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Mitogenome Diversity and Phylogeny of Felidae Species

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Abstract

As apex predators, felids (Felidae) face unresolved phylogenetic controversies due to their recent rapid speciation and remarkable morphological conservatism. Previous studies, often relying on a limited number of genetic markers, were constrained by insufficient data and conflicting phylogenetic signals, leaving these disputes unresolved. Therefore, establishing a robust phylogenetic framework based on larger-scale genomic data is crucial. This study integrated complete mitogenomes from 37 species representing all major felid genera to characterize genomic diversity, selection pressures, and phylogenetic relationships. Results revealed conserved gene content and arrangement patterns but significant intergenic variation in nucleotide composition, with the light-strand encoded *ND6* exhibiting pronounced strand-specific bias. Nucleotide diversity was highest in *ND4L* (Pi = 0.132) and *ATP6* (Pi = 0.131), suggesting their utility as novel markers for species delimitation and population studies. Selection pressure analysis indicated strong purifying selection on cytochrome oxidase subunits (e.g., *COX1* Ka/Ks = 0.00327) but relaxed constraints on *ATP8* (Ka/Ks = 0.12304). Phylogenies reconstructed from the complete 13PCGs + 2rRNAs dataset (showing high congruence between maximum likelihood and Bayesian methods) clearly delineated Felidae into two primary clades (Pantherinae and Felinae), confirming monophyly of all genera and positioning *Neofelis nebulosa* as the basal lineage within Pantherinae. Crucially, exclusion of *ND6* (12PCGs + 2rRNAs) yielded topologies congruent with the complete 13PCGs + 2rRNAs dataset, whereas single-gene or limited multi-gene datasets produced inconsistent trees (particularly at genus-level nodes). This demonstrates that near-complete mitogenomic data (≥12PCGs + 2rRNAs) are essential for reconstructing robust felid phylogenetic frameworks. Our study provides insights into carnivoran mitogenome evolution.

Keywords: Felidae; mitogenome; evolution; diversity; phylogeny

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1. Introduction

The family Felidae, commonly known as felids, belongs to the class Mammalia and the order Carnivora. Currently, there are approximately 40 extant species [1]. This family exhibits remarkable diversity, ranging from apex predators like the lion (*Panthera leo*), tiger (*P. tigris*), and snow leopard (*P. uncia*) to the human companion, the domestic cat (*Felis catus*).

Felids boast a near-global distribution, inhabiting every continent except Antarctica, and occupy diverse habitats including deserts, tropical rainforests, and high mountains [2–4]. Alarmingly, recent global research indicates that terrestrial carnivores, including felids, face unprecedented survival crises [5].

As apex predators within ecosystems, felids play crucial roles in regulating food webs and maintaining ecosystem structure and function, serving as vital indicators of ecosystem health. Beyond their significant ecological value, they possess rich cultural and symbolic importance. However, reconstructing felid phylogeny and taxonomy faces substantial challenges: the group underwent rapid and very recent speciation events, compounded by an incomplete fossil record [3,6]. This rapid and recent radiative evolutionary pattern has resulted in significant difficulties resolving relationships among taxa, making it one of the most contentious issues in current systematic biology [3,6–8]. Consequently, establishing a robust phylogenetic framework for Felidae is not only theoretically important for understanding evolutionary mechanisms but also provides essential scientific underpinning for effective conservation strategies.

All extant felids occupy highly similar ecological niches, leading to remarkable morphological conservatism with few discernible dental or skeletal synapomorphies [3]. Consequently, accurate classification based solely on morphology of extant species is challenging. Advances in molecular biology have revolutionized our understanding of felid phylogeny. The mitochondrial genome (mitogenome), a vital cytoplasmic genetic element, has long been instrumental in studies of species evolution, population genetics, and phylogenetics [9–16], owing to its maternal inheritance, high mutation rate, relatively small size (~14–20 kbp), and conserved gene content [17,18]. Over the past two decades, the rapid development and widespread adoption of high-throughput sequencing technologies have accelerated the accumulation of mitochondrial DNA data, leading to frequent reports of complete mitogenomes across various felid species [19–30]. This provides a timely opportunity for comparative genomic studies. Previously, numerous research teams have attempted to reconstruct the phylogenetic relationships among felids based on single or multiple mitochondrial genes. For instance, Agnarsson et al. utilized *Cytb* [31], Masuda et al. employed *rrnS* or *Cytb* [32], Janczewski et al. analyzed both *rrnS* and *Cytb* [6], Johnson and O'Brien focused on *rrnL* and *ND5* [33], Mattern and McLennan examined rrnS, rrnL, *ND5*, and *Cytb* [34], while Yu and Zhang incorporated *rrnS*, *rrnL*, *ND2*, *ND4*, *ND5*, and *Cytb* into their study [8]. Additionally, Wei et al. included *rrnS*, rrnL, *ND2*, *ND4*, *ND5*, *Cytb* as well as *ATP8* in their analysis [7,21]. Some research teams have also made similar attempts using complete mitogenomes as a basis for their studies [35–38]. Nevertheless, prior phylogenetic studies on felids primarily relied on single mitochondrial genes (e.g., *Cytb*, *rrnL*) or limited concatenated datasets. While providing foundational insights, the limited phylogenetic signal inherent in such approaches often resulted in poorly supported topologies, particularly at deeper nodes. Although recent analyses employing complete mitogenomes have improved resolution, there are still inconsistencies, partly due to differences in gene selection (e.g., exclusion of *ND6*, *rrnL* or *rrnS* genes) and different analytical methods. In addition, comprehensive assessments of mitochondrial genomic characteristics in felids—including nucleotide composition biases, nucleotide diversity, and selection pressures—are still relatively scarce, hindering our understanding of the molecular evolutionary mechanisms within this ecologically pivotal group.

This study integrates publicly available complete mitogenomes from 37 felid species, encompassing all major genera, to achieve three primary objectives: (1) Characterize mitogenomes diversity, including systematic analysis of genomic structural variation, nucleotide composition across genes, and nucleotide diversity; (2) Assess selective pressures on protein-coding genes (PCGs) using Ka/Ks ratio (ratio of nonsynonymous to synony-

mous nucleotide substitution rate); (3) Employ multi-gene concatenation strategies to reconstruct robust phylogenetic trees and identify the optimal dataset for resolving felid relationships. Our findings are expected to contribute new perspectives on understanding mitochondrial evolution within Carnivora.

2. Materials and Methods

2.1. Data Acquisition and Processing

This study analyzed publicly available mitochondrial genome data from GenBank (https://www.ncbi.nlm.nih.gov/genbank/ (accessed 28 February 2025)). Initial screening identified 1045 Felidae mitogenomes, from which 37 complete mitochondrial genomes (one per species) were selected as representative sequences for interspecific comparisons and phylogenetic tree reconstruction (Table 1). All sequences were retrieved and processed using PhyloSuite v1.2.3 [39,40].

Table 1. The Felidae mitogenome sequences used in this study.

Species	Accession ID	Species	Accession ID
Acinonyx jubatus	NC_005212	*Lynx pardinus*	NC_028319
Caracal caracal	NC_028306	*Lynx rufus*	NC_014456
Catopuma badia	NC_028300	*Neofelis nebulosa*	NC_008450
Catopuma temminckii	NC_027115	*Otocolobus manul*	NC_028323
Felis catus	NC_001700	*Panthera leo*	NC_028302
Felis chaus	NC_028307	*Panthera onca*	KM236783
Felis margarita	NC_028308	*Panthera pardus*	KP001507
Felis nigripes	NC_028309	*Panthera tigris*	NC_010642
Felis silvestris	NC_028310	*Panthera uncia*	PP646745
Leopardus colocolo	NC_028314	*Pardofelis marmorata*	NC_028303
Leopardus geoffroyi	NC_028320	*Prionailurus bengalensis*	NC_028301
Leopardus guigna	NC_028321	*Prionailurus iriomotensis*	LC375963
Leopardus jacobita	NC_028322	*Prionailurus planiceps*	NC_028312
Leopardus pardalis	NC_028315	*Prionailurus rubiginosus*	NC_028304
Leopardus tigrinus	NC_028317	*Prionailurus viverrinus*	NC_028305
Leopardus wiedii	NC_028318	*Profelis aurata*	NC_028299
Leptailurus serval	NC_028316	*Puma concolor*	NC_016470
Lynx canadensis	NC_028313	*Puma yagouaroundi*	NC_028311
Lynx lynx	NC_027083		

2.2. Mitogenomic Characteristics Analysis

(1) Nucleotide Composition and Variability Analysis

Multiple sequence alignments of individual genes and complete mitogenomes were performed using the MAFFT online program [41] (https://mafft.cbrc.jp/alignment/server/ (accessed on 5 May 2025)). The aligned sequences were analyzed with MEGA v11.0.9 [42] to determine nucleotide composition and variable sites. Nucleotide composition bias was evaluated by calculating AT-skew [(A − T)/(A + T)] and GC-skew [(G − C)/(G + C)] [43]. Additionally, nucleotide diversity (Pi) for each sequence set was computed using DnaSP v6.12.03 [44]. Data visualization was performed using the CNSknowall platform (https://cnsknowall.com/ (accessed on 20 May 2025)).

(2) Selection Pressure Analysis

For the PCGs sequence sets, after eliminating termination codons, nonsynonymous substitution rate (Ka) and synonymous substitution rate (Ks) were calculated using DnaSP v6.12.03 [44], followed by the computation of the Ka/Ks ratio. Data visualiza-

tion was performed using the CNSknowall platform (https://cnsknowall.com/ (accessed on 20 May 2025)).

2.3. Mitogenomic Phylogenetic Analysis

In this study, mitochondrial genome sequences from 37 felid species (Table 1) were selected to reconstruct the phylogenetic relationships within Felidae. Following previous studies [36,37], *Prionodon pardicolor* (NC_024569) from the family Prionodontidae was designated as the outgroup. To systematically evaluate the impact of sequence length on phylogenetic reconstruction, two single-gene datasets and eight multi-gene concatenation datasets were compared: *Cytb*, *rrnS*, *rrnS* + *Cytb*, *rrnL* + *ND5*, *ND5* + *Cytb* + 2rRNAs, *ND2* + *ND4* + *ND5* + *Cytb* + 2rRNAs, *ND2* + *ND4* + *ND5* + *Cytb* + *APT8* + 2rRNAs, 12PCGs, 12PCGs + 2rRNAs, and 13PCGs + 2rRNAs.

rRNAs were aligned using MAFFT v7.505 [45] with the "—auto" strategy and normal alignment mode. PCGs were aligned using the codon-aware program MACSE v2.06 [46], which preserves reading frame and allows incorporation of sequencing errors or sequences with frameshifts. All alignments were subsequently trimmed using trimAl v1.2rev57 [47] with the "-automated1" command. Final concatenation of gene alignments was conducted using PhyloSuite v1.2.3 [39,40].

Phylogenetic reconstruction was performed using both maximum likelihood (ML) and Bayesian inference (BI) approaches. The optimal partition model (Edge-linked) was selected using ModelFinder v2.2.0 [48] under the Bayesian Information Criterion (BIC), with results detailed in Table S1. ML analysis was inferred using IQ-TREE v2.2.0 [49] under Edge-linked partition model for 100,000 ultrafast bootstraps replicates [50]. BI analysis was conducted using MrBayes v3.2.7a [51] (partition model, two independent runs, 10 million generations, sampling every 1000 generations), with the first 25% of samples discarded as burn-in.

Phylogenetic trees were visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/ (accessed on 21 May 2025)) and Dendroscope v3.8.10 [52].

3. Results and Discussion

3.1. Nucleotide Composition and Bias

The complete mitogenomes of all 37 analyzed felid species maintained identical gene composition, each containing 37 coding genes (13 protein-coding genes, two rRNA genes, and 22 tRNA genes) and one major non-coding control region (CR), consistent with other mammalian species [13,53–55]. Among the 37 coding genes, nine (*ND6* and eight tRNA genes) were located on the light strand while the remaining 28 were encoded on the heavy strand. The gene arrangement followed the typical vertebrate-type pattern, showing identical organization to other mammals [56,57].

The complete mitogenomes of these 37 felid species ranged from 16,610 to 17,153 bp in length, with an average size of 16,817 bp. *Puma concolor* possessed the longest mitochondrial genome, while *Leopardus jacobita* exhibited the shortest among the analyzed felid species. This variation in total length of the whole mitogenome was primarily due to differences in the size of the CR, which ranged from 887 bp (*L. guigna*) to 1707 bp (*P. concolor*). The nucleotide composition of the heavy strand in felid mitogenomes exhibited a consistent pattern of A>T>C>G (Figure 1A), with the combined A + T content approaching 60% (Figure 1B). This observation aligns with the general characteristic of high AT bias commonly observed in vertebrate mitochondrial genomes [55,58–61], reinforcing the characteristic AT-rich nature of vertebrate mitochondrial DNA. Analysis of individual mitochondrial genes and regions revealed distinct nucleotide composition patterns (Figure 1A): *ATP6*, *ATP8*, *COX2*, and rrnL showed an A>T>C>G pattern, *COX1*, *COX3*, and *ND4L* exhibited T>A>C>G

pattern, *Cytb* displayed a unique C>A>T>G pattern, *ND1~5* and rrnS shared an A>C>T>G profile, and *ND6* was exceptional with T>G>A>C pattern. Furthermore, all protein-coding genes, rRNA genes, and the control region exhibited consistently higher A + T content than C + G content (Figure 1B). Notably, *ATP8* displayed the most extreme A + T bias (67.86%; range: 65.20%~70.10%), significantly exceeding the genomic average.

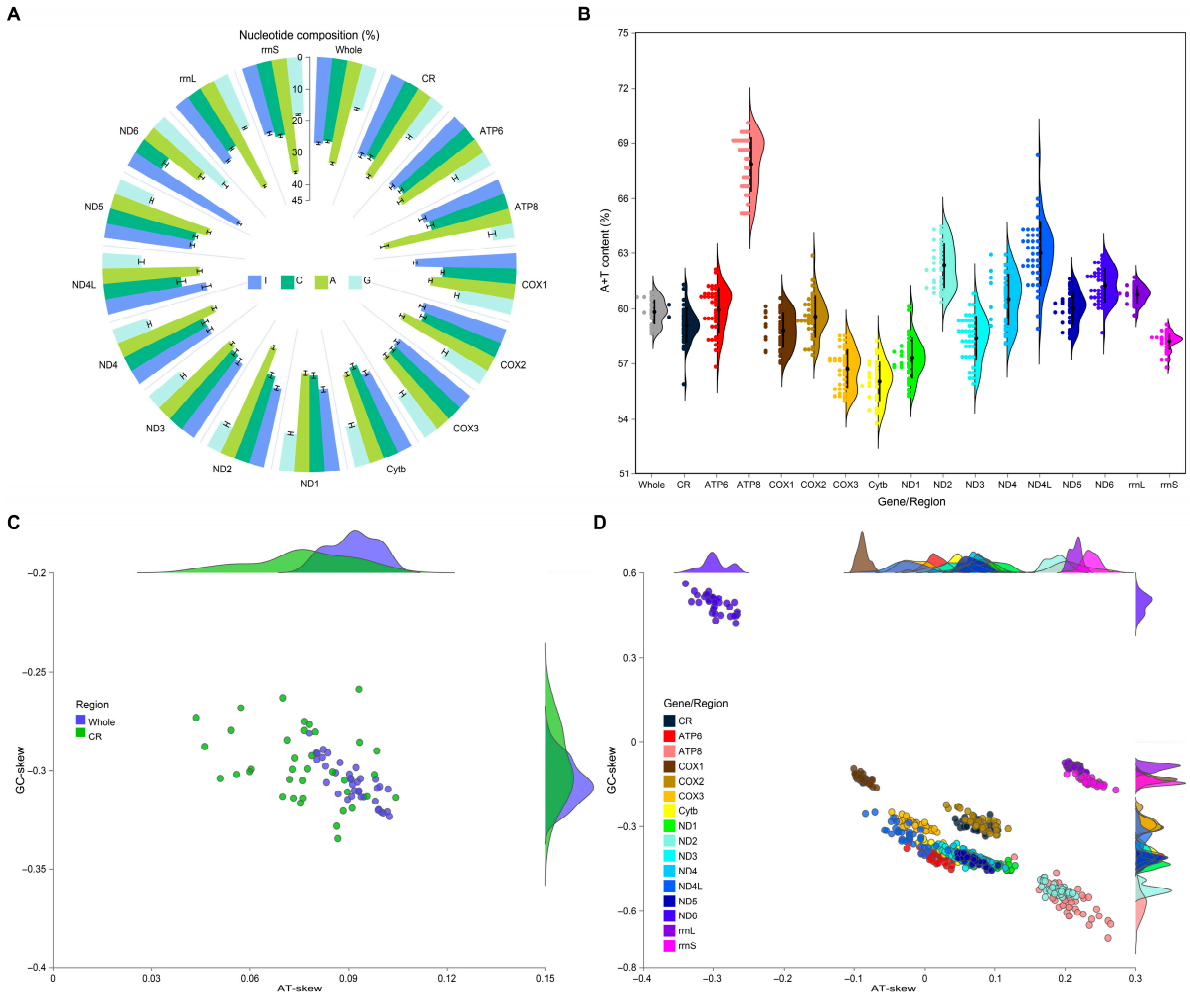


Figure 1. Nucleotide composition and skewness of mitochondrial genes in Felidae species. (**A**) Nucleotide composition; (**B**) A + T content; (**C**) GC-skew and AT-skew; (**D**) GC-skew and AT-skew.

Mitogenome-wide nucleotide skew analyses provide an effective approach to assess interspecific divergence in global nucleotide composition, while gene-level nucleotide skew patterns reveal distinct compositional biases among orthologous genes across taxa [55]. AT-skew and GC-skew are fundamental metrics for assessing nucleotide composition bias in mitogenomes. An AT-skew > 0 (or GC-skew > 0) indicates preferential usage of adenine over thymine (or guanine over cytosine), whereas negative values reflect the opposite trend. These asymmetries primarily arise from strand-specific mutational pressures during replication and transcription [62]. We also calculated the nucleotide skew of mitochondrial DNA in 37 felid species. The heavy strand of felid mitogenomes exhibited a negative GC-skew (−0.32365~−0.28130) but a positive AT-skew (0.07809~0.10251) (Figure 1C), demonstrating a significant predominance of cytosine over guanine and adenine over thymine, which reflects distinct strand-specific nucleotide composition bias. When examining individual genes or regions, *ND6* showed a positive GC-skew, whereas the other 12 protein-coding genes, two rRNA genes, and the CR displayed negative GC-skew values. The AT-skew appeared more complex: only *COX1* and *ND6* exhibited negative values, *ATP6*, *COX3*, *Cytb*

and *ND4L* fluctuated around zero, while CR and most other genes maintained positive values (Figure 1D). Notably, *ND6* exhibited a distinct clustering pattern in both GC-skew and AT-skew dimensions (Figure 1D), showing clear separation from all other protein-coding genes and rRNA genes. This pronounced divergence is strongly associated with its light-strand encoded nature. Notably, similar strand-specific compositional biases in *ND6* have been consistently documented across diverse vertebrate lineages, including avian [60,61], amphibian [63], and teleost species [64,65].

3.2. Nucleotide Variability

Statistical analysis of nucleotide variation sites in mitochondrial PCGs and rRNAs of felids revealed that all 15 genes exhibited high variability, with a total of 5285 variable sites detected (Figure 2A). Among these, *ND5* contained the highest number of variable sites (776), comprising 627 parsimony-informative sites and 149 singleton mutation sites. *ND4* and *COX1* ranked next, each containing over 500 polymorphic sites, whereas *ATP8* showed the fewest variable sites (89), including 67 parsimony-informative sites and 22 singletons. To account for length differences among mitochondrial genes, we further calculated the variable site proportion and nucleotide diversity for each gene. The results demonstrated that both the percentage of variable sites and nucleotide diversity were consistently higher in PCGs than in rRNAs (Figure 2B,C).

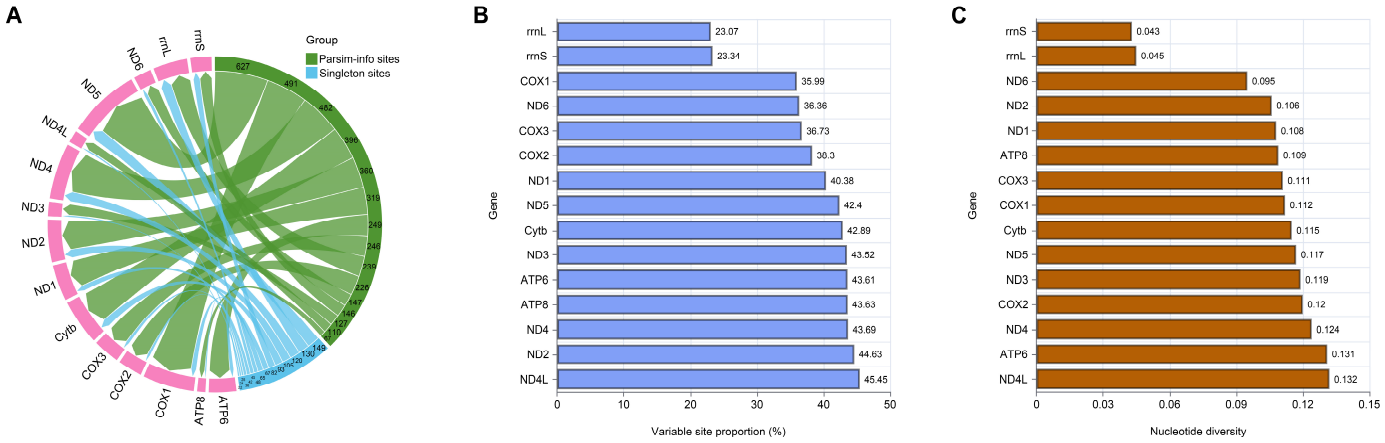


Figure 2. Nucleotide variations of 15 mitochondrial genes in Felidae species. (**A**) Number of variable sites; (**B**) Percentage of variable sites; (**C**) Nucleotide diversity levels across 37 felids.

In summary, *ND4L*, *ND2*, *ND4*, *ATP8*, and *ATP6* exhibited the highest proportions of variable sites (43.61%~45.45%), with *ND4L* and *ATP6* additionally displaying the highest nucleotide diversity (Pi = 0.132 and 0.131, respectively), suggesting these genes may experience accelerated mutation rates potentially linked to adaptive evolution. Although *ND5* displayed a high proportion of variable sites (42.40%), its intermediate nucleotide diversity (Pi = 0.117) suggests that its mutations are likely dominated by neutral evolution. In contrast, *rrnL* and *rrnS* exhibited the lowest proportions of variable sites (approximately 23%) and nucleotide diversity (0.043~0.045), consistent with their functional conservation where structural stability is crucial for translational fidelity. *COX1* demonstrated low variability (35.99%) and moderate diversity (0.112), reflecting its evolutionarily constrained role in respiratory chain function. The reduced variability (36.36%) and diversity (0.095) of *ND6* may stem from its light-strand transcription-associated mutation bias.

Although *COX1* has been widely adopted for closely related species discrimination [66,67] and population-level analyses [68–70], our findings suggest *ND4L* and *ATP6* represent superior markers for interspecific or intraspecific differentiation in felids, whereas

conserved genes (*COX1*, *rrnL*, *rrnS*) remain appropriate for higher-level (family/genus) phylogenetic reconstruction [71].

3.3. Selection Pressure

Our comparative analysis of selection pressures across 13 mitochondrial protein-coding genes in 37 felid species revealed distinct patterns of molecular evolution (Figure 3). *COX1* exhibited the lowest average Ka/Ks ratio (0.00327), followed by *COX2* and *COX3* (0.00974 and 0.01450, respectively) (Figure 3A). This pattern is consistent with observations in other animal groups [60,61], indicating that these cytochrome c oxidase subunits are under exceptionally strong purifying selection pressure in felids. In contrast, *ATP8* showed the highest average Ka/Ks ratio (0.12304), with *ND2* ranking second (0.07358) (Figure 3A), suggesting that, as in other taxa [60,61,72], these two genes experience relatively weaker selective constraints in felids. In addition, among the 13 mitochondrial protein-coding genes, *COX1* exhibited the lowest standard deviation (SD) of the Ka/Ks ratio (0.00267), followed by *COX2*, *COX3*, *ND5*, *ND4*, *ND1*, *Cytb*, *ATP6*, *ND6*, *ND4L*, *ND2*, and *ND3* (SDs: 0.00602, 0.00735, 0.00803, 0.00917, 0.01027, 0.01148, 0.01480, 0.01536, 0.01772, 0.01912, and 0.02101, respectively); whereas *ATP8* displayed the highest value at 0.05082. This indicates that there is relatively low interspecific selective pressure variation for *COX1* in felids while *ATP8* shows a greater degree of interspecific selective pressure variation. Furthermore, the heatmap analysis clearly illustrated that the differences in Ka/Ks ratios for *ATP8* among felid species are most pronounced compared to other genes (Figure 3B).

3.4. Matrilineal Molercular Phylogeny

In early matrilineal molecular phylogenetic studies, researchers predominantly employed single-gene markers (e.g., *COX1*, *rrnL*, *Cytb*) or multigene concatenation approaches [73,74]. With advancements in high-throughput sequencing technologies, whole-mitogenome analysis has gradually become the mainstream methodology. Currently, mitogenomes have been widely utilized in research areas such as phylogenetic relationship establishment and divergence time estimation [37,75–78]. The phylogenetic investigation of Felidae species has followed a similar trajectory. Initial studies predominantly relied on single or multiple mitochondrial genes, as exemplified by: Agnarsson et al. using *Cytb* [31]; Masuda et al. employing *rrnS* or *Cytb* [32]; Janczewski et al. utilizing *rrnS* and *Cytb* [6]; Johnson and O'Brien analyzing *rrnL* and *ND5* [33]; Mattern and McLennan examining *rrnS*, *rrnL*, *ND5*, and *Cytb* [34]; Yu and Zhang investigating *rrnS*, *rrnL*, *ND2*, *ND4*, *ND5*, and *Cytb* [8]; and Wei et al. incorporating *rrnS*, *rrnL*, *ND2*, *ND4*, *ND5*, *Cytb*, and *ATP8* [7,21]. In the past decade, other research teams have increasingly reconstructed felid phylogenetic relationships using complete mitochondrial genomes [35–38].

Phylogenetic analyses in the present study revealed that the BI tree constructed using 13PCG + 2rRNA scheme exhibited highly congruent topological relationships with the ML tree (Figures 4 and S1), providing internal consistency support for our conclusions. Our phylogenetic trees clearly delineated the evolutionary relationships of extant felids, which were divided into two major clades: Pantherinae and Felinae. The monophyly of all examined genera received strong support. All *Felis* species formed a highly supported monophyletic group, validating the traditional taxonomic status of this genus. *Prionailurus* species also constituted a well-supported monophyletic clade. The four *Lynx* species formed a distinct branch, with their unique morphological characteristics (e.g., short tails and ear tufts) being highly consistent with molecular evidence. Within *Leopardus*, significant adaptive differentiation was observed, with South American species forming two distinct subgroups: open-habitat adapters (e.g., Geoffroy's cat *L. geoffroyi*) and forest specialists (e.g., margay *L. wiedii*). Furthermore, the phylogenetic position of Pallas's cat *Otocolobus*

manul suggested a closer ancestral relationship with *Prionailurus* and *Felis*, providing new insights into the adaptive radiation of Asian plateau species. The clouded leopard *Neofelis nebulosa* was positioned as the sister group to *Panthera*, occupying the basal branch within Pantherinae, supporting its status as the earliest diverging extant big cat lineage.

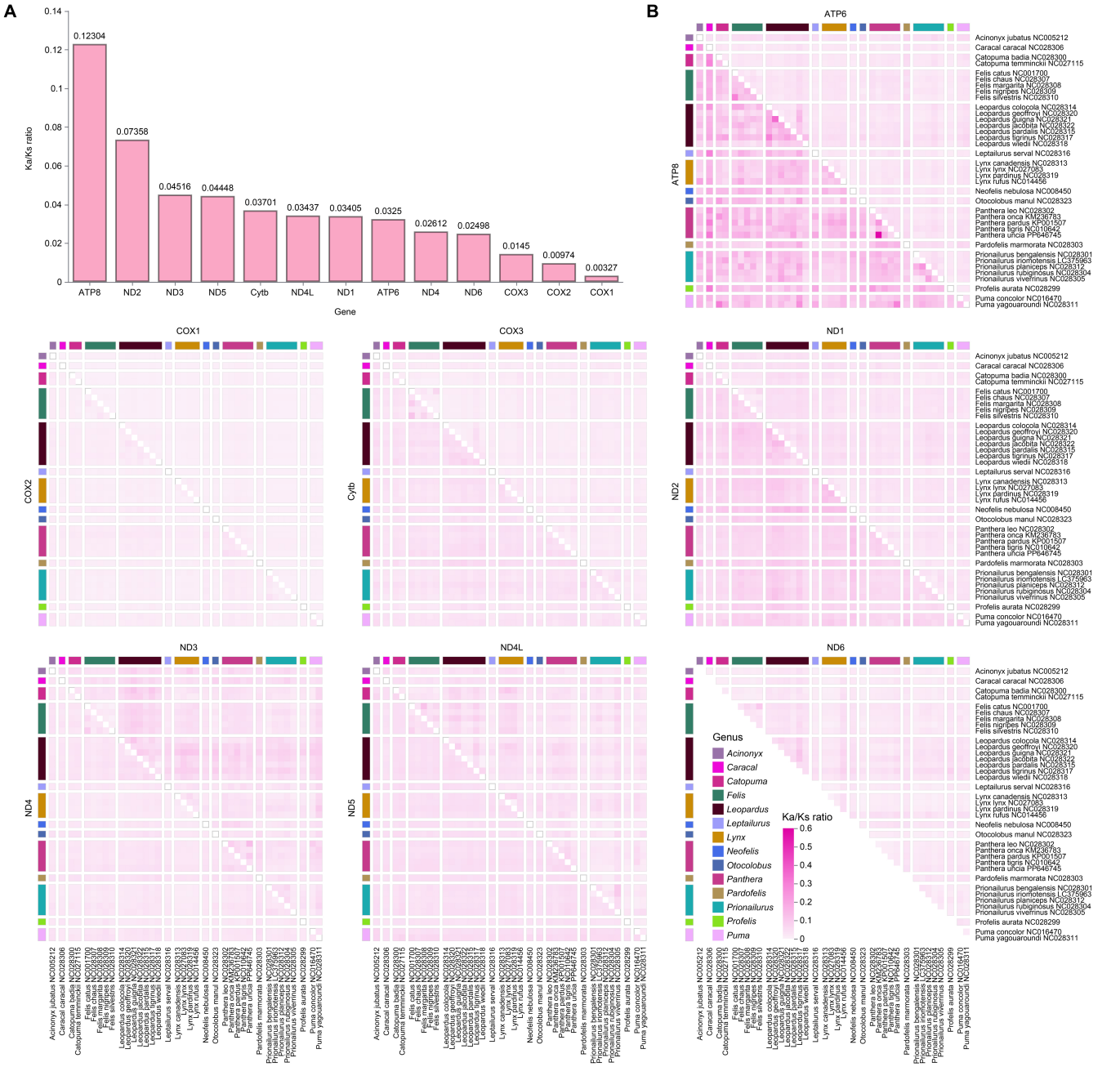


Figure 3. Ka/Ks ratios of mitochondrial protein-coding genes in Felidae species. (**A**) The mean Ka/Ks ratio among 37 felids; (**B**) Heatmap representation of Ka/Ks ratio across 37 felids.

More importantly, these results exhibit strong concordance with those of Rodrigues-Oliveira et al. [38] (who employed an identical mitochondrial 13PCGs + 2rRNAs scheme). Moreover, the phylogenetic framework of major clades remains robust even when compared to datasets with minor discrepancies (e.g., Hassanin et al. [37], which utilized only 12 PCGs, excluding the light strand-encoded *ND6*).

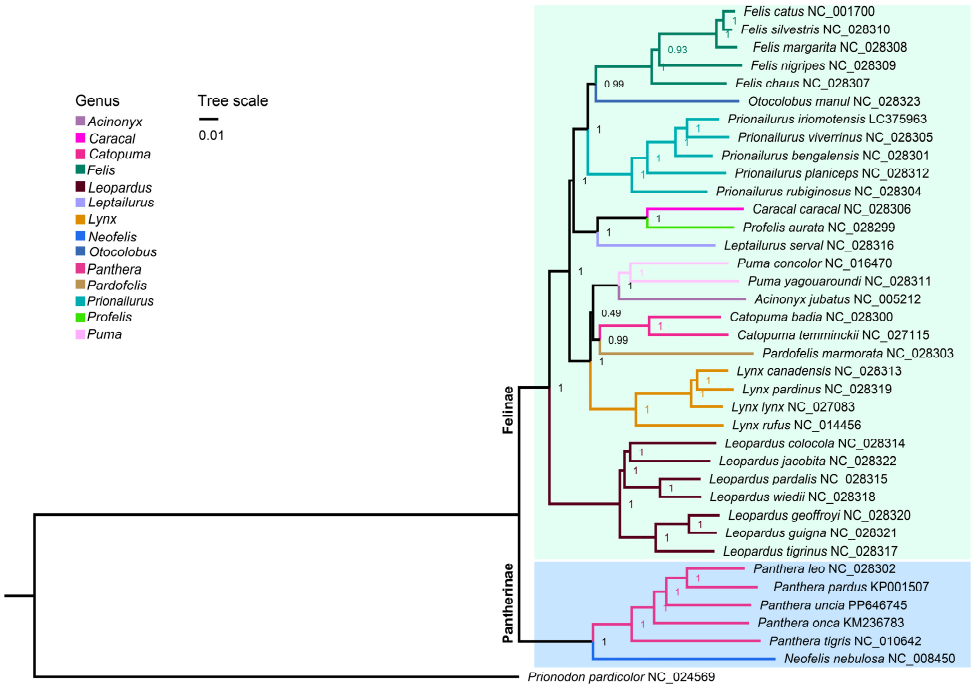


Figure 4. The Bayesian inference phylogeny of Felidae species based on the 13PCGs + 2rRNAs scheme. Numbers on nodes are the Bayesian posterior probabilities.

To further evaluate the impact of different mitochondrial gene combinations on phylogenetic reconstruction, we conducted detailed comparisons between the 13PCG + 2rRNA scheme and nine alternative single-gene or multi-gene concatenation schemes (including *Cytb*, *rrnS*, *rrnS* + *Cytb*, *rrnL* + *ND5*, *ND5* + *Cytb* + 2rRNAs, *ND2* + *ND4* + *ND5* + *Cytb* + 2rRNAs, *ND2* + *ND4* + *ND5* + *Cytb* + *APT8* + 2rRNAs, 12PCGs, and 12PCGs + 2rRNAs) regarding topological differences and nodal support values in ML trees (Figure S2). The results demonstrated that, with the exception of the 12PCG + 2rRNA dataset, phylogenetic trees reconstructed from other single-gene or multi-gene concatenation schemes exhibited topological conflicts to varying degrees. In particular, trees based on single gene showed significant inconsistencies at the genus-level nodes. Similar issues have been reported in previous studies. For instance, Masuda et al. constructed trees based on the *Cytb* and *rrnS* genes separately and found conflicting phylogenetic placements of *Otocolobus manul*: *Cytb* analysis positioned it at the base of the domestic cat lineage (i.e., genus *Felis*), whereas *rrnS* analysis grouped it within the pantherine lineage [32]. In contrast, the present study, utilizing a longer concatenated sequence of 13PCG + 2rRNA, supports the placement of *O. manul* at the base of the domestic cat lineage (i.e., genus *Felis*) (Figures 4 and S1). These discrepancies may stem from the limited phylogenetic information provided by single-gene markers (e.g., *Cytb*, *rrnS*), which often fail to resolve deep-level relationships. Long-sequence datasets are indispensable for achieving high resolution in deep phylogenetic inference, whereas shorter segments remain valuable for distinguishing closely related species or in resource-limited settings [78–80]. Intriguingly, the 12PCGs + 2rRNAs scheme showed high congruence with the 13PCGs + 2rRNAs scheme across all key nodes (bootstrap difference < 4%), whereas the 12PCGs scheme displayed topological discrepancies within *Felis* when compared with the 13PCGs + 2rRNAs scheme. This suggests that exclusion of the *ND6* gene had minimal impact on phylogenetic signals for the studied taxa, while the exclusion of 2rRNAs can introduce base into the phylogeny. These findings highlight that in felid phylogenetics, employing nearly complete mitochondrial genomic data (≥12PCGs + 2rRNAs) constitutes a prerequisite for ensuring topological reliability, with

practical selection needing to follow the "sufficient but economical" principle—prioritizing the most cost-effective sequence length scheme while maintaining necessary resolution.

Last but not least, in addition to maternal phylogenetic studies based on mitochondrial genes, research efforts have also been conducted using immunological markers [81], protein electrophoresis [82], and chemical signals [83], as well as investigations based on nuclear genes [84,85], and combined analyses of nuclear and mitochondrial genes [3,8,86]. Comparative examination reveals that phylogenetic trees derived from nuclear and mitochondrial data often yield conflicting topologies, likely due to incomplete lineage sorting and introgression at early stages of speciation [4], possibly compounded by insufficient data. Among all the relevant studies, the efforts of Johnson et al. [3], which incorporated 19 autosomal, 5 X-linked, and 6 Y-linked gene segments along with 8 mitochondrial gene fragments across all felid species, remains the most systematic and comprehensive to date.

In summary, the relatively recent divergence of felid species has posed considerable challenges for reconstructing their phylogenetic relationships, contributing to the ongoing ambiguity in their evolutionary history. Therefore, we contend that resolving these issues will require integrating multidimensional data—including joint mitochondrial and nuclear genomic analyses—and expanding sampling to encompass all felid species and even distinct geographic populations. Such efforts are essential to provide a more robust and well-supported evidence base for phylogenetic inference of felid species.

3.5. Limitations

Our comparative analysis revealed considerable heterogeneity in selective pressures among the 13 mitochondrial PCGs (Figure 3), a common phenomenon in evolutionary genomics. While this study provides a robust phylogenetic framework based on concatenated mitogenomic data, a pertinent question arising from our findings is whether and how this heterogeneity in selective constraints might impact phylogenetic inference. Gene-specific evolutionary rates and selective regimes can potentially lead to topological conflicts or biases in branch length estimates if not properly modeled. We acknowledge that our current study did not explicitly investigate the impact of these variable selective pressures on the phylogenetic reconstruction presented here.

4. Conclusions

This study provides a comprehensive analysis of mitogenome diversity and evolution across 37 felid species, yielding key insights into their molecular architecture and phylogeny. Although the felid mitochondrial genome has a conserved gene composition and arrangement pattern, there are obvious inter-gene differences in nucleotide composition, and the light chain encoding gene *ND6* shows obvious chain-specific bias. Nucleotide variability and diversity were highest in *ND4L* and *ATP6*, suggesting their utility as novel markers for species delimitation and population studies, whereas *COX1* and rRNAs showed stronger conservation, supporting their use for deeper phylogenetic inference. Selection pressure analysis revealed that *ATP8* and *ND2* experienced the weakest selective constraints (highest Ka/Ks ratios), consistent with their roles in energy metabolism adaptation, while cytochrome oxidase subunits (*COX1-3*) were under intense purifying selection. Phylogenetic reconstruction using the 13PCGs + 2rRNAs scheme resolved the Felidae into two primary clades (Pantherinae and Felinae) with strong nodal support, confirming the monophyly of all genera and clarifying contentious relationships—such as the basal position of *Neofelis nebulosa* within Pantherinae and the close affinity of *Otocolobus manul* with *Prionailurus* and *Felis*. Critically, comparative analyses of gene concatenation schemes revealed that datasets excluding *ND6* (12PCGs + 2rRNAs) produced topologies congruent with the complete 13PCGs + 2rRNAs scheme, whereas analyses based on single-gene or

limited multi-gene concatenations produced inconsistent tree topologies, particularly at genus-level nodes. These findings demonstrate that near-complete mitogenomic datasets (≥12PCGs + 2rRNAs) are essential for robust felid phylogenetics, achieving an optimal balance between resolution and practical efficiency.

Our findings not only refine the evolutionary framework of Felidae but also offer a benchmark for future conservation genomics, enabling precise species identification and management of these ecologically vital yet vulnerable carnivores.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d17090634/s1, Table S1: The partition and best-fit partition models used in this study. Figure S1: The Bayesian inference phylogeny of Felidae species based on the 13PCGs + 2rRNAs scheme. Numbers on nodes are the Bayesian posterior probabilities. Figure S2. Comparison of maximum likelihood phylogenies in Felidae across multi-gene concatenation schemes. Numbers on nodes are the bootstrap percentages.

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