BI phylogenies were reconstructed using a supermatrix of the 13 PCGs. The phylogeny showed that Lucanidae was a monophyletic group and *S. yunnanense* was sister to other Lucanidae (Fig. 6). Similarly, the genetic distance between this species and others was high (0.285–0.359) (Supp Table 1 [online only]). These results are consistent with previous studies based on morphological characters (Holloway 1960, 2007) and molecular data (Kim and Farrell 2015).

The species *P. confucius* and *P. blanchardi* were recovered as sister taxa (1.00 BPP, 83% MLB), and had low genetic distance (0.241; Supp Table 1 [online only]). This result is consistent with their traditional taxonomy. These two species have long been considered archetypal *Prosopocoilus*, sharing characters such as large, stout and shiny body, with straight and well developed mandibles in the males. However, the genus *Prosopocoilus* formed a polyphyly due to the distinctive positioning of *P. gracilis* (1.00 BPP, 100% MLB), sister to the pair *Dorcus paralleipipedus* and *Hemisodorcus rubrofemoratus*. The genetic distance among the three taxa of *Prosopocoilus* is 0.241, 0.287, and 0.296 (Supp Table 1 [online only]), whereas the value between *P. gracilis* and *D. paralleipipedus* is 0.267, and 0.260 with *H. rubrofemoratus* (Supp Table 1 [online only]). The status of *P. gracilis* has been revised often in morphological taxonomy. *P. gracilis* was first erected by Benesh (1950). Séguy (1954) established the genus *Epidorcus*, including *P. gracilis* and a congener, despite a lack of convincing generic characters (see Zhong et al. 2014). Actually, its genital characters are quite distinct, and much more similar to those of species of *Serrognathus* (Wan 2007). Huang and Chen (2013) resurrected the genus *Epidorcus* for *P. gracilis* and its congeners after morphological analysis although they acknowledged characters shared by *Epidorcus* and *Serrognathus* species.

In our study, the phylogenomic analysis supported the morphological conclusion, i.e., *P. gracilis* should be removed from *Prosopocoilus*. Instead, *P. gracilis* probably belongs in *Dorcus* (*s.l.*). This is just one of many issues warranting more comprehensive work in Lucanidae phylogenetics, including higher-level classification, doubtful monophylies of some key genera, and species/subspecies boundaries and nomenclature, all of which would benefit from more comprehensive genetic data.

Acknowledgments

We are very grateful to Dr. Hong–liang Shi (Beijing Forestry University, Beijing, P. R. China) for donation of fresh specimens. We would like to thank Professor Alfried Vogler (Natural History Museum, London, UK), Dr. Martijn Timmermans (Middlesex University, London, UK) for help during a visit from the corresponding author; to Professor Wan-zhi Cai, Dr. Hu Li (China Agricultural University, Beijing, P. R. China), Dr. Shu-jun Wei (Institute of Plant and Environmental Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing, P. R. China) and Dr. Jia-xing Huang (Institute of Apicultural Research, China Academy of Agricultural Science, Beijing, P. R. China) for their advice on this article. This study was supported by the National Natural Science Foundation of China (grant numbers 31201745, 31071954, and 31572311).

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

References Cited

Ahrens, D., J. Schwarzer, and A. P. Vogler. 2014. The evolution of scarab beetles tracks the sequential rise of angiosperms and mammals. Proc. R. Soc. 281: 203–212.

Arrow, G. J. 1950. The Fauna of India Including Pakistan, Ceylon, Burma and Malaya. Coleoptera, Lamellicornia, Lucanidae and Passalidae. Volume IV, pp.1–275. Taylor & Francis Ltd., London, UK.

Balke, M., I. Ribera, and A. P. Vogler. 2004. MtDNA phylogeny and biogeography of Copelatinae, a highly diverse group of tropical diving beetles (Dytiscidae). Mol. Phylogenet. Evol. 32: 866–880.

Bartolozzi, L., and E. Sprecher-Uebersax. 2006. Lucanidae. pp. 63–76. *In* I. Lobl and A. Smetana (eds.), Catalogue of Palaearctic Coleoptera, Vol. 3: Scarabaeoidea – Scirtoidea – Dascilloidea – Buprestoidea – Byrrhoidea,. Published by Apollo Books, Vester Skerninge, Denmark.

Benesh, B. 1950. Descriptions of new species of stagbeetles from Formosa and the Philippines (Coleoptera: Lucanidae). Pan-Pacific Ent. 26: 11–18.

Benesh, B. 1960. Lucanidae. Coleopterorum cataloguss Supplementa. 8, pp. 1–178. W. Junk, Gravenhage, Netherlands.

Cameron, S. L. 2014a. Insect mitochondrial genomics: Implication for evolution and phylogeny. Ann. Rev. Ent. 59: 95–117.

Cameron, S. L. 2014b. How to sequence and annotate insect mitochondrial genomes for systematic and comparative genomics research. Syst. Ent. 39: 400–411.

Cameron, S. L., and M. F. Whiting. 2008. The complete mitochondrial genome of the tobacco hornworm, *Manduca sexta*, (insecta: Lepidoptera: Sphingidae), and an examination of mitochodnrial gene variability within butterflies and moths. Gene. 408: 112–123.

Cheng, X. F., P. Zhang, D. N. Yu, K. B. Storey, and J. Y. Zhang. 2016. The complete mitochondrial genomes of four cockroaches (Insecta: Blattodea) and phylogenetic analyses within cockroaches. Gene. 586: 115–122.

Didier, R., and E. Séguy. 1953. Catalogue illustré des lucanides du globe. Texte encyclopédie entomologique serie A. 27: 1–223. Paul Lechevalier, Paris, France.

Du, C., S. He, X. Song, Q. Liao, X. Y. Zhang, and B. S. Yue. 2016. The complete mitochondrial genome of *Epicauta chinensis* (Coleoptera:Meloidae) and phylogenetic analysis among Coleopteran insects. Gene. 578: 274–280.

Fujita, H. 2010. The Lucanid Beetles of the World. Mushi-Sha's Iconographic Series of Insects. 6:1–472. Mushi-sha, Tokyo.

Grant, J. R., and P. Stothard. 2008. The CGView Server: a comparative genomics tool for circular genomes. Nucleic. Acids. Res. 36: W181–W184.

Hassanin, A., N. Léger, and J. Deutsch. 2005. Evidence for multiple reversals of asymmetric mutational constraints during the evolution of the mitogenome of Metazoa, and consequences for phylogenetics inferences. Syst. Biol. 54: 277–298.

Holloway, B. A. 1960. Taxonomy and phylogeny in the Lucanidae (Insecta: Coleoptera). Rec. Dom. Museum. 3: 321–365.

Holloway, B. A. 2007. Lucanidae (Insecta: Coleoptera). Fauna of New Zealand. 61. Pp.1–254. Wellington, New Zealand.

Hosoya, T., and K. Araya. 2005. Phylogeny of Japanese stag beetles (Coleoptera: Lucanidae) inferred from 16S mtrRNA gene sequences, with reference to the evolution of sexual dimorphism of mandibles. Zool. Sci. 22: 1305–1318.

Hosoya, T., M. Honda, and K. Araya. 2001. Genetic variations of 16S rRNA gene observed in *Ceruchus lignarius* and *Dorcus rectus rectus* (Coleoptera: Lucanidae). Entomol. Sci. 4: 335–344.

Howden, H. F. 1982. Larval and adult characters of Frickius Germain, its relationship to the Geotrupini, and a phylogeny of some major taxa in the Scarabaeoidea (Insecta: Coleoptera). Can. J. Zool. 60: 2713–2724.

Huang, H., and C. C. Chen. 2013. Stag beetles of China II, pp. 41–113. Formosa Ecological Company, Taiwan.

Huang, J. P., and C. P. Lin. 2010. Diversification in subtropical mountains: Phylogeography, Pleistocene demographic expansion, and evolution of polyphenic mandibles in Taiwanese stag beetle, *Lucanus formosanus*. Mol. Phylogenet. Evol. 57: 1149–1161.

Huelsenbeck, J. P., and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogenenetic trees. Biometrics. 17: 754–755.

Kim, S. I., and B. D. Farrell. 2015. Phylogeny of World Stag Beetles (Coleoptera: Lucanidae) Reveals a Gondwanan Origin of Darwin's Stag Beetle. Mol. Phylogenet. Evol. 6: 35–48.

Kim, M. J., G. Kim, S. R. Kim, and I. Kim. 2015. Complete mitochondrial genome of the two–spotted stag beetle, *Metopodontus blanchardi* (Coleoptera: Lucanidae). Mitochondr. DNA. 26: 307–309.

Krajcik, M. 2001. Lucanidae of the World. Catalogue - Part I, Checklist of the Stag Beetles of the World (Coleoptera: Lucanidae). M. Krajcik, Most.

Kubota, K., Y. Nagahata, H. Ikeda, N. Kubota, H. Otobe, and K. Umetsu. 2011. Diversification process of stag beetles belonging to the genus *Platycerus* Geoffroy (Coleoptera: Lucanidae) in Japan based on nuclear and mitochondrial genes. Entomol. Sci. 14: 411–427.

Lawrence, J. F., and A. F. Newton. 1995. Families and subfamilies of Coleoptera (with selected genera, notes, references and data on family-group names). *In* J., Pakaluk, S.A., Slipinski (eds.), Biology, phylogeny, and classification of Coleoptera: papers celebrating the 80th birthday of Roy A. Crowson, pp. 779–106. Museum i Instytut Zoologii PAN, Warszawa, Poland.

Li, T. 2014. Phylogenomic studies of Lygaeoidea (Hemiptera: Heteroptera) based on the mitochondrial genomes. Ph.D. dissertation, Nankai University, Tianjin.

Li, H., R. Shao, F. Song, X. Zhou, and Q. Yang. 2013. Mitochondrial genomes of two barklice, *Psococerastis albimaculata* and *Longivalvus hyalospilus* (Psocoptera: Psocomorpha): contrasting rates in mitochondrial gene rearrangement between major lineages of Psocodea. PLoS One. 8: e61685.

Lin, C., J. Huang, Y. Lee, and M. Chen. 2011. Phylogenetic position of a threatened stag beetle, *Lucanus datunensis* (Coleoptera: Lucanidae) in Taiwan and implications for conservation. Conservation Genetics. 12: 337–341.

Mckenna, D. D., D. Farrell, M. S. Caterino,C. W. Farnum,D. C. Hawks, D. R. Maddison, A. E. Seago, A. E. Z. Short, A. F. Newton, and M. K. Thayer. 2015. Phylogeny and evolution of Staphyliniformia and Scarabaeiformia: forest litter as a stepping stone for diversification of nonphytophagous beetles. Syst. Entomol. 40: 35–60.

Moritz, C., T. Dowling, and W. Brown. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Ann. Rev. Eco. Syst. 18: 269–292.

Nie, R. E., and X. K. Yang. 2014. Research progress in mitochondrial genomes of Coleoptera. Acta Ent. Sin. 57: 860–868.

Perna, N. T., and T. D. Kocher. 1995. Patterns of nucleotide composition at fourfold degenerate sites of animal mitochondrial genomes. J. Mol. Evol. 41: 353–358.

Posada, D. 2008. jModelTest: Phylogenetic model averaging. Mol. Biol. Evol. 25: 1253–1256.

Rambaut, A. 2016. FigTree: Tree Figure Drawing Tool, Version 1.4.3. Institute of Evolutionary Biology, University of Edinburgh, http://tree.bio.ed.ac.uk/.

San, M. D., J. Gower, R. Zardoya, and M. Wilkinson. 2006. A hotspot of gene order rearrangement by tandem duplication and random loss in the vertebrate mitochondrial genome. Mol. Biol. Evol. 23: 227–234.

Scholtz, C. H., J. Browne, and J. Kukalova-Peck. 1994. Glaresidae, archaeopteryx of the Scarabaeoidea. Syst. Entomol. 19: 45–173.

Séguy, E. 1954. Les hemisodorcites du Museum de Paris. Rev. Franc. Ent. 21: 184–194.

Sheffield, N. C., Song, S. L. Cameron, and M. F. Whiting. 2008. A Comparative Analysis of Mitochondrial Genomes in Coleoptera (Arthropoda: Insecta) and Genome Descriptions of Six New Beetles. Mol. Biol. Evol. 25: 2499–2509.

Sheffield, N. C., Song, S. L. Cameron, and M. F. Whiting. 2009. Nonstationary evolution and compositional heterogeneity in beetle mitochondrial phylogenomics. Syst. Biol. 58: 381–394.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87: 651–701.

Song, F., H. Li, P. Jiang, X. Zhou, J. P. Liu, C. H. Sun, A. P. Vogler, and W. Z. Cai. 2016b. Capturing the phylogeny of Holometabola with Mitochondrial Genome Data and Bayesian Site–Heterogeneous MixtureModels. Genome Biol. Evol. 8: 1411–1426.

Song, F., H. Li, R. Shao, A. Shi, X. S. Bai, X. R. Zheng, E. Heiss, and W. Z. Cai. 2016a. Rearrangement of mitochondrial tRNA genes in flat bugs (Hemiptera Aradidae). Sci. Rep. 6: 25725.

Stamatakis, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post–analysis of large phylogenies. Bioinformatics. 30: 1312–1313.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30: 2725–2729.

Timmermans, M. J. T. N., Dodsworth, C. Culverwell, L. Bocak, D. Ahrens, D. Littlewood, J. Pons, and A. P. Vogler. 2010. Why barcode? High-throughput multiplex sequencing of mitochondrial genomes for molecular systematics. Nucleic Acids. Res. 38: e197.

Timmermans, M. J. T. N., and A. P. Vogler. 2012. Phylogenetically informative rearrangements in mitochondrial genomes of Coleoptera, and monophyly of aquatic elateriform beetles (Dryopoidea). Mol. Phylogenet. Evol. 63: 299–304.

Tsai, C. L., Wan, and W. B. Yeh. 2014. Differentiation in stag beetles, *Neolucanus swinhoei* complex (Coleoptera: Lucanidae): four major lineages caused by periodical Pleistocene glaciations and separation by a mountain range. Mol. Phylogenet. Evol. 78: 245–259.

Tsai, C. L., and W. B. Yeh. 2016. Subspecific differentiation events of montane stag beetles (Coleoptera, Lucanidae) endemic to formosa island. PloS One. 11: e0156600.

Wan, X. 2007. Study on the Systematics of Lucanidae (Coleoptera: Scarabaeoidea). 1–343.

Wei, S. J., and X. X. Chen. 2011. Progress in research on the comparative mitogenomics of insects. Chin. J. Appli. Ent. 48: 1573–1585.

Wei, S. J., Shi, J. H. He, M. Sharkey, and X. X. Chen. 2009. The complete mitochondrial genome of *Diadegma semiclausum* (Hymenoptera: Ichneumonidae) indicates extensive independent evolutionary events. Genome. 52: 308–319.

Wu, Y. Y., Y. Y. Cao, J. Fang, and X. Wan. 2016. The first complete mitochondrial genome of stag beetle from China, *Prosopocoilus gracilis* (Coleoptera, Lucanidae). Mitochondr. DNA. 27: 1–2.

Wu, Q. L., Q. Li, Y. Gu, B. C. Shi, C. Achterberg, S. J. Wei, and X. X. Chen. 2014. The complete mitochondrial genome of *Taeniogonalos taihorina* (Bischoff) (Hymenoptera: Trigonalyidae) reveals a novel gene rearrangement pattern in the Hymenoptera. Gene. 543: 76–84.

Yang, J., F. Ye, and Y. Huang. 2016. Mitochondrial genomes of four katydids (Orthoptera: Phaneropteridae): New gene rearrangements and their phylogenetic implications. Gene. 575: 702–711.

Yuan, M. L., L. Zhang,L. Zhang,Z. L. Guo, Y. J. Liu, Y. Y. Shen, and R. F. Shao. 2016. High-level phylogeny of the Coleoptera inferred with mitochondrial genome sequences. Mol. Phylogenet. Evol. 104: 99–111.

Zhang, Z. Q. 2013. Phylum Arthropoda. Zootaxa. 3703: 17–26.

Zhong, F., M. Bai, and X. Wan. 2014. Taxonomic revision of *Prosopocoilus gracilis* (Saunders, 1854) and its allied species from China (Coleoptera, Lucanidae). Acta. Zootaxonomica Sinica. 39: 136–148.