Complete Mitochondrial Genomes and Phylogenetic Positions of Two Longicorn Beetles, *Anoplophora glabripennis* and *Demonax pseudonotabilis* (Coleoptera: Cerambycidae)

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Abstract: *Anoplophora glabripennis* (Motschulsky, 1854) and *Demonax pseudonotabilis* Gressitt & Rondon, 1970 are two commonly found longicorn beetles from China. However, the lack of sufficient molecular data hinders the understanding of their evolution and phylogenetic relationships with other species of Cerambycidae. This study sequenced and assembled the complete mitochondrial genomes of the two species using the next-generation sequencing method. The mitogenomes of *A. glabripennis* and *D. pseudonotabilis* are 15,622 bp and 15,527 bp in length, respectively. The mitochondrial gene content and gene order of *A. glabripennis* and *D. pseudonotabilis* are highly conserved with other sequenced longicorn beetles. The calculation of nonsynonymous (Ka) and synonymous (Ks) substitution rates in PCGs indicated the existence of purifying selection in the two longicorn beetles. The phylogenetic analysis was conducted using the protein-coding gene sequences from available mitogenomes of Cerambycidae. The two species sequenced in this study are, respectively, grouped with their relatives from the same subfamily. The monophyly of Cerambycinae, Dorcasominae, Lamiinae, and Necydalinae was well-supported, whereas Lepturinae, Prioninae, and Spondylidinae were recovered as paraphyletic.

Keywords: Cerambycidae; mitogenome; longicorn beetle; gene arrangement; phylogeny

1. Introduction

Cerambycidae (longicorn beetle) is one of the most speciose families of Coleoptera, comprising over 4000 genera and 35,000 species worldwide [1–3]. *Cerambycidae sensu stricto* (s.s.) usually consists of the eight subfamilies: Cerambycinae, Dorcasominae, Lamiae, Lepturinae, Necydalinae, Parandrinae, Prioninae, and Spondylidinae [4]. *Cerambycidae sensu lato* (s.l.) comprises *Cerambycidae s.s.*, Disteniidae, Oxypeltidae, and Vesperidae [5]. The adults of longicorn beetles are morphologically diverse and phytophagous, usually feeding on living plant tissue, pollen, fruit, or tree sap [6]. Larvae of longicorn beetles usually have reduced or sometimes absent legs and they are mostly internal borers of their host plants [7–10]. In cultivated ecosystems, e.g., forest farms and tea gardens, the longicorn beetles are nonnegligible pests causing significant economic damage to the host plants [11,12].

The phylogeny and early evolution of Cerambycidae have been comprehensively reviewed by Haddad & Mckenna (2016) [13]. The phylogeny of longicorn beetles, especially the monophyly of Cerambycidae s.s. and s.l., as well as the subfamily and tribe-level relationship, remains debatable due to the high species richness and highly variable morphological characters [5,14,15]. Haddad et al. (2018) [5] reconstructed the higher-level phylogeny of Cerambycidae with anchored hybrid enrichment of nuclear genes. Their results recovered a monophyletic Cerambycidae s.s. in most analyses and a polyphyletic
Cerambycidae s.l. as well as the monophyletic subfamilies of Cerambycidae s.s. except for the paraphyletic Cerambycinae [5]. Nie et al. (2020) [15] used 151 mitochondrial genomes (mitogenomes) representing all families of Chrysomeloidea and all subfamilies of Cerambycidae s.s. to explore the higher-level phylogeny of Chrysomeloidea, especially Cerambycidae and allied families. However, their study could not support the monophyly of Cerambycidae s.s. and all its subfamilies. The two subfamilies, Nectyalinae and Parandrinae, were considered as tribes Nectyalini and Parandrini, respectively [15]. Meanwhile, the mitogenomes of many important cerambycid clades remained poorly represented, which restricted the accuracy of the results.

The mitogenome is an informative molecular marker for taxonomic and evolutionary research and has become one of the most popular molecules used in current insect phylogenetic studies [16]. The development of next-generation sequencing techniques largely reduced the expense and experimental period to efficiently obtain the mitogenomes from all kinds of organisms. Diverse insect orders, such as Coleoptera [17,18], Lepidoptera [19], Hemiptera [20], etc., have combined the mitogenomes with dense taxon sampling to generate large-scale phylogenomic datasets for phylogenetic reconstruction and have revealed the strengths of mitogenomes in resolving the higher-level phylogenetic relationships. However, the available number of mitogenomes of Cerambycidae s.l. in the NCBI database is out of proportion to the remarkable species richness of longicorn beetles, which is a major impediment to better understanding the classification and evolution of this ecologically and economically significant group of insects.

To provide more genetic data for the longicorn beetles and investigate their phylogenetic relationships, this study sequenced and analyzed the mitogenomes of two commonly found longicorn beetles from China, A. glabripennis and D. pseudonotabilis [21,22]. Although the mitogenome of A. glabripennis (NC_008221) has been sequenced in a previous study [23], it is still very important to sequence more mitogenomes for the same species already listed in GenBank considering the existence of intraspecific variation of mitogenomes between different geographic populations [24]. Phylogenetic trees of Cerambycidae s.l. is constructed based on the newly sequenced as well as the known mitogenomic data to investigate the phylogenetic positions of the two newly sequenced species and provide more information for resolving the relationships within Cerambycidae s.l.

2. Materials and Methods

2.1. Sample Collection, DNA Extraction, and Mitogenome Sequencing

Adult specimens of A. glabripennis and D. pseudonotabilis were collected by Malaise traps set in the tea garden of Hongyan Town (29°59′31.42″ N, 103°10′34.45″ E), Mingshan County, Ya’an City, Sichuan Province of China, in 2016. The specimens were identified based on the morphological characteristics under a light microscope and were deposited in Sichuan Academy of Agricultural Sciences (specimen voucher: SAASCO1 (A. glabripennis) and SAASCO2 (D. pseudonotabilis)). All experiments and procedures for this study complied with the current animal ethics guidelines and did not involve any protected animals.

The total genomic DNA was extracted by E.Z.N.A. Tissue DNA Kit (Omega, Norcross, GA, USA). At least 1 µg of purified DNA was used to construct the TruSeq DNA library with an insert size of 400 bp according to standard protocols. The library was sequenced using the Illumina HiSeq 4000 platform (Personal Gene Technology Co., Ltd., Nanjing, China) with paired-end reads of 2 × 150 bp. A total of 21,616,708 and 22,096,010 raw reads were obtained for A. glabripennis and D. pseudonotabilis, respectively. Over 97.8% of bases in the raw reads were regarded as correctly identified with an accuracy rate above 99%. The unpaired, short, and low-quality raw reads were filtered by fastp [25] to obtain clean reads. The above quality-control and data-filtering process generated 21,584,444 and 22,059,322 high-quality reads for A. glabripennis and D. pseudonotabilis, respectively.
2.2. Mitogenome Assembly, Annotation, and Analyses

Before the assembly, the high-quality reads were trimmed again using BBduk with default settings implemented in Geneious Prime [26]. The high-quality reads of *A. glabripennis* and *D. pseudonotabilis* were, respectively, mapped to the reference mitogenome of the previously sequenced *A. glabripennis* (NC_008221) [23] and amplified bilaterally by Geneious Prime [26], with the parameters set as follows: 95% minimum overlap identity, 50 bp minimum overlap, and maximum ambiguity as 4. The completeness of each circular mitogenome was confirmed when both ends of the final assembled contigs overlapped (100% coverage). The assembled mitogenomes of *A. glabripennis* and *D. pseudonotabilis* were deposited in GenBank under the accession numbers OP096420 and OP096419, respectively.

The two mitogenomes were annotated in the MITOS web server [27]. The resultant gene boundaries of the protein-coding genes (PCGs) were checked manually by the NCBI’s ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/, accessed on 9 August 2022). The location and secondary structures of the transfer RNA (tRNA) and ribosomal RNA (rRNA) genes were predicted and visualized by MITOS. The mitogenome structure and GC skews were visualized by the CGView Server [28]. The nucleotide composition, skews, codon usage, and relative synonymous codon usage (RSCU) were calculated by MEGA11 [29]. The synonymous substitution rate (Ks) and nonsynonymous substitution rate (Ka) were calculated using KaKs_Calculator v2.0, with the mitogenome of *Aoria nigripes* (Baly, 1860) (Chrysomelidae) as the outgroup [30,31]. The alignment file of each PCG was uploaded to the Datamonkey web server for a more thorough exploration of selective pressure in PCGs of the two newly sequenced mitogenomes. BUSTED (Branch-Site Unrestricted Statistical Test for Episodic Diversification) was used to test whether each PCG has experienced positive selection [32]. FEL (fixed-effects likelihood) was employed to infer site-specific Ks and Ka values and detect the following four types of sites in each PCG: diversifying sites, purifying sites, neutral sites, and invariable sites [33]. Tandem repeats in the control regions were identified using the Tandem Repeats Finder web server [34]. The stem-loop structures in the control region were predicted by the Mfold web server [35].

2.3. Phylogenetic Analyses Methods

The phylogenetic relationships were reconstructed based on the nucleotide sequences of 13 PCGs derived from 186 mitogenomes of Cerambycidae s.l. (Table 1). Overall, 30 of the 186 mitogenomes were originally unannotated in GenBank; they were re-annotated by MITOS and manual homology alignments in this study. Other mitogenomes from GenBank that had incomplete set of 13 PCGs or incorrect PCG sequences were omitted from the dataset. The mitogenome of *A. nigripes* (Chrysomelidae) was used as the outgroup [31]. The 13 PCGs were, respectively, aligned using MUSCLE with a codon mode [36], followed by the deletion of stop codons and the concatenation of sequences by SequenceMatrix v1.7.8 [37]. The best-fit partitioning schemes and substitution models for each PCG region were determined by PartitionFinder v2.1.1 using the Bayesian information criterion (BIC) and a greedy search algorithm of all available models [38]. Phylogenies were inferred using maximum-likelihood (ML) and Bayesian inference (BI) methods. The best-fit model was GTR+I+G for two partitioned subsets: one subset included ND1, ND4, ND4L, and ND5; the other subset included the remaining 9 PCGs. IQ-Tree was used to perform the ML analysis under the edge-unlinked partition model for 5000 ultrafast bootstraps as well as the Shimodaira–Hasegawa-like approximate likelihood-ratio test [39–41]. The BI analysis was conducted by MrBayes v3.2.7 [42] with four independent Markov chains for 30 million generations and sampled every 100 generations. The first 25% of the trees were discarded as burn-in. FigTree v1.4.4 was used to edit and visualize the phylogenetic trees [43].
Table 1. Species used in this study.

| Family                | Subfamily               | Species                        | Genome Size (bp) | GenBank No.   |
|-----------------------|-------------------------|--------------------------------|------------------|---------------|
| Cerambycidae s.s.     | Cerambycinae            | *Allotraeus orientalis*        | 15,966           | NC_061181     |
|                       |                         | *Anoplistes halodendri*       | 15,697           | NC_053350     |
|                       |                         | *Aromia bungii*               | 15,652           | MW617355      |
|                       |                         | *A. bungii*                   | 15,760           | NC_053714     |
|                       |                         | *A. bungii*                   | 15,759           | OK393714      |
|                       |                         | *Chloridolum lameeri*         | 15,731           | MN420467      |
|                       |                         | *Chlorophorus annularis*      | 15,487           | NC_061058     |
|                       |                         | *Chlorophorus diadema*        | 15,398           | MN473096      |
|                       |                         | *Chlorophorus simillimus*     | 13,675           | KY796055      |
|                       |                         | *Clytobius davidis*           | 15,571           | MN473101      |
|                       |                         | *D. pseudonotabilis*          | 15,527           | OP096419      |
|                       |                         | *Epipedocera atra*            | 15,662           | NC_051944     |
|                       |                         | *Gnatolea eurifera*           | 15,281           | MN420473      |
|                       |                         | *Jebusaea hammerschmidtii*    | 15,619           | MZ054170      |
|                       |                         | *Massicus raddci*             | 15,858           | NC_023937     |
|                       |                         | *Megacyllene sp. KM-2017*     | 15,832           | MG193470      |
|                       |                         | *Molorchus minor*             | 15,685           | MN442323      |
|                       |                         | *Nadezhdiiella cantori*       | 16,049           | NC_061180     |
|                       |                         | *Neoplocederus obesus*        | 15,683           | NC_048951     |
|                       |                         | *Norta carinicolis*           | 15,602           | NC_044698     |
|                       |                         | *Obrium cantharinum*          | 15,632           | MN420489      |
|                       |                         | *Obrium sp. NS-2015*          | 15,680           | KT945156      |
|                       |                         | *Polyzonus fasciatus*         | 15,804           | MN442321      |
|                       |                         | *Purpuricenus litoratus*      | 15,744           | MN473112      |
|                       |                         | *Purpuricenus temminckii*     | 15,689           | MN527358      |
|                       |                         | *Pyrrhidium sanguineum*       | 16,203           | KX087339      |
|                       |                         | *P. sanguineum*               | 15,748           | MN442320      |
|                       |                         | *Rhytidodera bowringii*       | 15,278           | MN420472      |
|                       |                         | *Semanotus bifasciatus*       | 13,837           | KY765550      |
|                       |                         | *S. bifasciatus*              | 16,051           | MN095416      |
|                       |                         | *Stenodrass sp. N127*         | 15,333           | MN473097      |
|                       |                         | *Trichoderus campestris*      | 13,696           | KY773688      |
|                       |                         | *T. campestris*               | 15,737           | MN473098      |
|                       |                         | *Turacoclytus namaqanensis*    | 15,565           | NC_060874     |
|                       |                         | *Xoanodera maculata*          | 15,767           | NC_061182     |
|                       |                         | *Xylotrichus graii*           | 15,540           | NC_030792     |
|                       |                         | *Xylotrichus magnicollis*     | 13,692           | KY773690      |
|                       |                         | *Xyloctroca globosa*          | 15,707           | NC_045097     |
|                       |                         | *Zoedes fulgaratus*           | 15,885           | MW858149      |
| Dorcasominae          |                         | *Apatophysis sieversi*        | 15,278           | MN420474      |
|                       |                         | *Dorcasomus pinheyi*          | 16,040           | MN474345      |
|                       |                         | *Tsivoka simplicicollis*      | 16,700           | MN420488      |
| Lamiinae              |                         | *Acanthocinus griseus*        | 15,600           | MN473099      |
|                       |                         | *Agapanthia amurensis*        | 15,512           | MW617354      |
|                       |                         | *Agapanthia daurica*          | 14,282           | KY773692      |
|                       |                         | *A. daurica*                  | 17,153           | MN473114      |
|                       |                         | *Aegelasta perplexa*          | 15,552           | NC_053905     |
|                       |                         | *Anaesthetis testacea*        | 15,169           | MN420492      |
|                       |                         | *Annananum lunulatum*         | 15,610           | NC_046851     |
|                       |                         | *Anoplophora chinensis*       | 15,871           | MN882586      |
|                       |                         | *A. chinensis*                | 15,805           | NC_029230     |
|                       |                         | *A. glabripennis*             | 15,774           | NC_008221     |
|                       |                         | *Anoplophora horsfieldi*      | 15,796           | MN248534      |
|                       |                         | *A. horsfieldi*               | 15,837           | NC_059864     |
|                       |                         | *A. glabripennis*             | 15,622           | OP096420      |
| Family                  | Subfamily | Species                        | Genome Size (bp) | GenBank No.   |
|------------------------|-----------|-------------------------------|------------------|---------------|
| Apomecyna saltator     |           |                               | 14,949           | NC_056277     |
| Apriona germanii       |           |                               | 14,858           | NC_056838     |
| Apriona swainsoni      |           |                               | 15,412           | NC_033872     |
| Aristobia reticulator  |           |                               | 15,838           | NC_042151     |
| Aulaconotus atronotatus|           |                               | 14,491           | MW858150      |
| Batocera davidi        |           |                               | 15,554           | MN420468      |
| Batocera lineolata     |           |                               | 15,420           | MF521888      |
| B. lineolata           |           |                               | 15,418           | NC_022671     |
| B. lineolata           |           |                               | 16,158           | NC_062817     |
| B. lineolata           |           |                               | 16,158           | NC_022671     |
| Blephephaeus succinctor|           |                               | 15,554           | NC_044697     |
| Cobetura sp. KM-2017   |           |                               | 15,912           | MG193463      |
| Eupithecia metallescens|           |                               | 15,072           | KY796053      |
| Glenea cantor          |           |                               | 15,514           | NC_043883     |
| Glenea licenti         |           |                               | 15,435           | MN473117      |
| Glenea paraornata      |           |                               | 15,510           | MN420483      |
| Glenea relicta         |           |                               | 15,486           | MN420484      |
| Heteroglenea nigromaculata|       |                               | 15,502           | MN420485      |
| Jamesia sp. KM-2017    |           |                               | 17,430           | MG193322      |
| Lamiinae sp. 1 ACP-2013|         |                               | 15,737           | MH789723      |
| Lamiinae sp. 2 ACP-2013|         |                               | 15,440           | MH789720      |
| Lamiinae sp. 4 ACP-2013|         |                               | 15,504           | MH789721      |
| Lamiinae sp. 4 ACP-2013|         |                               | 15,554           | MH836614      |
| Menesia sulphurata     |           |                               | 15,551           | MN473119      |
| Moechotypa diphydis    |           |                               | 15,493           | MW617356      |
| Monochamus alternatus  |           |                               | 14,649           | JX987292      |
| M. alternatus          |           |                               | 14,189           | MW585152      |
| M. alternatus          |           |                               | 15,874           | NC_024652     |
| M. alternatus          |           |                               | 15,880           | NC_050066     |
| Monochamus sartor urussovii| |                   | 14,359           | KY773691      |
| Monochamus sparsatus   |           |                               | 16,029           | NC_053906     |
| Monochamus sutor       |           |                               | 14,350           | KY773689      |
| Niphona lateraliplagiata|        |                               | 15,902           | MN473100      |
| Oberea diversipes      |           |                               | 15,499           | NC_053945     |
| Oberea formosana       |           |                               | 15,675           | MN473118      |
| Oberea yaoshana        |           |                               | 15,829           | MK863509      |
| Olenecamptus bilobus   |           |                               | 15,262           | NC_051945     |
| Olenecamptus suboblitatus|        |                               | 13,854           | KY796054      |
| Paraglenea fortunei    |           |                               | 15,401           | MN442322      |
| P. fortunei            |           |                               | 15,496           | NC_056837     |
| Parmena novaki         |           |                               | 15,668           | MN420491      |
| Psacothia hilaris      |           |                               | 15,856           | NC_013070     |
| Pseudechthistatus      |           |                               | 16,419           | OP006455      |
| Pseudechthistatus hei  |           |                               | 16,103           | NC_065262     |
| Pterolophia sp. ZY-2019|        |                               | 16,063           | NC_046999     |
| Saperda tetrastigma   |           |                               | 15,563           | MZ955033      |
| Serixia sedata         |           |                               | 14,714           | MN420487      |
| Thermistis crococinecta|           |                               | 15,503           | NC_044700     |
| Thyestilla gebleri     |           |                               | 15,503           | MN420486      |
| T. gebleri             |           |                               | 15,505           | NC_034752     |

**Lepturinae**

| Anastrangalia sequensi |           |                               | 16,269           | NC_038090     |
| Brachytia interrogationis|        |                               | 18,165           | KX087246      |
| Cortodera humeralis    |           |                               | 15,928           | KX087264      |
### Table 1. Cont.

| Family           | Subfamily | Species                  | Genome Size (bp) | GenBank No.  |
|------------------|-----------|--------------------------|------------------|--------------|
|                  |           | Gaurotes virginea       | 15,775           | MN473081     |
|                  |           | Grammoptera ruficornis   | 16,458           | MN473080     |
|                  |           | Leptura aethiops        | 15,690           | MN420475     |
|                  |           | Leptura annularis       | 16,530           | MN420469     |
|                  |           | Leptura arcuata         | 14,382           | KY796051     |
|                  |           | Oxytnirus cursor        | 15,797           | MN473085     |
|                  |           | Pachyta bicuneata       | 13,894           | KY763551     |
|                  |           | Peithona prionoides     | 13,636           | MN473095     |
|                  |           | Pidonia lurida          | 15,668           | MN473083     |
|                  |           | Rhagium fortescostatum  | 16,274           | MN473103     |
|                  |           | Rhamnusium bicolor      | 15,527           | MN473084     |
|                  |           | Rutpela maculata        | 17,437           | OW386295     |
|                  |           | Sachalinobia koltzei    | 15,809           | MN473113     |
|                  |           | Stenurella nigra        | 16,504           | KX087348     |
|                  |           | Stictoleptura succedanea| 14,381           | KY796052     |
|                  |           | Teledapalus zoalotichini| 16,651           | MN473111     |
|                  |           | Stenocorus meridianus   | 16,227           | MN473082     |
|                  |           | Xylosteus spinolae      | 15,708           | MN473086     |
|                  |           | Neocydalis major        | 15,598           | MN473087     |
|                  |           | Ulochaetes vacca        | 15,593           | MN473110     |
|                  | Necydalinae | Necydalis major     | 15,598           | MN473087     |
|                  |           |                       |                  |              |
|                  | Parandrinae | Papuandra araucariae   | 15,475           | MN420477     |
|                  | Prioninae  | Aegoliopis marginae     | 16,759           | MN420471     |
|                  |           | Aegosoma palladium      | 15,668           | MN473115     |
|                  |           | Aegosoma sinicum       | 15,658           | KY773686     |
|                  |           | A. sinicum             | 15,658           | NC_038089    |
|                  |           | Aesa media              | 15,714           | MK614538     |
|                  |           | Agrionome spinicollis   | 15,633           | MK614550     |
|                  |           | Analophus paralleus    | 15,722           | MK614551     |
|                  |           | Archetypus frenchi     | 16,156           | MK614554     |
|                  |           | Bifdopriornus rufus     | 15,590           | MK614537     |
|                  |           | Brephilydia jejuna      | 15,659           | MK614541     |
|                  |           | Cacodacnus planicollis  | 15,671           | MK614543     |
|                  |           | Callipogon reticus     | 15,742           | NC_037969    |
|                  |           | Cenemopilis australis   | 15,675           | MK614536     |
|                  |           | Cenemopilis edulis      | 13,161           | MK614556     |
|                  |           | Dorysus buquetii       | 15,778           | MN420481     |
|                  |           | Dorysus granulosus      | 15,858           | MN829437     |
|                  |           | Dorysthenes paradoxus   | 15,922           | NC_037927    |
|                  |           | Eboraphyllus mittldeni  | 15,776           | MK614546     |
|                  |           | Emnephylus aeneipennis  | 16,505           | MK614545     |
|                  |           | Euryynussa australis    | 15,612           | MK614547     |
|                  |           | Goeffmontellia queenslanda | 15,628           | MK614544     |
|                  |           | Hermerius prionoides    | 13,696           | MK614542     |
|                  |           | Houwa angulata         | 15,626           | MK614532     |
|                  |           | Megopsis sinica        | 15,689           | NC_045407    |
|                  |           | Neptodes costipennis    | 15,935           | MN420482     |
|                  |           | multicarinatus         |                  |              |
|                  |           | Olethrius laesipennis   | 15,690           | MK614533     |
|                  |           | Papunya picta          | 15,737           | MK614539     |
|                  |           | Paulhutchinsonia pilosicollis | 15,846           | NC_048496    |
|                  |           | Phaolus metallicus     | 15,997           | MK614535     |
|                  |           | Phlygethesis sp. N135   | 15,000           | MN473102     |
|                  |           | Priorygramma closteroides| 15,854           | NC_062855    |
|                  |           | Pseudopilipes inexpectatus | 15,651           | MK614549     |
|                  |           | Rhipidocerus australiasi| 15,721           | MK614540     |
|                  |           | Sarmydus sp. N117      | 15,720           | MN473091     |
3. Results and Discussion

3.1. Genome Structure and Composition

The assembled complete mitogenomes of *A. glabripennis* and *D. pseudonotabilis* are circular DNA molecules of 15,622 bp and 15,527 bp in length (Figure 1), respectively, which is within the range of the sequenced mitogenomes of Cerambycidae in GenBank (Table 1). Due to the presence of a shorter COX1 gene, the newly obtained *A. glabripennis* mitogenome is slightly shorter than the previously sequenced mitogenome (15,774 bp) based on samples from Hebei Province [31]. Both newly sequenced mitogenomes contain the standard set of 37 mitochondrial genes (13 PCGs, 22 tRNA genes, and 2 rRNA genes) as all other longicorn beetles. The gene order is identical to all other species of Cerambycidae as well as the ancestral mitogenome type of *Drosophila yakuba* Burla, 1954 [14,44,45]. Among the 37 genes, 23 (9 PCGs and 14 tRNAs) genes are on the majority strand (J-strand), while the remaining 4 PCGs, 8 tRNAs, and 2 rRNA genes are on the minority strand (N-strand).

A total of nine gene overlapping regions were found in the *A. glabripennis* mitogenome with a total of 29 bp in length, and the longest overlapping sequence (8 bp) was located between trnCys and trnTyr. In the *D. pseudonotabilis* mitogenome, there are 12 overlapping regions with a total of 21 bp in length, and the longest overlapping sequences were only 4 bp in length. The universally found 7 bp overlapping regions between ATP8 and ATP6, as well as NAD4 and NAD4L in Cerambycidae and many other insects [14,15], are restricted to the overlapping between NAD4 and NAD4L in the *A. glabripennis* mitogenome, which might be resulted from the different annotation methods. In addition to the overlapping regions, multiple intergenic spacers are scattered throughout both mitogenomes (Tables 2 and 3). The base composition is 38.8% A, 14.2% C, 9.2% G, and 37.8% T for the *A. glabripennis* mitogenome and 39.7% A, 14.5% C, 10.5% G, and 35.3% T for *D. pseudonotabilis*. The two mitogenomes are highly skewed towards A and T nucleotides, with an A + T content of 76.6% in *A. glabripennis* and 75.0% in *D. pseudonotabilis* (Table 1).
Table 2. Mitochondrial genome organization of A. glabripennis.

| Gene      | Position (bp) | Size (bp) | Direction | Intergenic Nucleotides | Anti- or Start/Stop Codons | A + T% |
|-----------|---------------|-----------|-----------|------------------------|-----------------------------|-------|
| trnLeu2 (L1) | 1–67         | 67        | Forward   | 0                      | GAT                         | 61.2  |
| trnGln (Q)   | 69–137       | 69        | Reverse   | 1                      | TTG                         | 78.3  |
| trnMet (M)    | 137–205      | 69        | Forward   | −1                     | CAT                         | 72.5  |
| ND2          | 206–1216     | 1011      | Forward   | 0                      | ATT/TAA                     | 77.6  |
| trnTrp (W)    | 1215–1282    | 68        | Forward   | −2                     | TCA                         | 76.5  |
| trnCys (C)    | 1275–1336    | 62        | Reverse   | −8                     | GCA                         | 74.2  |
| trnTyr (Y)    | 1338–1402    | 65        | Reverse   | 1                      | GTA                         | 69.2  |
| COXI         | 1403–2819    | 1417      | Forward   | 0                      | ATC/T                       | 68.1  |
| trnLeu2 (L2)  | 2820–2884    | 65        | Forward   | 0                      | TAA                         | 73.8  |
| COX2         | 2885–3572    | 688       | Forward   | 0                      | ATC/T                       | 72.1  |
| trnLys (K)    | 3573–3641    | 69        | Forward   | 0                      | CTT                         | 68.1  |
| trnAsp (D)    | 3642–3707    | 66        | Forward   | 0                      | GTC                         | 86.4  |
| ATP8         | 3708–3863    | 156       | Forward   | 0                      | ATT/TAG                     | 86.5  |
| ATP6         | 3860–4531    | 672       | Forward   | −4                     | ATA/TAA                     | 75.1  |
| COX3         | 4531–5319    | 789       | Forward   | −1                     | ATG/TAA                     | 70.6  |
| trnGly (G)    | 5322–5385    | 64        | Forward   | 2                      | TCC                         | 85.9  |
| ND3          | 5383–5739    | 357       | Forward   | −3                     | ATA/TAG                     | 79.0  |
| trnAla (A)    | 5738–5802    | 65        | Forward   | −2                     | TGC                         | 81.5  |
| trnArg (R)    | 5803–5864    | 62        | Forward   | 0                      | TCG                         | 74.2  |
| trnAsn (N)    | 5864–5927    | 64        | Forward   | −1                     | GTT                         | 75.0  |
| trnSer1 (S1)  | 5928–5994    | 67        | Forward   | 0                      | GCT                         | 76.1  |
| trnGlu (E)    | 5995–6057    | 63        | Forward   | 0                      | TTC                         | 87.3  |
| trnPhe (F)    | 6060–6123    | 64        | Reverse   | 2                      | GAA                         | 82.8  |
| ND5          | 6124–7840    | 1717      | Reverse   | 0                      | ATT/T                        | 78.3  |
| trnHis (H)    | 7841–7903    | 63        | Reverse   | 0                      | GTG                         | 84.1  |
| ND4          | 7904–9236    | 1333      | Reverse   | 0                      | ATG/T                        | 79.3  |
| ND4L         | 9230–9517    | 288       | Reverse   | −7                     | ATG/TAA                      | 83.0  |
| trnThr (T)    | 9520–9583    | 64        | Forward   | 2                      | TGT                         | 82.8  |
Table 2. Cont.

| Gene      | Position (bp) | Size (bp) | Direction | Intergenic Nucleotides | Anti– or Start/Stop Codons | A + T% |
|-----------|---------------|-----------|-----------|------------------------|-----------------------------|-------|
| trnPro (P) | 9584–9647     | 64        | Reverse   | 0                      | TGG                         | 78.1  |
| ND6       | 9650–10,153   | 504       | Forward   | 2                      | ATT/TAA                     | 85.1  |
| CYTB      | 10,159–11,292 | 1134      | Forward   | 5                      | ATA/TAA                     | 72.2  |
| trnSer2 (S2) | 11,296–11,364 | 69        | Forward   | 3                      | TGA                         | 81.2  |
| ND1       | 11,382–12,332 | 951       | Reverse   | 17                     | TTG/TAG                     | 76.3  |
| trnLeu1 (L1) | 12,334–12,398 | 65        | Reverse   | 1                      | TAG                         | 78.5  |
| rrnL      | 12,399–13,670 | 1272      | Reverse   | 0                      |                             | 80.1  |
| trnVal (V) | 13,671–13,739 | 69        | Reverse   | 0                      | TAC                         | 75.4  |
| rns       | 13,740–14,518 | 779       | Reverse   | 0                      |                             | 78.6  |
| Control Region | 14,519–15,622 | 1104    | Forward   | 0                      |                             | 79.3  |

Table 3. Mitochondrial genome organization of *D. pseudonotabilis*.

| Gene      | Position (bp) | Size (bp) | Direction | Intergenic Nucleotides | Anti– or Start/Stop Codons | A + T% |
|-----------|---------------|-----------|-----------|------------------------|-----------------------------|-------|
| trnIle (I) | 1–66          | 66        | Forward   | 0                      | GAT                         | 72.7  |
| trnGln (Q) | 64–132        | 69        | Reverse   | –3                     | TTG                         | 81.2  |
| trnMet (M) | 132–200       | 69        | Forward   | –1                     | CAT                         | 65.2  |
| ND2       | 201–1211      | 1011      | Forward   | 0                      | ATA/TAA                     | 76.2  |
| trnTrp (W) | 1210–1274     | 65        | Forward   | –2                     | TCA                         | 73.8  |
| trnCys (C) | 1274–1339     | 66        | Reverse   | –1                     | GCA                         | 72.2  |
| trnTyr (Y) | 1341–1405     | 65        | Reverse   | 1                      | GTA                         | 66.2  |
| COX1      | 1440–2940     | 1501      | Forward   | 34                     | ATT/T                        | 67.0  |
| trnLeu2 (L2)| 2941–3005    | 65        | Forward   | 0                      | TAA                         | 72.3  |
| COX2      | 3006–3692     | 687       | Forward   | 0                      | ATA/TAT                      | 70.7  |
| trnLys (K) | 3694–3764     | 71        | Forward   | 1                      | CTT                          | 70.4  |
| trnAsp (D) | 3768–3837     | 70        | Forward   | 3                      | GTC                          | 82.9  |
| ATP8      | 3847–3993     | 147       | Forward   | 9                      | ATA/TAG                      | 85.0  |
| ATP6      | 3990–4661     | 672       | Forward   | –4                     | ATA/TAA                      | 74.3  |
| COX3      | 4661–5447     | 787       | Forward   | –1                     | ATG/T                        | 69.5  |
| trnGly (G) | 5448–5510     | 63        | Forward   | 0                      | TCC                          | 84.1  |
| ND3       | 5511–5862     | 352       | Forward   | 0                      | ATT/T                        | 76.1  |
| trnAla (A) | 5863–5925     | 63        | Forward   | 0                      | TGC                          | 77.8  |
| trnArg (R) | 5925–5989     | 65        | Forward   | –1                     | TCG                          | 73.8  |
| trnAsn (N) | 5989–6053     | 65        | Forward   | –1                     | GTT                          | 73.8  |
| trnSer1 (S1)| 6054–6120   | 67        | Forward   | 0                      | GCT                          | 74.6  |
| trnGlu (E) | 6121–6186     | 66        | Forward   | 0                      | TTC                          | 86.4  |
| trnPhe (F) | 6190–6256     | 67        | Reverse   | 3                      | GAA                          | 79.1  |
| ND5       | 6257–7973     | 1717      | Reverse   | 0                      | ATT/T                        | 77.4  |
| trnHis (H) | 7974–8037     | 64        | Reverse   | 0                      | GTG                          | 84.4  |
| ND4       | 8037–9368     | 1332      | Reverse   | –1                     | ATA/TAA                      | 76.4  |
| ND4L      | 9365–9643     | 279       | Reverse   | –4                     | ATG/TAA                      | 79.9  |
| trnThr (T) | 9646–9709     | 64        | Forward   | 2                      | TGT                          | 84.4  |
| trnPro (P) | 9709–9774     | 66        | Reverse   | –1                     | TGG                          | 75.8  |
| ND6       | 9776–10,273   | 498       | Forward   | 1                      | ATA/TAA                      | 81.7  |
| CYTB      | 10,273–11,409 | 1137      | Forward   | –1                     | ATG/TAA                      | 68.1  |
| trnSer2 (S2) | 11,411–11,479 | 69        | Forward   | 1                      | TGA                          | 78.3  |
| ND1       | 11,497–12,447 | 951       | Reverse   | 17                     | TTG/TAG                      | 75.8  |
| trnLeu1 (L1) | 12,449–12,512 | 64        | Reverse   | 1                      | TAG                          | 75.0  |
| rrnL      | 12,513–13,778 | 1266      | Reverse   | 0                      |                              | 78.8  |
| trnVal (V) | 13,779–13,846 | 68        | Reverse   | 0                      | TAC                          | 77.9  |
| rns       | 13,847–14,620 | 774       | Reverse   | 0                      |                              | 76.5  |
| Control Region | 14,621–15,527 | 907      | Forward   | 0                      |                              | 82.1  |
3.2. Protein-Coding Genes

The PCGs have identical arrangement and similar size between the two mitogenomes and also other cerambycids. Most PCGs of the two species start with the standard ATN start codons (ATA, ATC, ATG, and ATT), whereas ND1 of both mitogenomes begins with the special codon TTG (Tables 2 and 3), which was similar to all other published Cerambycidae mitogenomes [14,15]. Most PCGs of each mitogenome have the complete termination codon TAN (TAA, TAT, or TAG), whereas four PCGs (COX1, COX2, ND4, and ND5) of A. glabripennis and four PCGs (COX1, COX3, ND3, and ND5) of D. pseudonotabilis end with an incomplete stop codon T. These incomplete stop codons are considered to be caused by the post-transcriptional polyadenylation [46] and can be completed by the addition of 3′ nucleotide residues to the neighboring mitochondrial genes.

The relative synonymous codon usage (RSCU) values indicate the most frequently used codon is TTA (Leu) for both mitogenomes (Figure 2), which appears to be a common feature of other sequenced longicorn beetles [14]. ATP8 of both mitogenomes has the highest A + T content among the 13 PCGs (Tables 2 and 3). The Ka/Ks ratios for each PCG of each mitogenome are calculated to assess the selective pressure of the two cerambycid species (Figure 3A). The evolutionary rate of ND6 was the highest among the 13 PCGs. The Ka/Ks ratios of all the 13 PCGs calculated by KaKs_Calculator v2.0 were below 1, which suggests the existence of purifying selection in the two species (Figure 3A). The results of Ka/Ks calculation were similar to a recent mitogenomic work [47], which used DnaSP for the calculation. The gene-wide BUSTED analysis based on the likelihood-ratio test found no evidence of episodic diversifying selection in the PCGs. The site-specific FEL analysis detected ND4 and ND4L each had one codon site under diversifying positive selection at \( p \leq 0.1 \) (Figure 3B). Nearly one-third of each PCG’s codon sites were under purifying selection at \( p \leq 0.1 \). The calculation of KaKs_Calculator v2.0 was consistent with the results of FEL analysis that the PCGs with lower Ka/Ks ratios tended to have more purifying codon sites (Figure 3).

3.3. Transfer RNAs, Ribosomal RNAs, and Control Region

The two mitogenomes both contain the complete set of 22 tRNA genes typical of metazoan mitogenomes. These tRNAs range in size from 62 to 69 bp, which was consistent with previously sequenced mitogenomes of Cerambycidae [15]. The highest A + T content is found in trnGlu of both mitogenomes (Tables 2 and 3). Most of the tRNAs have typical cloverleaf secondary structures, whereas the dihydrouridine (DHU) arm of trnSer1 is shortened in both mitogenomes (Figure 4), which is a common phenomenon in hexapods and metazoan mitogenomes [48]. Numerous mismatched base pairs are found in the secondary structures of tRNA genes, and all of them are G-U pairs.

The large ribosomal RNA (rrnL) gene and small ribosomal RNA (rrnS) gene are found in the conserved location between trnLeu1 and the control region (Tables 2 and 3). The rrnL gene is 1272 bp long in A. glabripennis and 1266 bp long in D. pseudonotabilis, with an A + T content of 80.1% and 78.8%, respectively. The rrnS gene is 779 bp long in A. glabripennis and 774 bp long in D. pseudonotabilis, with an A + T content of 78.6% and 76.5%, respectively.

The control region (CR) is the longest non-coding area in the two mitogenomes (Figure 1) and is functional in the regulation, transcription, and replication processes of the mitogenomes [49]. The CR of A. glabripennis is 1104 bp long and has an A + T content of 79.3%; the CR of D. pseudonotabilis is 907 bp long and has an A + T content of 82.1% (Tables 2 and 3). In the CR of A. glabripennis, 5.2 copies of 57 bp long tandem repeat “AAAATTTCACTCAGCTCTCCGCTATATAAATAATCGCTACCTTTTCAAATTTCCCTA” are detected near the 5′ end of this region. A total of 22 standard (single stem with single loop) and another 7 more complicated stem-loop structures are predicted in the CR of A. glabripennis (Figure S1). There are 17 standard and 4 complicated stem-loop structures in the CR of D. pseudonotabilis (Figure S2). However, no tandem repeats are found in the CR of D. pseudonotabilis. Functions of these secondary structures are unclear.
Figure 2. Relative synonymous codon usage (RSCU) of PCGs in *A. glabripennis* (A) and *D. pseudonotabilis* (B).
3.3. Transfer RNAs, Ribosomal RNAs, and Control Region

The two mitogenomes both contain the complete set of 22 tRNA genes typical of metazoan mitogenomes. These tRNAs range in size from 62 to 69 bp, which was consistent with previously sequenced mitogenomes of Cerambycidae [15]. The highest A + T content is found in \textit{trnGlu} of both mitogenomes (Tables 2 and 3). Most of the tRNAs have typical cloverleaf secondary structures, whereas the dihydrouridine (DHU) arm of \textit{trnSer1} is shortened in both mitogenomes (Figure 4), which is a common phenomenon in hexapods and metazoan mitogenomes [48]. Numerous mismatched base pairs are found in the secondary structures of tRNA genes, and all of them are G–U pairs.

3.4. Phylogenetic Analyses

The phylogenetic positions of \textit{A. glabripennis} and \textit{D. pseudonotabilis} are reconstructed based on the combined mitochondrial gene set of 13 PCGs. The ML and BI analyses generated similar tree topology (Figures 5 and S3). The phylogenetic results are largely congruent with the recent comprehensive mitogenomic phylogenetic study of Nie et al. (2020) [15]. The monophyly of \textit{Cerambycidae s.s.} is not well-supported in both ML and BI trees due to the inclusion of other families of Chrysomeloidea (Figure 5), which is similar to the results of Haddad et al. (2018) [5] and Nie et al. (2020) [15]. The positions of Disteniidae and Oxypeltidae are variable, and Oxypeltidae is recovered as the sister group to all other taxa in the BI tree (Figure S3). The phylogenetic position of Disteniidae remains uncertain, and this family has been recovered as the sister group to various other members of \textit{Cerambycidae s.l.} based on either molecular or morphological datasets [5,9,15,50–57]. The monophyly of \textit{Cerambycidae s.s.} is still one of the most debatable subjects in the phylogeny and evolution of Chrysomeloidea [5].

The monophyly of \textit{Cerambycinae}, \textit{Dorcasominae}, \textit{Lamiinae}, and \textit{Necydalinae} is well-supported in both ML and BI analyses (Figures 5 and S3). The subfamily \textit{Parandrini} is placed within \textit{Prioninae} and should be treated as a tribe of \textit{Prioninae}, as suggested in previous studies [15,58]. Similarly, \textit{Necydalinae} is nested in \textit{Lepturinae} and should be regarded as a tribe of \textit{Lepturinae} [15,50]. \textit{Spondylidinae} is rendered paraphyletic by the species of \textit{Vesperidae}, which differs from the monophyletic condition in Haddad et al. (2018) [5] and Nie et al. (2020) [15]. The non-monophyletic condition of \textit{Vesperidae} has also been recovered based on morphological and molecular characters [54,55,59–61]. The two species sequenced in this study are, respectively, grouped with their relatives from the same subfamily.
Although numerous contributions have been made to explore the higher-level phylogeny of longhorn beetles, there are still some debatable points to be solved: the monophyly of Cerambycidae s.l. and s.s.; the relative relationship between Cerambycidae s.s., Disteniidae, Oxypeltidae, and Vesperidae; and the monophyly and relationship of subfamilies in Cerambycidae s.l., especially within Cerambycidae s.s. The incongruence between different molecular phylogenetic studies could be attributed to the usage of different molecular types, sample sizes, and analytical methods. The taxonomic misidentification of sequenced samples in online databases such as GenBank could also lead to bizarre tree topology, especially for those clades with few taxa. Most main clades of Cerambycidae s.l. still lack sufficient molecular data to clarify their phylogenetic positions. The sequencing of more mitogenomes, optimization of datasets and substitution models, and the supplement of nuclear genes are expected to improve the resolution of mitochondrial phylogenetic reconstruction of Cerambycidae s.l. in future works.

Figure 4. Secondary structures of tRNA genes in the mitogenomes of *A. glabripennis* and *D. pseudonotabilis*. The identity of each tRNA gene is represented by the abbreviation of the related amino acid.
4. Conclusions

In this study, we sequenced and analyzed the mitogenomes of two longicorn beetles, which are important pests of cultivated ecosystems in China. The structure and content of the two mitogenomes are conserved in comparison to other sequenced mitogenomes of...
Cerambycidae, but the intraspecific mitogenomic variation is also detected. The monophyly of four subfamilies was supported by the phylogenetic analysis based on the nucleotide sequence of PCGs. The results provided basic genetic information for understanding the phylogeny and evolution of longicorn beetles.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13101881/s1, Figure S1: Predicted stem-loop structures in the control region of A. glabripennis; Figure S2: Predicted stem-loop structures in the control region of D. pseudonotabilis; Figure S3: Bayesian inference phylogeny of Cerambycidae s.l. inferred from mitogenomic data. Numbers at the nodes are posterior probabilities.

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