Mitochondrial genome of Diploderma micangshanense and its implications for phylogeny of the genus Diploderma

Yanping Li, Yongming Wang, Yinfong Bai, Yunyun Lv and Jianli Xiong

Key Laboratory of Sichuan Province for Fishes Conservation and Utilization in the Upper Reaches of the Yangtze River, College of Life Sciences, Neijiang Normal University, Neijiang, China; College of Animal Science and Technology, Henan University of Science and Technology, Luoyang, China

ABSTRACT

The lizard Diploderma micangshanense, which belongs to the family Agamidae is endemic to China. Here, we determined the complete mitogenome of D. micangshanense using an Illumina Hiseq X Ten sequencer. This mitogenome’s structure is a typical circular molecule of 16,467 bp in length, consisting of 13 protein-coding genes, 22 transfer RNA genes, 2 ribosomal RNA genes, and a control region. The overall base composition of D. micangshanense is 34.1% A, 23.64% T, 13.62% C, and 28.64% G with a slight AT bias of 57.74%. Most mitochondrial genes except ND6 and seven tRNAs were encoded on the heavy strand. Notably, the trnP gene was encoded on the heavy strand instead of its typical light strand position, providing an example of gene inversion in vertebrate mitogenomes. Phylogenetic analysis indicated that D. micangshanense had a close relationship with D. zhaoermii.

Introduction

Animals in the genus Japalura sensu lato are important components of species diversity in Agamidae, and are widely distributed in East Asia and the Himalayas (Manthey 2008). Recently, the current taxonomy of Japalura sensu lato has been redefined as four genera, including Japalura sensu stricto, Pseudocalotes, Cnemidophorus, and the resurrected genus Diploderma (Wang et al. 2019). Almost all the species of the original Japalura sensu lato, have been assigned to Diploderma, except for J. bapoensis, which has been reclassified to genus Pseudocalotes, and two species recorded from southern Tibet, J. andersoniana and J. tricarinata, which are still remain in Japalura sensu stricto. Currently, there are 27 species belonging to Diploderma; of these, 22 are specifically distributed in China, while D. polygonatum is distributed in China and Japan, and the remaining four species are distributed in Vietnam, Myanmar and mainland Southeast Asia.

Diploderma micangshanense is distributed in Sichuan, Shaanxi, Shanxi, Gansu and Henan Provinces. It bears Least Concern status on the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species (IUCN 2020). However, the available genetic data for this species remains scarce. Mitochondrial DNA has many valuable features including relatively conserved gene content and organization, lack of genetic recombination, maternal inheritance, and relatively fast evolutionary rate. Hence, partial or complete mitochondrial genes have been used for species identification (Hebert et al. 2003; Chambers and Hebert 2016), and to determine molecular phylogenetic and evolutionary relationships (Leavitt et al. 2017; Medina et al. 2018; Shahamat et al. 2020). In this study, we assemble and annotate the mitochondrial genome of D. micangshanense, and determine its genomic structure and base composition. We also reconstruct the phylogenetic relationships within the genus Diploderma using the mitochondrial sequence ND2 obtained here and from NCBI. This study not only improves understanding of genomic information and phylogenetic of Diploderma, but is also conducive to the conservation genetics of D. micangshanense.

Materials and methods

Sample collection

Samples were collected from Luoning County, Henan Province, China (34°16′48″N, 111°43′5″E). Muscle samples were preserved in 95% ethanol, and voucher samples were deposited in the Museum of Henan University of Science and Technology (contact with Jianli Xiong, xiongjl@haust.edu.cn) under the voucher number HNUSTM20200824. Sampling was performed according to Chinese animal protection laws.

DNA extraction and sequencing

Genomic DNA was extracted from muscle tissue using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany).
DNA integrity, purity and concentration were assessed with an Agilent 5400 fragment analyzer (Agilent Technologies, Santa Clara, CA, U.S.A.). After the DNA sample was qualified, and the template size is 21.578 ng/ul, it was randomly disrupted with a Covaris ultrasonicator (Covaris Inc., Woburn, MA, USA), and then the library was constructed through several steps: end repair and phosphorylation, adding A-tailing, ligating index adapter, purification, denaturing and PCR amplification. After the library was constructed, a Qubit 2.0 (Life Technologies, Singapore) was used to quantify and dilute the library. We then employed an Agilent 2100 Bioanalyzer (Agilent) to detect inserted fragments in the library. Finally, the effective concentration of the library was accurately quantified by q-PCR to ensure the library quality. After that, different libraries were pooled into the flow cell according to the effective concentration and target drop-off data. Illumina paired-end sequencing was conducted with an Illumina HiSeq X Ten sequencer (Illumina, San Diego, CA, USA).

Mitochondrial genome assembly and annotation

The raw data contained adapter information, low-quality bases, and undetected bases (indicated by N), which would interfere with subsequent analysis. We therefore filtered the raw data using the following criteria: (1) Filtered out reads containing adapter sequences; (2) removed paired reads, when the content of N in a single-ended sequence exceeded 10%; (3) Base with quality no more than 5 was regarded as low-quality base based on phred + 33. If in a sequence more than half were low-quality bases, this sequence, along with the paired one was...
discarded. The remaining clean data was used for mitochondrial genome assembly with MitoZ v.2.4 using default parameters (Meng et al. 2019). Clade and required taxa were set to Chordata and Japalura, respectively. The assembled genome was annotated using MitoZ v.2.4 with Diploderma flaviceps (NC_039541.1) as the reference (Liu et al. 2019).

**Phylogenetic analysis and genetic distance estimate**

To examine the evolutionary status of *D. micangshanensis*, we used ND2 regions of *Diploderma* species for phylogenetic inference with *Pseudocalotes flavigula* as the outgroup. Of the 27 valid species currently recognized in genus *Diploderma*, we covered 19 species for which ND2 sequences were available so far, including *D. zhaoermii* (*n* = 1), *D. micangshanensis* (*n* = 3), *D. varcoae* (*n* = 1), *D. dymondi* (*n* = 1), *D. swinhonis* (*n* = 1), *D. polygonatum* (*n* = 1), *D. makii* (*n* = 1), *D. luei* (*n* = 1), *D. brevipes* (*n* = 1), *D. splendidum* (*n* = 1), *D. flaviceps* (*n* = 1), *D. yunnanense* (*n* = 2), *D. chapaense* (*n* = 2), *D. yulonense* (*n* = 1), *D. batangensis* (*n* = 2), *D. vela* (*n* = 1), *D. slowinski* (*n* = 1), *D. laeviventre* (*n* = 1), and *D. swild* (*n* = 1). Multiple codon-based alignments were conducted with MEGA v.7 (Kumar et al. 2016) with the MUSCLE module, and each alignment was further manually corrected. Firstly, the genetic distances of these 19 species were calculated using Kimura 2-parameter (K2P) model (Kimura 1980) with MEGA v.7, which showed the intraspecific genetic distance, and confidence was assessed with 1000 bootstrap replications. Subsequently, we predicted the best nucleotide substitution model using jModeltest v.2 (Darriba et al. 2012) with Bayesian Information Criterion (BIC). We used IQ-tree v.1.6.2 (Nguyen et al. 2015) to construct phylogenetic topologies based on maximum likelihood (ML) and Bayesian inference (BI), using an HKY + F + I + G4 model. Node support of the trees was inferred by bootstrapping with 1000 replications. Trees were graphically visualized and edited with FigTree v1.4.0 (Rambaut and Drummond 2012).

**Results and discussion**

A total of 22,652,258 raw reads was generated and it has been deposited to NCBI database (see additional details in Data availability statement). After assembly, the complete mitogenome of *D. micangshanensis* was obtained (accession number: MW242820), with a total length of 16,467 bp, similar to other agamid species (Liu et al. 2019). The mitogenome of *D. micangshanensis* consists of 13 protein-coding genes (*ND1, ND2, COI, COII, ATP8, ATP6, COIII, ND3, ND4L, ND4, ND5, ND6, and Cyt b*), 22 transfer RNA (tRNA) genes, 2 ribosomal RNA genes, and a control region (Figure 1). The outermost layer of Figure 1 is gene structure, where orange-yellow indicates the rRNA genes, orange-red indicates the tRNA genes, light green indicates the 13 protein-coding genes, and the

---

**Table 1. Characteristics of 37 genes in the mitochondrial genome of Diploderma micangshanensis.**

| Gene/Element | From | To | Length (bp) | Start | Stop | Intergenic nucleotides | Strand |
|--------------|------|----|-------------|-------|------|------------------------|--------|
| tmF          | 528  | 324| 67         | -1    | H    |                         |        |
| s-rRNA/ 12S rRNA | 324  | 1170| 847        | -2    | H    |                         |        |
| tmV          | 1169 | 1234| 66         | -1    | H    |                         |        |
| l-rRNA/16S rRNA | 1234 | 2729| 1496       | +33   | H    |                         |        |
| tmL (uaa)    | 2763 | 2837| 75         |       | H    |                         |        |
| ND1          | 2841 | 3809| 969        | ATG   | TAG  | -5                     | H      |
| tmQ          | 3805 | 3876| 72         | +2    | L    |                         |        |
| tmI          | 3879 | 3949| 71         | 0     | H    |                         |        |
| tmM          | 3950 | 4014| 65         | 0     | H    |                         |        |
| ND2          | 4015 | 5043| 1029       | ATA   | TAG  | -2                     | H      |
| tmW          | 5042 | 5113| 72         | +3    | H    |                         |        |
| tmA          | 5117 | 5184| 68         | +9    | L    |                         |        |
| tmH          | 5194 | 5265| 72         | +21   | L    |                         |        |
| tmC          | 5287 | 5337| 51         | 0     | L    |                         |        |
| tmY          | 5338 | 5400| 63         | 0     | L    |                         |        |
| COI          | 5401 | 6978| 1578       | ATG   | AGA  | -5                     | H      |
| tmS (uga)    | 6974 | 7044| 71         | +2    | L    |                         |        |
| tmD          | 7047 | 7115| 69         | +3    | H    |                         |        |
| COII         | 7119 | 7805| 687        | ATG   | AGG  | +6                     | H      |
| tmK          | 7799 | 7865| 67         | +1    | H    |                         |        |
| ATP8         | 7867 | 8028| 162        | GTG   | TAA  | -10                    | H      |
| ATP6         | 8019 | 8701| 683        | ATG   | T    | -1                     | H      |
| COIII        | 8701 | 9485| 785        | ATG   | TA- | -1                     | H      |
| tmG          | 9485 | 9551| 67         | +4    | H    |                         |        |
| ND3          | 9556 | 9892| 337        | ATG   | T    | 0                      | H      |
| tmR          | 9893 | 9963| 71         | 0     | H    |                         |        |
| ND4L         | 9964 | 10,254| 291       | ATG   | TAA  | -7                     | H      |
| ND4          | 10,248| 11,615| 1368     | ATG   | AGG  | +7                     | H      |
| tmH          | 11,623| 11,685| 63       | 0     | H    |                         |        |
| tmS (gcu)    | 11,686| 11,743| 58       | +4    | H    |                         |        |
| tmL (uag)    | 11,748| 11,818| 71       | 0     | H    |                         |        |
| ND5          | 11,819| 13,597| 1779    | ATA   | TAA  | -4                     | H      |
| ND6          | 13,594| 14,100| 507     | ATG   | TAG  | 0                      | L      |
| tmE          | 14,101| 14,168| 68      | +2    | L    |                         |        |
| cyt b        | 14,171| 15,303| 1133    | ATG   | T    | -1                     | H      |
| tmT          | 15,303| 15,370| 68      | 0     | H    |                         |        |
| tmP          | 15,371| 15,437| 67      | 0     | H    |                         |        |
remainder is the control region. Most genes are transcribed from the heavy strand (2 rRNAs, 12 protein-coding genes and 15 tRNAs); only eight genes, including one protein-coding gene (ND6) and seven tRNAs (trnQ, trnA, trnN, trnC, trnY, trnS and trnE), are encoded on the light strand. Notably, the trnP gene is encoded on the heavy strand instead of its typical light strand position, providing an example of gene inversion in vertebrate mitogenomes. *D. micangshanensis* shares the same gene arrangement type (inverted trnP gene) with other Draconinae species, indicating a single occurrence of the trnP inversion in the ancestral draconine lineage (Liu et al. 2019).

As is the case with other agamid mitogenomes, the overall base composition of *D. micangshanensis* is 34.1% A, 23.64% T, 13.62% C, and 28.64% G, with a slight AT bias of 57.74%. There are 11 overlapping regions totaling 40 bp (varying from 1 to 10 bp) and 14 intergenic spacer regions totaling 100 bp (varying from 1 to 33 bp). Almost all protein-coding genes (PCGs) start with the typical ATA/ATG initiation

---

**Figure 2.** Phylogenetic relationships of species in genus *Diploderma* inferred by Bayesian Inference and Maximum Likelihood analyses, based on the mitochondrial ND2 gene fragment. Numbers on the branches from left to right are Bayesian posterior probabilities obtained by BI and ML bootstrap values, respectively. Posterior probabilities less than 0.60 and bootstrap values under 60% are not shown.
codons whereas ATP8 starts with GTG. Most PCGs are terminates with the typical TAA/TAG/AGG/AGA codons, except for ATP6, COIII, ND3, and Cyt b, which are characterized by incomplete stop codons (T or TA). The 22 tRNA genes are interspersed along the genome, with the length varying from 51 to 75 bp. The 12S and 16S rRNA genes are 847 and 1496 bp, respectively. They are located between trnF and trnL (uaa) and are separated by trnV (Table 1). The D-loop region is located between trnP and trnF.

Genetic distance shows the D. micangshanensis in this study has the closest distance with the D. micangshanensis deposited on NCBI (Table S1), which confirms that the sequenced specimen in this study indeed belongs to D. micangshanensis. The two methods (BI and ML) generated a consistent phylogenetic topology that D. micangshanensis in this study clustered together with the individuals deposited in GenBank and displayed a closest relationship with D. zhaoermii (Figure 2). Additionally, the topology of Diploderma divide into two major clades (Clade A and B in Figure 2), and the D. micangshanensis locate into Clade A. The placement of D. micangshanensis was also supported by Wang et al. 2019. Thus, our study further verify and confirm the phylogenetic position of D. micangshanensis with molecular data. In summary, our study provides a new resource for understanding whole mitochondrial genome of D. micangshanensis, which will promote the molecular study on this species.

Acknowledgments
The authors sincerely thank Dr Yunhai Yi for her kind revising the manuscript. We also thank two anonymous reviewers for their valuable comments that improved the quality of this article.

Disclosure statement
No potential conflict of interest was reported by the author(s).

Funding
This work was supported by the grants of the National Natural Science Foundation of China [NSFC 31471971]; and Open Project of Ecological Security and Protection Key Laboratory of Sichuan Province [ESP2006].

Data availability statement
The genome sequence data that support the findings of this study are openly available in GenBank of NCBI at (https://www.ncbi.nlm.nih.gov/) under the accession no. MW242820. The associated BioProject, SRA, and Bio-Sample numbers are PRJNA675949, SRR13022469, and SAMN16736307 respectively.

References
Chambers EA, Hebert PD. 2016. Assessing DNA barcodes for species identification in North American reptiles and amphibians in natural history collections. PLOS One. 11(4):e0154363.
Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 9(8):772.
Hebert PD, Cywinska A, Ball SL, Dewaard JR. 2003. Biological identifications through DNA barcodes. Proc Biol Sci. 270(1512):313–321.
IUCN. 2020. The IUCN Red List of Threatened Species. Version 2020.1. [accessed 2020 November 10]. https://www.iucnredlist.org.
Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 16(2):111–120.
Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 33(7): 1870–1874.
Leavitt DH, Marion AB, Hollingsworth BD, Reeder TW. 2017. Multilocus phylogeny of alligator lizards (Elgaria, Anguidae): testing mtDNA introgression as the source of discordant molecular phylogenetic hypotheses. Mol Phylogenet Evol. 110:104–121.
Liu J, Yu J, Zhou M, Yang J. 2019. Complete mitochondrial genome of Japalura flaviceps: deep insights into the phylogeny and gene rearrangements of Agamidae species. Int J Biol Macromol. 125: 423–431.
Manthey U. 2008. Terralog: Agamid lizards of Southern Asia, Draconinae 2, Leiolepidinae. Frankfurt/M: Chimaira.
Medina CD, Avila LJ, Sites JW, Jr, Santos J, Morando M. 2018. Alternative methods of phylogenetic inference for the Patagonian lizard group Liolaemus elongatus-krieji (Iguania: Liolaemini) based on mitochondrial and nuclear markers. Mol Phylogenet Evol. 120:158–169.
Meng G, Li Y, Yang C, Liu S. 2019. MitoZ: a toolkit for animal mitochondrial genome analysis, annotation and visualization. Nucleic Acids Res. 47(11):e63.
Nguyen L-T, Schmidt HA, Von Haeseler A, Minh BQ. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol. 32(1):268–274.
Rambaut A, Drummond A. 2012. Molecular evolution, phylogenetics and epidemiology. FigTree version 1.4.
Shahamat A-A, Rastegarpouyani E, Rastegar-Pouyani N, Yousefkhani SSH, Wink M. 2020. Molecular phylogeny and infraspecific differentiation of the Trapelus agilis species complex in Iran (Squamata: Agamidae) inferred from mitochondrial DNA sequences. PeerJ. 8: e8295.
Wang K, Ren J, Jiang K, Wu J, Yang C, Xu H, Messanger K, Lei K, Yu H, Yang J. 2019. Revised distributions of some species in the genus Diploderma (Reptilia: Agamidae) in China. Sichuan Journal of Zoology. 38:481–495.