



the genetic diversity and evolutionary background of *G. liboensis*. This study contributes to the growing genomic resources for gekkonid reptiles and aids future phylogenetic and conservation research.

**Keywords:** *Gekko (Japonigekko) liboensis*, Gekkonidae, mitogenome, phylogeny

## 1. INTRODUCTION

The genus *Gekko* Laurenti, 1768, belongs to the family Gekkonidae and is one of the most recognized gecko genera. It comprises a broadly spread group of nocturnal gekkonid lizards, currently encompassing approximately 90 known species classified into seven subgenera [1-2]. Among these, *Japonigekko* is the most diverse, with 21 species recorded in China [2], including four karst-dwelling species: *G. adleri* Nguyen et al. 2013, *G. kwangsiensis* Yang 2015, *G. liboensis* Zhou and Li 1982, and *G. paucituberculatus* Wang et al. 2024 [3].

*Gekko liboensis*, commonly known as the Libo Gecko, is endemic to China, occurring in Guizhou and Guangxi [4]. The species was originally described from a single female specimen collected in Libo, Guizhou [5]. In 1999, three additional specimens (two females and one juvenile) were reported [6]. However, due to its strong morphological similarity to *G. hokouensis* Pope, 1927, and the absence of male specimens, the taxonomic validity of *G. liboensis* remained uncertain for many years [7]. This uncertainty persisted until 2015, when Jono et al. (2015) confirmed its validity based on the morphological characteristics of 31 newly collected specimens from the type locality [4]. Despite these morphological studies, molecular data for the species remain unavailable, limiting further exploration of its genetic characteristics and molecular identification. This gap in knowledge restricts a deeper understanding of its phylogenetic relationships and evolutionary significance.

Currently, although 36 species have been described in the subgenus *Japonigekko* [2], only eight complete mitochondrial genomes, representing five species (*Gekko chinensis*, *G. japonicus*, *G. hokouensis*, *G. subpalmatus*, and *G. swinhonis*) [8-13], have been deposited in GenBank, none of which belong to karst-dwelling species. Here, by employing the next-generation sequencing technology, we present the first complete mitogenome of *G. liboensis*, marking the first mitogenome record for a karst-adapted species within *Japonigekko*. This study aims to enhance our understanding of the mitogenome

structure, phylogenetic relationship, and taxonomic status of *G. liboensis*.

## 2. MATERIALS AND METHODS

### 2.1 Sampling and DNA Extraction

After morphological identification, based on characteristics such as a larger body size, the presence of one cloacal spur on each side, tubercles on the dorsal surface but absent on the limbs, and the absence of developed interdigital webbing [4-5], a voucher specimen of *Gekko liboensis* (SWU 0010068) was collected by hand from the Guizhou Maolan National Nature Reserve, Libo, Guizhou, China (25°15'26.3"N, 108°1'13.98"E; altitude 500 m a.s.l.). The specimen was preserved at Southwest University, Chongqing, China, while muscle tissue was isolated and preserved in 95% ethanol. Genomic DNA was extracted using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China).

### 2.2 Genome Sequencing and Annotation

A paired-end library with an insert size ranging from 300 to 800 bp was constructed and sequenced on the Illumina NovaSeq 6000 platform at Wuhan Benagen Technology Company Limited (Beijing, China) using paired-end 150-bp chemistry. The sequencing target depth for the sample was 25 ×.

The quality of the demultiplexed raw paired-end reads was evaluated using FastQC v0.12.0 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were then processed using Trimmomatic v0.39 [14] to generate a cleaned dataset. This process involved trimming Illumina adapters, removing reads that fell below the quality threshold (sliding window set to 5:20), and discarding short cleaned reads (<50 bp) or those lacking either the forward or reverse pair. The cleaned reads were then assembled into the mitochondrial genome sequence using MitoFinder v1.4.2 [15], followed by annotation to generate the complete mitogenome sequence. A mitochondrial genome annotation file of the closely related species *Gekko swinhonis* (JQ906550) was downloaded from NCBI and used as a reference. Proksee [16], a specialized online genome visualization tool, was used to generate the circular mitogenome map of *G. liboensis*.

### 2.3 Phylogenetic Analysis

To analyze the phylogeny of *Gekko liboensis* within the subgenus *Japonigekko*, mitogenomes of five species and mitochondrial 16S rRNA and ND2

genes (concatenated into one contig for each species) of 16 species were downloaded from GenBank nucleotide database. *Gekko gekko*, belonging to the subgenus *Gekko* [1, 17], was used as the outgroup. In total, nine complete mitochondrial genomes, including *G. liboensis*, were used for mitogenome-based phylogenetic analysis, while 25 concatenated 16S rRNA and ND2 sequences were employed for mitochondrial gene based phylogenetic analysis (Supplementary Table S1). All sequences were assembled and aligned by MEGA X v10.1 [18].

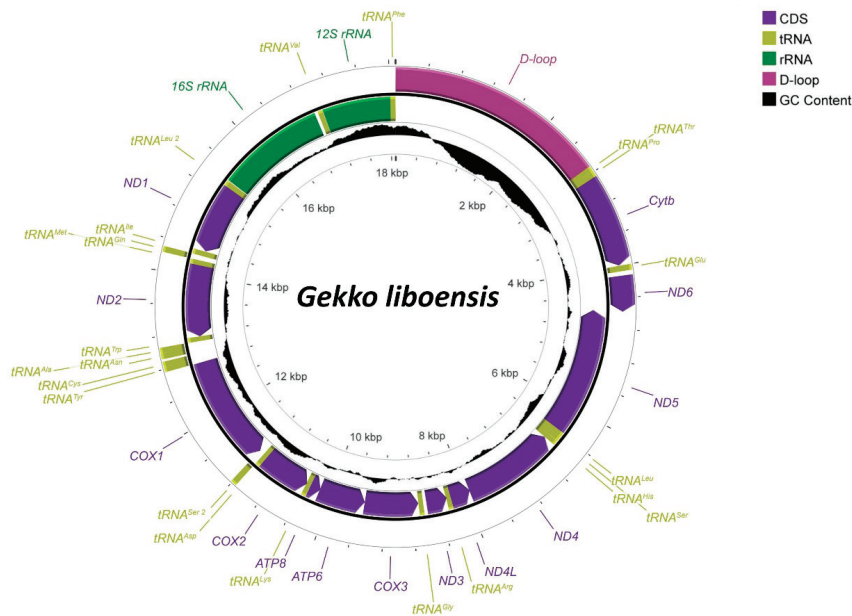
Using the above two sets of genetic data, firstly, the Maximum Likelihood (ML) analyses were conducted on the IQ-TREE webserver [19-20], with the best-fitting substitution model and 10,000 ultrafast bootstrap replications (UFB) [21]. Strong support was assigned to nodes with UFB values greater than or equal to 95 [22]. Then Bayesian Inference (BI) analyses were conducted on the two mitochondrial gene sequences using MrBayes v3.2.7 [23]. The posterior probability distributions were generated using four incrementally heated Markov chains run for 20 million generations, with trees sampled every 1000 generations. After discarding the initial 25% of trees as burn-in, Bayesian posterior probabilities (BPPs) were estimated from the remaining sampled trees. Convergence was confirmed by a mean split frequency standard deviation below 0.01, effective

sample sizes exceeding 200, and potential scale reduction factors approaching 1. BPP values of 0.95 or higher were regarded as providing significant support for the nodes [24-25]. Prior to phylogenetic analyses, PartitionFinder v2 [26] was used to determine the optimal nucleotide substitution model for 16S rRNA and ND2.

### 3. RESULTS

#### 3.1 Characteristics of *Gekko liboensis* Mitogenome

The whole mitochondrial genome of the *Gekko liboensis* is circular and has a total length of 18,079 bp (GenBank accession number PV066215). Its gene composition and arrangement pattern are consistent with those observed in other *Japonigekko* species [8-13]. The mitogenome contains the typical 37 genes: 13 protein-coding genes (PCGs), 22 tRNA genes, two rRNA genes, and a single control region (Figure 1). Among the PCGs, ten are initiated by the standard ATG start codon, while ND2 starts with an ATT codon. The ND6 and ND3 genes begin with incomplete stop codons, G-- and A--, respectively. Seven PCGs (ND6, ND4, ND4L, ATP6, ATP8, ND2 and ND1) terminate with a TAA stop codon, while three (Cytb, ND3 and COX2) end in TAG and two (ND5 and COX1) in AGA. The COX3 gene, however, possesses an incomplete stop codon (A--) (Table 1).



**Figure 1.** Circular map of *Gekko liboensis* mitochondrial genome. Different colors indicate different types of genes and regions. The black inner circle represents the GC content of the mitogenome.

**Table 1.** Characteristics of the mitochondrial genome of *Gekko liboensis*.

Gene/region	Start	End	Length(bp)	Spacer (+)	Start codon	Stop codon
				Overlap (-)		
<i>D-loop</i>	1	2719	2719	1		
<i>tRNA<sup>Pro</sup></i>	2720	2789	70	1		
<i>tRNA<sup>Thr</sup></i>	2790	2858	69			
<i>Cytb</i>	2858	3997	1140	4	ATG	TAG
<i>tRNA<sup>Glu</sup></i>	4001	4072	72	57		
<i>ND6</i>	4129	4584	456	-15	G--	TAA
<i>ND5</i>	4569	6377	1809	2	ATG	AGA
<i>tRNA<sup>Leu</sup></i>	6379	6450	72	1		
<i>tRNA<sup>Ser</sup></i>	6451	6513	63	2		
<i>tRNA<sup>His</sup></i>	6515	6582	68	2		
<i>ND4</i>	6584	7942	1359	-6	ATG	TAA
<i>ND4L</i>	7936	8232	297	3	ATG	TAA
<i>tRNA<sup>Arg</sup></i>	8235	8298	64	-9		
<i>ND3</i>	8289	8591	303	49	A--	TAG
<i>tRNA<sup>Gly</sup></i>	8640	8706	67	2		
<i>COX3</i>	8708	9490	783		ATG	A--
<i>ATP6</i>	9490	10170	681	-9	ATG	TAA
<i>ATP8</i>	10161	10316	156	4	ATG	TAA
<i>tRNA<sup>Lys</sup></i>	10320	10385	66	-10		
<i>COX2</i>	10375	11073	699	1	ATG	TAG
<i>tRNA<sup>Asp</sup></i>	11074	11139	66	3		
<i>tRNA<sup>Ser 2</sup></i>	11142	11217	76	-6		
<i>COX1</i>	11211	12758	1548	5	ATG	AGA
<i>tRNA<sup>Tyr</sup></i>	12763	12825	63	2		
<i>tRNA<sup>Cys</sup></i>	12827	12891	65	30		
<i>tRNA<sup>Asn</sup></i>	12921	12993	73	1		
<i>tRNA<sup>Ala</sup></i>	12994	13062	69			
<i>tRNA<sup>Trp</sup></i>	13062	13128	67			
<i>ND2</i>	13128	14159	1032	1	ATT	TAA
<i>tRNA<sup>Met</sup></i>	14160	14228	69			
<i>tRNA<sup>Gln</sup></i>	14228	14299	72	-1		
<i>tRNA<sup>Ile</sup></i>	14298	14370	73	6		
<i>ND1</i>	14376	15338	963	1	ATG	TAA
<i>tRNA<sup>Leu 2</sup></i>	15339	15413	75	12		
<i>16S rRNA</i>	15425	16938	1514	46		
<i>tRNA<sup>Val</sup></i>	16984	17051	68	5		
<i>12S rRNA</i>	17056	18006	951	1		
<i>tRNA<sup>Phe</sup></i>	18007	18079	73			

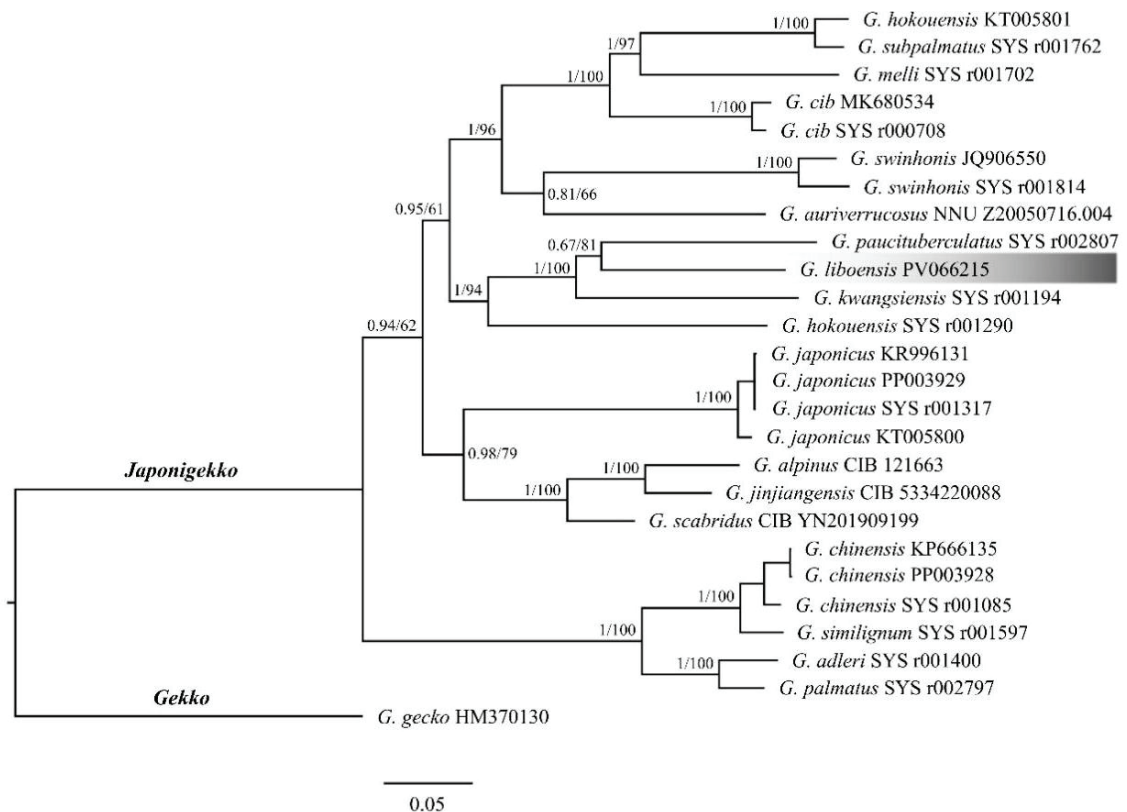
On the heavy strand, the *ND6* gene along with eight tRNA genes (*tRNA<sup>Pro</sup>*, *tRNA<sup>Glu</sup>*, *tRNA<sup>Ser</sup>*, *tRNA<sup>Tyr</sup>*, *tRNA<sup>Cys</sup>*, *tRNA<sup>Asn</sup>*, *tRNA<sup>Ala</sup>*, and *tRNA<sup>Gln</sup>*) are located, while the other genes are encoded on the light strand. The *12S* and *16S rRNA* genes, which are 951 bp and 1514 bp in size, respectively, are positioned between the *rRNA<sup>Phe</sup>* and *tRNA<sup>Leu</sup>* genes, with the *tRNA<sup>Val</sup>* gene serving as a separator. The length of tRNAs ranges from 63 to 76 bp. Moreover, the control region (*D-loop*), spanning 2719 bp, shows an A+T content of 62.9%. It is situated between the *tRNA<sup>Phe</sup>* and *tRNA<sup>Pro</sup>*. The overall base composition of the genome is as follows: 26.0% A, 31.8% T, 14.6% C, and 27.6% G, resulting in an A+T content of 57.8%, indicating a slight AT bias.

### 3.2 Phylogenetic Relationship

The outcome of the mitogenome-based phylogenetic analyses strongly support the placement of *Gekko liboensis* within the subgenus *Japonigekko*

(Supplementary Figure S1). Due to the limited available data, the phylogenetic relationships between *G. liboensis* and other species cannot be reliably inferred from the currently published mitochondrial genome sequences.

The concatenated nucleotides dataset of *16S rRNA* and *ND2* genes is 1574 bp in length per species, with GTR + G identified as the best-fitting substitution model. Phylogenetic trees constructed using these sequences from 25 species reveal that *Gekko liboensis* is closely related to *G. paucituberculatus*, which subsequently groups with *G. kwangsiensis* (Figure 2). However, two inconsistencies are observed: 1.) the mitogenome of *G. hokouensis* (KT005801) clusters with the mitochondrial genes of *G. subpalmatus* (SYS r001762), instead of grouping with *G. hokouensis* (SYS r001290). and 2.) the mitogenome of *G. subpalmatus* (MK680534) groups with the mitochondrial genes of *G. cib* (SYS r000708), rather than with *G. subpalmatus* (SYS r001762). Aside from these discrepancies, the



**Figure 2.** Bayesian Inference tree of the subgenus *Japonigekko* based on concatenated mitochondrial *16S rRNA* and *ND2* genes. Numbers before the slashes represent Bayesian posterior probabilities (BPP), while those after slashes denote ultrafast bootstrap replications (UFB). The scale bar represents 0.05 nucleotide substitutions per site.

phylogenetic relationships among other species within *Japonigekko* are consistent with previously reported phylogenies based on the same mitochondrial genes [3, 27].

#### 4. DISCUSSION AND CONCLUSION

By employing next-generation sequencing technology, we have obtained and annotated the first complete mitochondrial genome of *Gekko liboensis*. Our findings reveal that the mitogenomic gene order and composition in Libo Gecko are identical to those of the five previously reported species within the subgenus *Japonigekko* [8-13], all of which were also included in the phylogenetic tree construction in our study. Furthermore, this study provides the first sequenced mitogenome of a karst-dwelling species within *Japonigekko*. This novel genomic data serves a valuable resource for future taxonomic and phylogenetic studies.

The phylogenetic analysis performed in this study, which integrated mitogenomes and nucleotide sequences of the *16S rRNA* and *ND2* genes, demonstrated that relationships within the *Japonigekko* group were largely consistent with earlier studies [3, 27-28]. However, two exceptions were observed in the mitochondrial genomes of *Gekko subpalmatus* (MK680534) and *G. hokouensis* (KT005801). The mitochondrial genome of *G. subpalmatus* (MK680534), reported by Luo et al. (2019), was based on a sample collected from Chengdu, Sichuan Province, China [12]. Following this study, Lyu et al. (2021) revealed that the *G. subpalmatus* complex comprises three evolutionarily distinct taxa. Among them, populations from Zhejiang in eastern China represent *G. subpalmatus*, while those from the Sichuan Basin in southwestern China have been identified as *G. cib* [29]. Therefore, based on these taxonomic revisions and our phylogenetic analyses, we consider that *G. subpalmatus* (MK680534) is *G. cib* (MK680534), and that the name change reflects a taxonomic update rather than the re-identification of a distinct species. For *G. hokouensis* (KT005801), the sample was collected from Luxi Island, Wenzhou, Zhejiang, China [10]. It is not geographically distinguishable from *G. subpalmatus*. In our phylogenetic analyses based on two mitochondrial genes, *G. hokouensis* samples did not cluster together. Instead, *G. hokouensis* (KT005801) formed a well-supported clade with *G. subpalmatus* (SYS r001762). In the absence of morphological data, this pattern may indicate either species misidentification or mitochondrial introgression, and further investigation is warranted.

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#### CONFLICT OF INTEREST STATEMENT

The authors declare that they hold no competing interests.

#### DECLARATION OF GENERATIVE AI IN PREPARATION OF MANUSCRIPT

During the preparation of this work, the authors used ChatGPT-4 to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as necessary and take full responsibility for the publication's content.

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