The complete mitochondrial genome of stag beetle *Lucanus cervus* (Coleoptera: Lucanidae) and phylogenetic analysis

Dan Chen[1], Jing Liu[1], Luca Bartolozzi[2] and Xia Wan[1]

[1] School of Resources and Environmental Engineering, Anhui University, Hefei, Anhui, China
[2] Department of Entomology, Natural History Museum of the University of Florence, Zoological Section "La Specola", Natural History Museum of the University of Florence, Florence, Italy

ABSTRACT

Background: The stag beetle *Lucanus cervus* (Coleoptera: Lucanidae) is widely distributed in Europe. Habitat loss and fragmentation has led to significant reductions in numbers of this species. In this study, we sequenced the complete mitochondrial genome of *L. cervus* and reconstructed phylogenetic relationships among Lucanidae using complete mitochondrial genome sequences.

Methods: Raw data sequences were generated by the next generation sequencing using Illumina platform from genomic DNA of *L. cervus*. The mitochondrial genome was assembled by IDBA and annotated by MITOS. The aligned sequences of mitochondrial genes were partitioned using PartitionFinder 2. Phylogenetic relationships among 19 stag beetle species were constructed using Maximum Likelihood (ML) method implemented in IQ-TREE web server and Bayesian method implemented in PhyloBayes MPI 1.5a. Three scarab beetles were used as outgroups.

Results: The complete mitochondrial genome of *L. cervus* is 20,109 bp in length, comprising 13 protein-coding genes, 22 transfer RNA genes, two ribosomal RNAs and a control region. The $A + T$ content is 69.93% for the majority strand. All protein-coding genes start with the typical ATN initiation codons except for *cox1*, which uses AAT. Phylogenetic analyses based on ML and Bayesian methods shown consistent topologies among Lucanidae.

INTRODUCTION

Many species from the family of Lucanidae (stag beetles) are facing conservation concerns in many regions of the world. Some stag beetles are used as flagship species to raise public awareness of their protection (*New, 2018*). Understanding the phylogenetic relationships among species of Lucanidae will be helpful to reveal the evolutionary history of this special group and conduct conservation in practice. Previous studies have tried to construct the phylogenetic relationships among Lucanidae using partial mitochondrial and nuclear genes (*Hosoya & Araya, 2005*; *Kim & Farrell, 2015*). With the development of next-generation sequencing, it is possible to get more molecular markers for phylogenetic analysis to get more robust relationships (*Fagua et al., 2018*; *Li et al., 2015*).

Family	Species	GenBank Accession number	References
Table 1 GenBank accession numbers of species used in this study.			
Lucanidae	*Cyclommatus vitalisi*	MF037205	*Liu et al. (2017)*
	Dorcus curvidens hopei	MF612067	*Chen et al. (2018)*
	Dorcus hansi	MF621709	Direct submission
	Dorcus parallelipipedus	KT876887	*Linard et al. (2016)*
	Lucanus cervus	MN580549	This study
	Lucanus fortunei	MF614013	Direct submission
	Lucanus mazama	FJ613419	*Sheffield et al. (2009)*
	Macrodorcas seguyi	MF612068	*Chen et al. (2018)*
	Neolucanus maximus	MF401425	Direct submission
	Nigidionus parryi	KP987576	Direct submission
	Nigidius sp	JX412771	Direct submission
	Odontolabis cuvera fallaciosa	MF908524	*Wang et al. (2018)*
	Prosopocoilus blanchardi	KF364622	*Kim et al. (2015)*
	Prosopocoilus confucius	KU552119	*Lin et al. (2017)*
	Prosopocoilus gracilis	KP735805	*Wu et al. (2015)*
	Pseuderhaetus sinicus	KP987575	Direct submission
	Prismognathus prossi	MF614014	*Jing et al. (2018)*
	Rhaetus westwoodi	MG159815	*Jing et al. (2018)*
	Serrognathus platymelus	MF612070	Direct submission
	Sinodendron yunnanense	KP735804	*Lin et al. (2017)*
Scarabaeidae	*Cheirotonus jansoni*	NC023246	*Shao et al. (2014)*
	Protaetia brevitarsis	NC023453	*Kim et al. (2014)*
	Rhopaea magnicornis	NC013252	*Cameron et al. (2009)*

The mitochondrial genome is a double-stranded circular molecule. Due to its features of material inheritance, stability of gene content and easy sequencing, it has been widely used in phylogenetic inference in many groups of animal from higher levels to lower levels (*Li et al., 2016*; *Zheng et al., 2018*). Currently, 20 mitochondrial genomes were used from the family of Lucanidae, representing four tribes, 14 genera (Table 1). Compared to the number of known species in Lucanidae, the sequenced mitochondrial genomes are still limited.

The stag beetle *L. cervus* is one of the species that is in a protected status in many European countries. This species has been included in Annex II of the EU Habitats Directive since 1992 (Council Directive 92/43/EEC of 21 May 1992 on the Conservation of Natural Habitats and of Wild Fauna and Flora) (*Bardiani et al., 2017*), and the European Union finances "Life" Projects to protect the species every year. Nevertheless, populations of this stag beetle have dramatically declined in recent years in several European countries, mainly due to fragmentation and loss of suitable habitats (*Campanaro et al., 2016*). *L. cervus* is the largest European beetle and is widely distributed in Europe (*Harvey et al., 2011*). It is noteworthy that *L. cervus* spends most of its life in larval and

pupal stage; its saproxylic larva feeds in rotten wood or roots in deciduous broad-leaved forests, in lowland and medium-altitude areas (*Tini et al., 2017*). Previous works revealed that the species shows a similar life history across its range, but the habitat conditions in different European countries (such as climate, temperature, humidity, and food availability) significantly affects the larval life duration and also the adult body size (*Harvey et al., 2011*). The larvae show a marked increase in size in favorable conditions; the adult, especially in male specimens, can show strong morphological variability in size and shape (*Romiti et al., 2017*).

In certain areas of the S. Mediterranean region (e.g., Latium in Italy), *L. cervus* can be morphologically confused and can even have hybrids with the congeneric *L. tetraodon* (a species not included in protection lists). In some areas of Central Italy, *L. cervus* has a sympatric occurrence with the closely related species *L. tetraodon*, and individuals with a mosaic of morphological traits can be found, making the species assignment on a simple morphological basis nearly impossible. More or less similar problems can raise at the south-eastern borders of the Palearctic distribution of *L. cervus* (e.g., in Turkey or Near East) where populations of other closely related species of *Lucanus* converge (*Cox et al., 2013*).

In this study, we sequenced the complete mitochondrial genome of *L. cervus* using next-generation sequencing; we inferred phylogenetic relationships among 20 stag beetle species. The aim of this study is to contribute to research on phylogeny of the family Lucanidae and to provide genomic information that could be useful for better management and conservation strategies that impact on this species.

MATERIALS AND METHODS

Sample collection and DNA extraction

The voucher specimen of *L. cervus* was collected in Ukraine in July 2017 and deposited in the Museum of Anhui University with the accession number Lu166. Total genomic DNA was extracted from the muscle of *L. cervus* using the Qiagen DNAeasy Kit. The sequence was submitted to GenBank and assigned Accession Number MN580549 and converts into graphical maps utilized Organellar Genome DRAW in the GenBank format (*Greiner, Lehwark & Bock, 2019*).

Polymerase chain reaction amplification, and sequencing

Polymerase chain reaction (PCR) amplification reactions for *cox1*, *cytb*, and *rrnl* were carried out in 25 μL volumes containing 2 μL template DNA, 12.5 μL 2 × EasyTaq SuperMix (+dye), 1 μM of each primer (forward and reverse), and 8.5 μL sterile double-distilled water. Three fragments were amplified using common primers for *L. cervus* (Table 2). The PCR amplifications were performed under the conditions as previously described in *Lin et al. (2017)*. Sanger sequencing was used to obtain the fragments of *cox1*, *cytb* and *rrnl*. A library was prepared using Truseq nano DNA kit (Illumina) with an insert size of 450 bp, and sequenced on the Illumina HiSeq 2,000 platformat Berry Genomics, Beijing, China. Raw reads were trimmed using Trimmomatic, with matched to the adaptor sequence >15 bp, poly-Ns (>15 bp Ns) or >75 bp bases with

Table 2 Information of the 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, Ribera & Vogler (2004)*
	Cytb–R2	AAAAGAAARTATCATTCAGGTTGAAT	*Balke, Ribera & Vogler (2004)*
rrnl	16S–F1	CCGGTTTGAACTCAGATCATG	*Hosoya, Honda & Araya (2001)*
	16S–R1	TAATTTATTGTACCTTGTGTATCAG	*Hosoya, Honda & Araya (2001)*

quality score ≤3 (*Bolger, Lohse & Usadel, 2014*) and we obtained 98,22,905 clean 250 bp paired-end reads with the Q30 = 93.25.

Genome assembly, annotation and analysis

High-quality reads were de novo assembled using IDBA-UD (*Peng et al., 2012*) with the parameters: minimum k value 80, similarity threshold 98%, and maximum k value 240. The *cox1* (555 bp), *cytb* (404 bp) and *rrnl* (865 bp) fragments of *L. cervus* were used to identify mitochondrial assemblies using BLAST searches with ≥98% similarity (*Altschul et al., 1990*). To verify the accuracy of the assembly, clean reads were mapped onto the obtained fragments using "Map to reference" option of Geneious Prime 2019.1.1 (https://www.geneious.com) with maximum mismatches per read 2%; maximum ambiguity two; minimum overlap identity 97%; minimum overlap 100 bp; and no gaps. When genome was assembled in full length, the two ends of the contig overlapped, indicating circular organization of the mitochondrial genome. Finally, we obtained the mitochondrial genome of *L. cervus* with the mean coverage of 252.

The mitochondrial genome sequence was annotated using the MITOS web server (*Bernt et al., 2013*). tRNA genes and their secondary structures were inferred using tRNAscan-SE v2.0 (*Lowe & Chan, 2016*). In addition to 16S ribosomal RNA (*rrnl*, lrRNA), and 12S ribosomal RNA (*rrns*, srRNA), *trnS*1 (TCT) also determined according to sequence similarity with related species because of can't identified by tRNAscan-SE. The codon usage, nucleotide compositions of PCGs were calculated with MEGA 7 (*Kumar, Stecher & Tamura, 2016*). Composition skew analysis was conducted according to formulas AT skew = $(A − T)/(A + T)$ and GC skew = $(G − C)/(G + C)$ (*Perna & Kocher, 1995*).

Phylogenetic analysis

We retrieved 19 complete or near complete mitogenomic sequences from GenBank (Table 1) and added a newly sequenced *L. cervus* generating a dataset of 20 species. Three mitochondrial genomes from the family Scarabaeidae were used as outgroups. Twenty species represent 14 genera of Lucanidae. The lacked genes were treated as missing data in mitochondrial genomes of *Dorcus parallelipipedus* (lack of *rrnl*), *Nigidius* sp. (lack of *rrns*), and *Nigidionus parryi* (lack of *nad2*, *cox1*, *rrns*, *rrnl*). The dataset containing nucleotide sequences of 13 PCGs-condon12 with the third position removed and two rRNA genes (*rrns* and *rrnl*) of 22 species. Each PCG was aligned individually based on codon-based multiple alignments using MEGA 7 (*Kumar, Stecher & Tamura, 2016*). PCG12 was

produced by removing the third codon with MEGA 7 (*Kumar, Stecher & Tamura, 2016*). Two rRNA genes (*rrnl* and *rrns*) was aligned individually using MAFFT 7 server with the G-INS-i strategy (*Katoh, Rozewicki & Yamada, 2017*; *Kuraku et al., 2013*), and we used Gblocks v0.91b Server (*Castresana, 2000*; *Talavera & Castresana, 2007*) to select conserved blocks from multiple alignments. Final, we concatenated alignments using Geneious Prime 2019.1.1 (https://www.geneious.com). The PCG12RNA dataset was partitioned using PartitionFinder 2 (*Lanfear et al., 2017*) for maximum likelihood (ML) analyses.

Bayesian inference (BI) and ML analyses were conducted using PhyloBayes MPI 1.5a (*Lartillot, Lepage & Blanquart, 2009*) and IQ-TREE web server, respectively. The BI analysis was carried out on the CIPRES Science Gateway (*Miller, Pfeiffer & Schwartz, 2011*). We chose the site-heterogeneous mixture model (CAT + GTR) (*Song et al., 2016*). Two independent chains starting from a random tree were run for 20,000 cycles, with trees being sampled every 10 cycles. The initial 25% trees of each MCMC run were discarded as burn-in. A consensus tree was computed from the remaining 1,500 trees combined from two runs, and the two runs converged at a maxdiff of less than 0.1. The best scheme for ML analyses see Table S1. For ML analyses, the "Auto" option was set under optimal evolutionary models, and the phylogenetic trees were constructed using an ultrafast bootstrap approximation approach with 10,000 replicates. Phylogenetic trees were viewed and edited in Figtree v1.4.3.

RESULTS

Genome organization and base composition

The complete mitochondrial genome of *L. cervus* is 20,109 bp in length. It is a closed circular molecule (Fig. 1), consisting of 22 tRNAs, two ribosomal RNA genes (*rrnl* and *rrns*), 13 PCGs and one control region as other typical stag beetles (*Lin et al., 2017*; *Liu et al., 2019*; *Wu et al., 2015*). Overall the mitogenome consisted of 37.48% *A*, 32.45% *T*, 19.05% *C* and 11.02% *G* with a highly biased *A* + *T* content of 69.93% (Table 3). Compared with *L. mazama* and *L. foutunei*, *L. cervus* has higher *A* + *T* content, especially in the control region. But the *A* + *T* content of *L. cervus* is at a medium level within the Lucanidae which has the variable base composition (*Chen et al., 2018*). Additionally, there is a negative *GC* skew, and a positive *AT* skew of *L. cervus* as other stag beetles (*Chen et al., 2018*; *Lin et al., 2017*; *Liu et al., 2019*, *2017*; *Jing et al., 2018*; *Wu et al., 2015*). The bias of base composition has important reference value for studying the mechanism of mitochondrial genome replication and transcription (*Wei et al., 2010*).

PCGs and codon usage

In PCGs, four (*nad4*, *nad4l*, *nad5*, *nad1*) of the 13 PCGs were coded on the N-strand, with the other nine genes (*cox1*, *cox2*, *cox3*, *atp8*, *atp6*, *nad2*, *nad3*, *nad6*, and *cytb*) were coded on the J-strand. Among the 13 PCGs, the longest was the *nad5* gene and the shortest was the *atp8* gene. The start codons of *cox1* are AAT whereas other 12 PCGs are ATN codons (Table 4). Seven of the 13 PCGs shared the typical termination codons TAA and TAG, while others use *TA* residue or a single *T* as the terminator codons (Table 4). It is
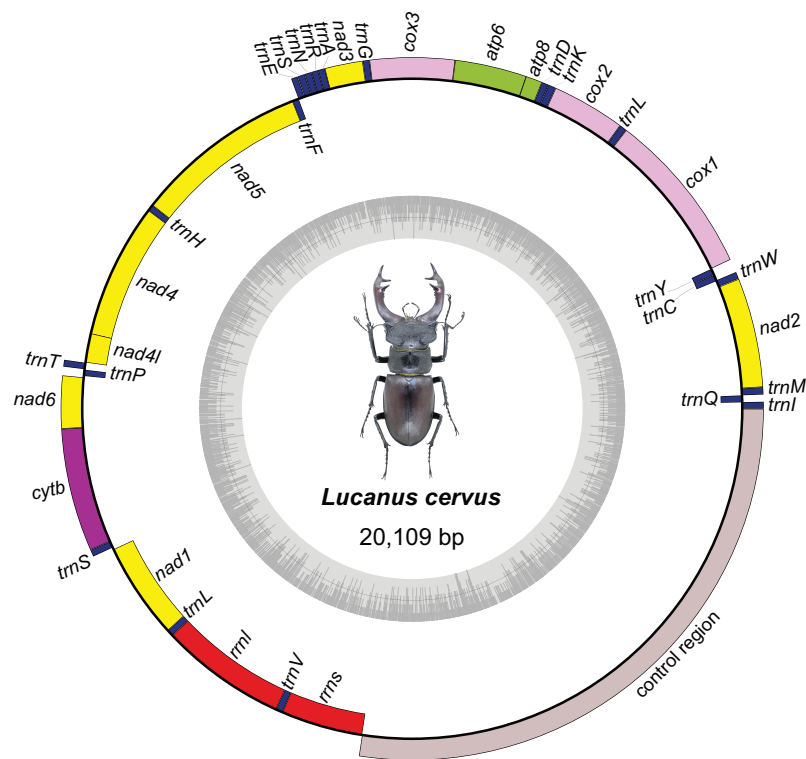
Figure 1 Mitochondrial genome map of *Lucanus cervus*. Different types of genes are represented by different colors in the genome map: *nad1*, *nad2*, *nad3*, *nad4*, *nad4l*, *nad5*, and *nad6* refer to nicotinamide adenine dinucleotide subunit indicated by yellow; *cox1*, *cox2*, and *cox3* refer to cytochrome oxidase subunit indicated by pink; *atp6* and *atp8* refer to ATP synthase F0 subunit indicated by green; *cytb* refers to cytochrome B indicated by purple; *rrnl* and *rrns* refers to the large and small subunit of ribosomal RNA genes respectively, and both of them indicated by red; all transfer RNA indicated by deep blue; control region indicated by gray.   Full-size ⊡ DOI: 10.7717/peerj.8274/fig-1

generally accepted that incomplete codon structures signal a halt of protein translation in insects and other invertebrates (*Cheng et al., 2016*).

tRNA and rRNA genes

All of the 22 tRNAs had a total length of 1,426 bp and range from 61 to 71 bp (Table 4), eight of the 22 tRNA-coding genes were located on the N-strand and others were located on the J-strand. Secondary structures predicted by the tRNA scan-SE suggested that all the tRNA genes in *L. cervus* adopted a typical clover-leaf structure, expect for *trnS1* (TCT) is absent due to the deficiency of the dihydrouridine arm, which is a typical feature of metazoan mitochondrial genomes (*Cameron, 2014*). The length of *rrnl* and *rrns* were 1,252 bp and 806 bp, respectively (Table 4).

Control region

The control region of *L. cervus* was located between *trnI* and *rrns* genes as normally with the length of 5516 bp; The $A + T$ contents, *AT*-skew and *GC*-skew is 70.59%, 0.14 and -0.23, respectively. There were seven poly-*T* (≥ 7) stretch, 24 poly-*A* (≥ 7) stretch, and one poly-*TA* (≥ 7) sequence in the long control region. Furthermore, two tandem repeats were

Table 3 Base composition of different regions of the mitochondrial genome of *Lucanus cervus*.

Region	Length (bp)	T (%)	C (%)	A (%)	G (%)	A + T (%)	AT skew	GC skew
atp6	668	38.62	20.36	29.49	11.53	68.11	−0.13	−0.28
atp8	156	37.82	19.87	35.90	6.41	73.72	−0.03	−0.51
cox1	1,531	34.81	20.18	29.33	15.68	64.14	−0.09	−0.13
cox2	684	36.40	20.61	30.70	12.28	67.11	−0.08	−0.25
cox3	785	35.54	21.66	28.41	14.39	63.95	−0.11	−0.20
cytb	1,141	36.02	22.26	29.18	12.53	65.21	−0.10	−0.28
nad1	951	26.18	19.87	43.43	10.52	69.61	0.25	−0.31
nad2	1,014	37.18	21.89	31.56	9.37	68.74	−0.08	−0.40
nad3	354	37.57	19.49	31.64	11.30	69.21	−0.09	−0.27
nad4l	1,334	25.64	19.42	45.65	9.30	71.29	0.28	−0.35
nad4l	288	26.74	15.28	48.96	9.03	75.69	0.29	−0.26
nad5	1,715	27.52	19.01	43.79	9.68	71.31	0.23	−0.33
nad6	498	38.35	18.47	33.94	9.24	72.29	−0.06	−0.33
rrnl	1,244	34.41	18.49	39.31	7.80	73.71	0.07	−0.41
rrns	806	35.86	17.37	38.21	8.56	74.07	0.03	−0.34
rRNAs	2,050	34.98	18.05	38.88	8.10	73.85	0.05	−0.38
tRNAs	1,426	34.22	15.5	38.78	11.5	73	0.06	−0.15
13PCGs	11,119	32.65	20.16	35.82	11.37	68.47	0.05	−0.28
Control region	5,516	30.49	18.11	40.10	11.29	70.59	0.14	−0.23
Whole genome	20,109	32.45	19.05	37.48	11.02	69.93	0.07	−0.27

found by online Tandem repeats finder (*Benson, 1999*), both of which were repeated three times; the length of the consensus sequence was 272 bp, 278 bp, respectively.

Phylogenetic analysis

Including the newly sequenced mitochondrial genome of *L. cervus*, a total of twenty mitochondrial genomes from Lucanidae were used for phylogenetic analysis to analyze their phylogenetic relationships. Both BI and ML analyses consistently showed phylogenetic relationships among Lucanidae (Fig. 2). *Sinodendron yunnanense* is an early branch in Lucanidae (BPP = 1, MLB = 100). Lucanini was not monophyletically supported, and the earliest branch lineage was comprised of genera *Neolucanus* and *Odontolabis*. *Prismognathus* and *Cyclommatus* were closely related to *Lucanus*, they clustered into a lineage and sister to Dorcini. Additionally, *L. cervus* and *L. fortunei* clustered into a lineage (BPP = 0.7, MLB = 73) and sister to *L. mazama* (BPP = 1, MLB = 100).

DISCUSSION

The control region is the most important non-coding region in the mitochondrial genome with extremely abundant A + T content, and the length variation is very large (*Zhang & Hewitt, 1997*; *Zhang, Szymura & Hewitt, 1995*), even among species having a close genetic relationship. *L. cervus* has a control region with the length of 5,516 bp, obviously longer than other Lucanini species reported in our previous work (*Chen et al., 2018*;

Table 4 **Characteristics of the mitochondrial genome of *Lucanus cervus*.** J and N indicates the Majority strand and Minority strand of mt genome, respectively.

Gene	Strand	Region	Length (bp)	Start codon	Stop codon	Anticodon	Intergenic nucleotides (bp)
trnI	J	1–64	64			GAT	0
trnQ	N	62–130	69			TTG	−3
trnM	J	130–197	68			CAT	−1
nad2	J	198–1,211	1,014	ATC	TAG		0
trnW	J	1,214–1,279	66			TCA	2
trnC	N	1,272–1,336	65			GCA	−8
trnY	N	1,337–1,400	64			GTA	0
cox1	J	1,402–2,932	1,531	AAT	T		1
trnL (CUN)	J	2,933–2,998	66			TAA	0
cox2	J	2,999–3,682	684	ATA	TAA		0
trnK	J	3,683–3,752	70			CTT	0
trnD	J	3,753–3,815	63			GTC	0
atp8	J	3,816–3,971	156	ATT	TAA		0
atp6	J	3,968–4,635	668	ATA	TA		−4
cox3	J	4,635–5,419	785	ATG	TA		−1
trnG	J	5,419–5,480	62			TCC	−1
nad3	J	5,481–5,834	354	ATT	TAG		0
trnA	J	5,833–5,897	65			TGC	−1
trnR	J	5,897–5,960	64			TCG	−1
trnN	J	5,960–6,023	64			GTT	−1
trnS (AGN)	J	6,024–6,090	67			TCT	0
trnE	J	6,092–6,153	62			TTC	1
trnF	N	6,152–6,214	63			GAA	−2
nad5	N	6,214–7,928	1,715	ATT	TA		−1
trnH	N	7,929–7,990	62			GTG	0
nad4	N	7,990–9,323	1,334	ATG	TA		−1
nad4l	N	9,317–9,604	288	ATG	TAA		−7
trnT	J	9,607–9,670	64			TGT	2
trnP	N	9,675–9,736	62			TGG	4
nad6	J	9,741–10,238	498	ATG	TAA		4
cytb	J	10,238–11,378	1,141	ATG	T		−1
trnS (UCN)	J	11,379–11,443	65			TGA	0
nad1	N	11,463–12,413	951	ATT	TAG		19
trnL (CUN)	N	12,414–12,476	63			TAG	0
rrnl	N	12,477–13,720	1,244				0
trnV	N	13,721–13,788	68			TAC	−8
rrns	N	13,788–14,593	806				−1
Control region	N	14,594–20,109	5,516				0
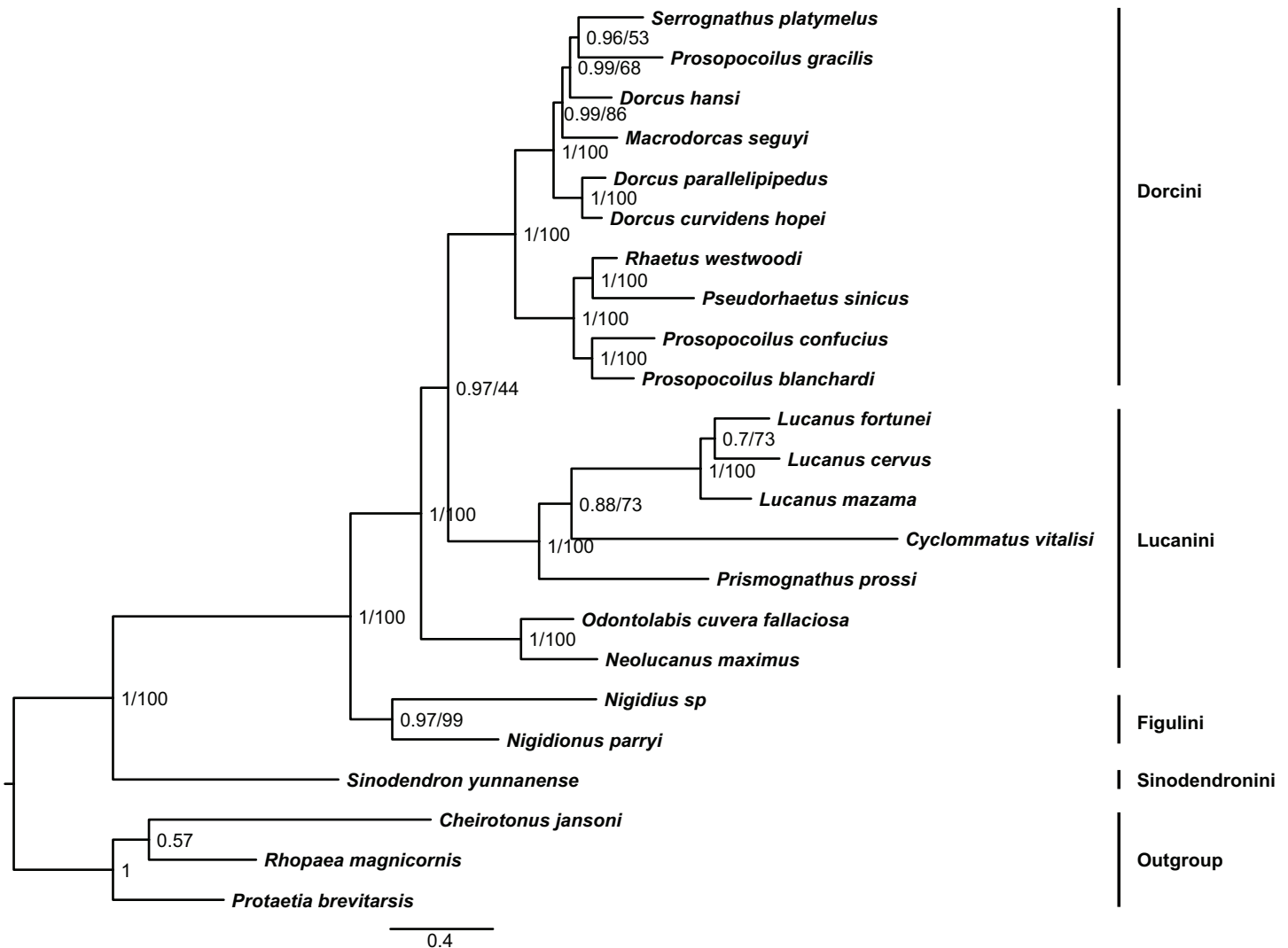
Figure 2 Combination of the BI and ML. Phylogenetic relationships among the Lucanidae inferred from Bayesian inference and Maximum Likelihood methods based on PCG12RNA dataset. We selected 19 stag beetles as ingroup and three other scarab species (*Rhopaea magnicornis*, *Protaetia brevitarsis* and *Cheirotonus jansoni*) as outgroup. These two methods obtained consistent topologies in the ingroup. The line with 0.4 is a scale for scaled branches of BI and shows the evolutionary distance of each species. Values near the the node show the posterior probability of both methods of corresponding node. Full-size 🖾 DOI: 10.7717/peerj.8274/fig-2

Lin et al., 2017; *Liu et al., 2019*, *2017*; *Jing et al., 2018*; *Wu et al., 2015*), causing its total mitochondrial genome to be significantly longer than others.

This study presents consistent phylogenetic relationships basing BI and ML methods. Overall, there was no apparent effect of long-branch attraction within the ingroup. Most of our findings based on BI and ML were congruent with the phylogenetic analyses based on a combination of several mitochondrial genes and nuclear ribosomal genes (*Kim et al., 2015*). However, there were controversial relationships, especially in the lower taxonomic levels. For example, the phylogenetic status of genera *Neolucanus* and *Odontolabis* in our results were different from previous results generated from multiple fragments (*Kim et al., 2015*). *Prosopocoilus gracilis* has been classified into *Dorcus* (s.l.) rather than *Prosopocoilus*

(*Lin et al., 2017*). Our results showed that mitochondrial genomes sequences are powerful in relationships inference within Lucanidae. The sequencing and assembly of the mitochondrial genome will facilitate future works of mitochondrial genome sequencing (*Bourguignon et al., 2017*; *Crampton-Platt et al., 2015*). Increased taxa sampling and genome sequencing will help to resolve the classification problems within Lucanidae.

CONCLUSIONS

In our study, the phylogenomic analysis supports the morphological conclusion on relationships of Lucanidae. Although our data could not solve all the phylogenetic relationships within Lucanidae, this study can be helpful for future research on the Lucanidae phylogeny.

ACKNOWLEDGEMENTS

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

Grant Disclosures

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Dan Chen conceived and designed the experiments, performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Jing Liu conceived and designed the experiments, analyzed the data, prepared figures and/or tables, approved the final draft.
- Luca Bartolozzi conceived and designed the experiments, authored or reviewed drafts of the paper, approved the final draft, provide writing guidance and suggestions for revision.
- Xia Wan contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft, provide samples and experimental conditions.

REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215(3)**:403–410 DOI 10.1016/S0022-2836(05)80360-2.

Balke M, Ribera I, Vogler AP. 2004. MtDNA phylogeny and biogeography of Copelatinae, a highly diverse group of tropical diving beetles (Dytiscidae). *Molecular Phylogenetics and Evolution* **32**:866–880.

Bardiani M, Chiari S, Maurizi E, Tini M, Toni I, Zauli A, Campanaro A, Carpaneto GM, Audisio P. 2017. Guidelines for the monitoring of *Lucanus cervus*. *Nature Conservation* **20(2)**:37–78 DOI 10.3897/natureconservation.20.12687.

Benson G. 1999. Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Research* **27(2)**:573–580 DOI 10.1093/nar/27.2.573.

Bernt M, Donath A, Jühling F, Externbrink F, Florentz C, Fritzsch G, Pütz J, Middendorf M, Stadler PF. 2013. MITOS: improved de novo metazoan mitochondrial genome annotation. *Molecular Phylogenetics and Evolution* **69(2)**:313–319 DOI 10.1016/j.ympev.2012.08.023.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* **30(15)**:2114–2120 DOI 10.1093/bioinformatics/btu170.

Bourguignon T, Lo N, Šobotník J, Ho SY, Iqbal N, Coissac E, Leen M, Jendryka MM, Sillam-Dussès D, Krížková B, Roisin Y, Evans TA. 2017. Mitochondrial phylogenomics resolves the global spread of higher termites, ecosystemengineers of the tropics. *Molecular Biology and Evolution* **34(3)**:589–597.

Cameron SL, Sullivan J, Song HJ, Miller KB, Whiting MF. 2009. A mitochondrial genome phylogeny of the Neuropterida (lace-wings, alderflies and snakeflies) and their relationship to the other holometabolous insect orders. *Zoologica Scripta* **38**:575–590.

Cameron SL. 2014. Insect mitochondrial genomics: implications for evolution and phylogeny. *Annual Review of Entomology* **59(1)**:95–117 DOI 10.1146/annurev-ento-011613-162007.

Campanaro A, Zapponi L, Hardersen S, Méndez M, Al Fulaij N, Audisio P, Bardiani M, Carpaneto GM, Corezzola S, Della Rocca F, Harvey D, Hawes C, Kadej M, Karg J, Rink M, Smolis A, Sprecher E, Thomaes A, Toni I, Vrezec A, Zauli A, Zilioli M, Chiari S. 2016. A European monitoring protocol for the stag beetle, a saproxylic flagship species. *Insect Conservation and Diversity* **9(6)**:574–584 DOI 10.1111/icad.12194.

Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17(4)**:540–552 DOI 10.1093/oxfordjournals.molbev.a026334.

Chen YJ, Liu J, Cao YY, Zhou S, Wan X. 2018. Two new complete mitochondrial genomes of Dorcus stag beetles (Coleoptera, Lucanidae). *Genes & Genomics* **40(8)**:873–880 DOI 10.1007/s13258-018-0699-8.

Cheng X-F, Zhang L-P, Yu D-N, Storey KB, Zhang J-Y. 2016. The complete mitochondrial genomes of four cockroaches (Insecta: Blattodea) and phylogenetic analyses within cockroaches. *Gene* **586(1)**:115–122 DOI 10.1016/j.gene.2016.03.057.

Cox K, Thomaes A, Antonini G, Zilioli M, De Gelas K, Harvey D, Solano E, Audisio P, McKeown N, Shaw P, Minetti R, Bartolozzi L, Mergeay J. 2013. Testing the performance of a fragment of the COI gene to identify western Palaearctic stag beetle species (Coleoptera, Lucanidae). *ZooKeys* **365**:105–126 DOI 10.3897/zookeys.365.5526.

Crampton-Platt A, Timmermans MJTN, Gimmel ML, Kutty SN, Cockerill TD, Vun Khen C, Vogler AP. 2015. Soup to tree: the phylogeny of beetles inferred by mitochondrial metagenomics of a bornean rainforest sample. *Molecular Biology and Evolution* **32(9)**:2302–2316 DOI 10.1093/molbev/msv111.

Fagua G, Condamine FL, Brunet BMT, Clamens AL, Laroche J, Levesque RC, Cusson M, Sperling FAH. 2018. Convergent herbivory on conifers by Choristoneura moths after boreal forest formation. *Molecular Phylogenetics and Evolution* **123**:35–43 DOI 10.1016/j.ympev.2018.01.013.

Greiner S, Lehwark P, Bock R. 2019. OrganellarGenomeDRAW (OGDRAW) version 1.3.1: expanded toolkit for the graphical visualization of organellar genomes. *Nucleic Acids Research* **47(W1)**:W59–W64 DOI 10.1093/nar/gkz238.

Harvey DJ, Gange AC, Hawes CJ, Rink M, Abdehalden M, Al Fulaij N, Asp T, Ballerio A, Bartolozzi L, Brustel H, Cammaerts R, Maria Carpaneto G, Cederberg B, Chobot K, Cianferoni F, Drumont A, Ellwanger G, Ferreira S, Grosso-Silva JM, Gueorguiev B, Harvey W, Hendriks P, Istrate P, Jansson N, Jelaska LŠ, Jendek E, Jović M, Kervyn T, Krenn HW, Kretschmer K, Legakis A, Lelo S, Moretti M, Merkl O, Megia Palma R, Neculiseanu Z, Rabitsch W, Merino Rodríguez S, Smit JT, Smith M, Sprecher-Uebersax E, Telnov D, Thomaes A, Thomsen PF, Tykarski P, Vrezec A, Werner S, Zach P. 2011. Bionomics and distribution of the stag beetle, *Lucanus cervus* (L.) across Europe. *Insect Conservation and Diversity* **4(1)**:23–38 DOI 10.1111/j.1752-4598.2010.00107.x.

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

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

Jing L, Zhou S-J, Chen Y-J, Wan X. 2018. Mitogenome of the monotypic genus *Rhaetus* (Coleoptera: Scarabaeidae: Lucanidae). *Journal of Entomological Science* **53(4)**:503–513 DOI 10.18474/JES17-122.1.

Katoh K, Rozewicki J, Yamada KD. 2017. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics* **20(4)**:1160–1166 DOI 10.1093/bib/bbx108.

Kim SI, Farrell BD. 2015. Phylogeny of world stag beetles (Coleoptera: Lucanidae) reveals a Gondwanan origin of Darwin's stag beetle. *Molecular Phylogenetics and Evolution* **86**:35–48 DOI 10.1016/j.ympev.2015.02.015.

Kim MJ, Im HH, Lee KY, Han YS, Kim I. 2014. Complete mitochondrial genome of the whiter-spotted flower chafer, *Protaetia brevitarsis* (Coleoptera: Scarabaeidae). *Mitochondrial DNA* **25**:177–178 DOI 10.3109/19401736.2013.792064.

Kim MJ, Kim K-G, Kim SR, Kim I. 2015. Complete mitochondrial genome of the two-spotted stag beetle, *Metopodontus blanchardi* (Coleoptera: Lucanidae). *Mitochondrial DNA* **26(2)**:307–309 DOI 10.3109/19401736.2013.825788.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33(7)**:1870–1874 DOI 10.1093/molbev/msw054.

Kuraku S, Zmasek CM, Nishimura O, Katoh K. 2013. aLeaves facilitates on-demand exploration of metazoan gene family trees on MAFFT sequence alignment server with enhanced interactivity. *Nucleic Acids Research* **41(W1)**:W22–W28 DOI 10.1093/nar/gkt389.

Lanfear R, Frandsen PB, Wright AM, Senfeld T, Calcott B. 2017. PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution* **34**:772–773.

Lartillot N, Lepage T, Blanquart S. 2009. PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. *Bioinformatics* **25(17)**:2286–2288 DOI 10.1093/bioinformatics/btp368.

Li H, Shao R, Song N, Song F, Jiang P, Li Z, Cai W. 2015. Higher-level phylogeny of paraneopteran insects inferred from mitochondrial genome sequences. *Scientific Reports* **5(1)**:1–10 DOI 10.1038/srep08527.

Li Q, Wei S-J, Tang P, Wu Q, Shi M, Sharkey MJ, Chen X-X. 2016. Multiple lines of evidence from mitochondrial genomes resolve phylogenetic relationships of parasitic wasps in braconidae. *Genome Biology and Evolution* **8(9)**:2651–2662 DOI 10.1093/gbe/evw184.

Lin Z-Q, Song F, Li T, Wu Y-Y, Wan X. 2017. New mitogenomes of two Chinese stag beetles (Coleoptera, Lucanidae) and their implications for systematics. *Journal of Insect Science* **17(2)**:63 DOI 10.1093/jisesa/iex041.

Liu J, Cao Y, Zhou S, Chen Y, Wan X. 2019. Complete mitochondrial genome of *Prismognathus prossi* (Coleoptera: Lucanidae) with phylogenetic implications. *Entomologica Fennica* **30(1)**:1–7 DOI 10.33338/ef.79901.

Liu J, Li CG, You S, Wan X. 2017. The first complete mitogenome of *Cyclommatus* stag beetles (Coleoptera: Lucanidae) with the phylogenetic implications. *Entomotaxonomia* **39**:294–299 DOI 10.11680/entomotax.2017035.

Linard B, Arribas P, Andújar C, Crampton-Platt A, Vogler AP. 2016. Lessons from genome skimming of arthropod-preserving ethanol. *Molecular Ecology Resources* **16**:1365–1377 DOI 10.1111/1755-0998.12539.

Lowe TM, Chan PP. 2016. tRNAscan-SE On-line: integrating search and context for analysis of transfer RNA genes. *Nucleic Acids Research* **44(W1)**:W54–W57 DOI 10.1093/nar/gkw413.

Miller MA, Pfeiffer W, Schwartz T. 2011. The CIPRES science gateway: a community resource for phylogenetic analyses. In: *Proceedings of the 2011 TeraGrid Conference: Extreme Digital Discovery, 18–21 July 2011, Salt Lake City, Utah*. New York: ACM.

New TR. 2018. Insect flagships and indicators in forests. In: New TR, ed. *Forests and Insect Conservation in Australia*. Cham: Springer International Publishing, 111–139.

Peng Y, Leung HCM, Yiu SM, Chin FYL. 2012. IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics* **28(11)**:1420–1428 DOI 10.1093/bioinformatics/bts174.

Perna NT, Kocher TD. 1995. Patterns of nucleotide composition at fourfold degenerate sites of animal mitochondrial genomes. *Journal of Molecular Evolution* **41(3)**:353–358 DOI 10.1007/BF01215182.

Romiti F, De Zan LR, Piras P, Carpaneto GM. 2017. Shape variation of mandible and head in *Lucanus cervus* (Coleoptera: Lucanidae): a comparison of morphometric approaches. *Biological Journal of the Linnean Society* **120(4)**:836–851 DOI 10.1093/biolinnean/blw001.

Shao LL, Huang DY, Sun XY, Hao JS, Cheng CH, Zhang W, Yang Q. 2014. Complete mitochondrial genome sequence of *Cheirotonus jansoni* (Coleoptera: Scarabaeidae). *Genetics and Molecular Research* **13**:1047–1058 DOI 10.4238/2014.February.20.6.

Sheffield NC, Song H, Cameron SL, Whiting MF. 2009. Nonstationary evolution and compositional heterogeneity in beetle mitochondrial phylogenomics. *Systematic Biology* **58**:381–394 DOI 10.1093/sysbio/syp037.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. 1994. Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene Sequences and a Compilation of Conserved Polymerase Chain Reaction Primers. *Annals of the Entomological Society of America* **87**:651–701.

Song F, Li H, Jiang P, Zhou X, Liu J, Sun C, Vogler AP, Cai W. 2016. Capturing the phylogeny of holometabola with mitochondrial genome data and Bayesian site-heterogeneous mixture models. *Genome Biology and Evolution* **8(5)**:1411–1426 DOI 10.1093/gbe/evw086.

Talavera G, Castresana J. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* **56(4)**:564–577 DOI 10.1080/10635150701472164.

Tini M, Bardiani M, Campanaro A, Chiari S, Mason F, Maurizi E, Toni I, Audisio P, Carpaneto GM. 2017. A stag beetle's life: sex-related differences in daily activity and behaviour of *Lucanus cervus* (Coleoptera: Lucanidae). *Journal of Insect Conservation* **21(5–6)**:897–906 DOI 10.1007/s10841-017-0029-5.

Wang Q, Liu J, Lin ZQ, Wan X. 2018. The complete mitochondrial genome of *Odontolabis fallaciosa* (Coleoptera: Lucanidae) with its phylogenetic implications. *Zoological Systematics* **43(3)**:268–275 DOI 10.11865/zs.201831.

Wei S-J, Shi M, Chen X-X, Sharkey MJ, Van Achterberg C, Ye G-Y, He J-H. 2010. New views on strand asymmetry in insect mitochondrial genomes. *PLOS ONE* **5(9)**:e12708 DOI 10.1371/journal.pone.0012708.

Wu YY, Cao YY, Fang J, Wan X. 2015. The first complete mitochondrial genome of stag beetle from China, *Prosopocoilus gracilis* (Coleoptera, Lucanidae). *Mitochondrial DNA* **27**:2633–2634 DOI 10.3109/19401736.2015.1041129.

Zhang D-X, Hewitt GM. 1997. Insect mitochondrial control region: a review of its structure, evolution and usefulness in evolutionary studies. *Biochemical Systematics and Ecology* **25(2)**:99–120 DOI 10.1016/S0305-1978(96)00042-7.

Zhang D-X, Szymura JM, Hewitt GM. 1995. Evolution and structural conservation of the control region of insect mitochondrial DNA. *Journal of Molecular Evolution* **40(4)**:382–391 DOI 10.1007/BF00164024.

Zheng B-Y, Cao L-J, Tang P, Van Achterberg K, Hoffmann AA, Chen H-Y, Chen X-X, Wei S-J. 2018. Gene arrangement and sequence of mitochondrial genomes yield insights into the phylogeny and evolution of bees and sphecid wasps (Hymenoptera: Apoidea). *Molecular Phylogenetics and Evolution* **124**:1–9 DOI 10.1016/j.ympev.2018.02.028.