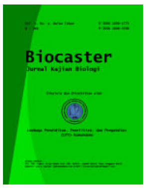

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INSIGHTS INTO EVOLUTIONARY RELATIONSHIPS OF PROBOSCIS MONKEYS USING IN SILICO MITOCHONDRIAL DNA ANALYSIS

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ABSTRACT: This study investigates the evolutionary position of the proboscis monkey (*Nasalis larvatus*) within the Colobinae subfamily using in silico phylogenetic analysis of three mitochondrial protein-coding genes (COI, COII, and COIII). Seventy sequences retrieved from GenBank were aligned and analyzed using the Maximum Likelihood method with 1,000 bootstrap replicates. All genes exhibited conserved lengths and strong AT bias (59,21-60,68%), reflecting typical mitochondrial genome characteristics shaped by asymmetric replication and purifying selection. Genetic distance analyses revealed high intraspecific consistency in *Nasalis larvatus*, while demonstrating marker dependent variation in phylogenetic resolution. COII and COIII provided clearer intergeneric separation compared to the more conserved COI marker. Phylogenetic reconstruction showed topological variation among markers, however, a consistent evolutionary signal emerged, with *Nasalis larvatus* clustering robustly with Simias concolor across all gene trees. This pattern supports the monophyly of the odd nosed monkey clade and aligns with genome scale phylogenomic evidence. Overall, this study highlights the limitations of single gene analyses and emphasizes the importance of multi marker or genome scale approaches for resolving complex evolutionary relationships. These findings provide foundational insights into the evolutionary history of *Nasalis larvatus* and reinforce the relevance of mitochondrial markers in primate conservation genetics.

Keywords: Colobinae, Genetic Distance, Mitochondrial DNA, *Nasalis larvatus*, Phylogenetics.

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INTRODUCTION

The proboscis monkey (*Nasalis larvatus*), endemic to Borneo, inhabits wetland and riverine forest ecosystems where it depends on specific microclimatic conditions, temperatures of 25,8–26,9°C, humidity of 97–99%, and the presence of key vegetation such as *Shorea blangeran* (Santoso et al., 2023). Yet habitat loss has severely constrained the species' range: only 11,54% of Borneo remains highly suitable, barely 54% of surveyed areas provide adequate habitat, and just 43% of those fall within protected zones (Bernard et al., 2025; Sakti et al., 2024) Agricultural expansion, urbanization, and infrastructure development continue to intensify these pressures, making robust genetic and evolutionary data essential for informed conservation strategies. Without conservation, habitat fragmentation may reduce connectivity and genetic diversity.

Understanding primate evolutionary relationships also offers broader biological insights. Comparative genomic analyses across primate lineages have revealed that significant innovations in the nervous, skeletal, and digestive systems originated in ancestral lineages and involved thousands of positively selected genes (Shao et al., 2023), while behavioral studies have addressed the evolution of cognition, cooperation, and social complexity (Sandel et al., 2024). Mitochondrial DNA (mtDNA) has become a central molecular tool in this context, valued for its maternal inheritance, elevated mutation rate, and cost effectiveness in species delineation and phylogenetic reconstruction. However, the utility of mtDNA is not without conceptual complexity: recent work has challenged traditional assumptions of strict maternal inheritance, selective neutrality, and the absence of recombination, underscoring the need for careful interpretation of mitochondrial markers in evolutionary and conservation studies.

Among mitochondrial genes, the cytochrome c oxidase subunits COI, COII, and COIII are particularly relevant to primate phylogenetics and cellular metabolism. Phylogenetic studies have shown that while individual mitochondrial genes may not fully represent the entire mitochondrial genome, certain gene combinations such as CYB and COIII offer strong resolving power (Finnegan et al., 2025). Beyond phylogenetic utility, COIII plays a critical role in translational regulation. Baleva et al. (2022) demonstrated that the PTCD2 protein specifically regulates COIII translation within mitochondria, with its deletion markedly reducing translation efficiency and compromising cytochrome c oxidase activity. These findings highlight the functional and evolutionary significance of mitochondrial protein-coding genes in primates.

Despite the widespread use of multi-gene mitochondrial approaches to assess genetic diversity, population structure, and evolutionary relationships in other species, this methodology has not yet been applied to *Nasalis larvatus*, leaving a notable gap in our understanding of its maternal lineage and evolutionary placement. Prior studies on endangered species have demonstrated the substantial resolving power of mitochondrial markers for phylogenetic reconstruction and haplotype diversity assessment (Thakur et al., 2025). To address this gap, the present study examines the evolutionary position of *Nasalis larvatus* within the Colobinae subfamily through in silico phylogenetic analysis of three mitochondrial protein-coding genes (COI, COII, and COIII), using curated publicly available sequences. In doing so, this work provides foundational insights into the species' evolutionary history while evaluating the effectiveness of mtDNA as a tool for primate genetics and conservation.

METHODS

Mitochondrial gene sequences for COI, COII, and COIII from *Nasalis larvatus* were retrieved from the GenBank (NCBI) database using targeted keyword searches for each gene. For intra subfamily comparison, homologous sequences from other Colobinae genera (Trachypithecus, Presbytis, Semnopithecus, Pygathrix, Rhinopithecus, Colobus, Piliocolobus, and Procolobus) were obtained using similar search criteria. Priority was given to sequences derived from complete mitogenomes or full length gene records to ensure consistency and minimize information loss. Two Cercopithecinae species from the genera Macaca and Papio were selected as outgroups to root the phylogenetic trees. A complete list of sequences used in this study is provided in Table 1.

Retrieved sequences were aligned using the multiple sequence alignment function in MEGA 12 (Kumar et al., 2024). Alignments were inspected and manually adjusted where necessary to maximize positional homology across taxa. Phylogenetic trees were reconstructed using the Maximum Likelihood (ML) method implemented in MEGA 12 (Zou et al., 2024). Branch support was assessed through bootstrap analysis with 1,000 replicates. The resulting trees were exported and refined for visual presentation using Tree Visualization by One Table (tvBOT) (Xie et al., 2023).

RESULT AND DISCUSSION

Nucleotide Composition

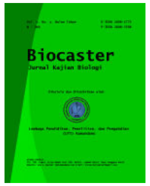
Analysis of 70 sequences revealed conserved fragment lengths across the three cytochrome c oxidase subunit genes: 1,545 bp (COI), 684 bp (COII), and 784 bp (COIII). All three genes exhibited a pronounced AT bias, with AT content ranging from 59,21% to 60,68% (Table 1). Thymine and adenine were the most abundant nucleotides in each gene, while guanine was consistently the least frequent (13,75–15,99%). Average nucleotide compositions were as follows: COI (T = 32,13%, A = 27,24%, C = 24,64%, G = 15,99%), COII (T = 29,28%, A = 31,40%, C = 25,50%, G = 13,82%), and COIII (T = 31,31%, A = 27,90%, C = 27,04%, G = 13,75%).

The high AT content in mitochondrial genomes is due to a process called asymmetric strand displacement replication. This process causes changes in the DNA and affects the whole genome (Sanchez-Contreras et al., 2021). Studies of over 6,000 vertebrate species show that the way genes are oriented affects their composition, highlighting the role of replication in shaping mitochondrial genomes (Gomes-Dos-Santos et al., 2023). The low guanine content in COI, COII, and COIII genes is typical of reptilian mitochondrial genomes, resulting from mutation pressure and selective constraints (Chakraborty et al., 2022). Although specific strand mechanisms were not detailed in that study, the bias fits with models of mtDNA evolution. Purifying selection on these genes limits changes that alter amino acids but allows changes that do not affect protein function, especially at third codon positions. This balance keeps oxidative phosphorylation proteins working while allowing genetic diversity for detailed phylogenetic studies.

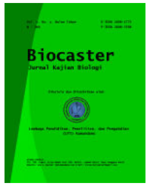
In addition to nucleotide composition bias, codon usage patterns in mitochondrial protein-coding genes are also strongly influenced by the elevated AT content. AT-rich codons are preferentially utilized in COI, COII, and COIII genes, particularly at synonymous third codon positions, where substitutions are less likely to alter amino acid sequences. This codon bias contributes to translational efficiency and may reflect adaptive optimization of mitochondrial gene expression in reptiles. Furthermore, the conserved fragment lengths observed across all sampled sequences indicate strong functional constraints on cytochrome c oxidase subunits, as these proteins play essential roles in the electron transport chain and ATP production.

Table 1. The Nucleotide Composition of the COI, COII, and COIII Gene from 70 Samples.

No.	Species	Accession Number	COI					COII					COIII				
			T	C	A	G	Total	T	C	A	G	Total	T	C	A	G	Total
1	*Nasalis larvatus*	KM889667.1	32	25	27	16	1545	29	26	32	13	684	32	26	28	14	784
2	*Nasalis larvatus*	NC_008216.1	32	25	27	16	1545	29	26	32	13	684	32	27	28	14	784
3	*Nasalis larvatus*	DQ355298.1	32	25	27	16	1545	29	26	32	13	684	32	27	28	14	784
4	*Nasalis larvatus*	JF293094.1	32	25	27	16	1545	29	26	32	13	684	32	26	28	14	784
5	*Trachypithecus phayrei*	NC_056326.1	32	24	28	16	1545	30	25	32	13	684	31	27	29	13	784
6	*Trachypithecus phayrei*	MT806066.1	32	25	27	16	1545	30	24	31	14	684	31	27	29	13	784
7	*Trachypithecus laotum*	NC_056330.1	32	25	28	16	1545	30	25	32	13	684	32	26	28	14	784
8	*Trachypithecus laotum*	MT806070.1	32	25	28	16	1545	30	25	32	13	684	32	26	28	14	784
9	*Trachypithecus geei*	NC_056324.1	32	25	28	16	1545	30	26	31	14	684	31	28	28	14	784
10	*Trachypithecus geei*	MT806030.1	32	25	28	16	1545	30	26	31	14	684	31	28	28	14	784
11	*Trachypithecus poliocephalus*	MT806068.1	32	25	27	16	1545	30	25	32	13	684	32	27	28	13	784
12	*Trachypithecus poliocephalus*	MT806067.1	32	25	28	16	1545	29	26	32	13	684	32	27	28	13	784
13	*Trachypithecus germaini*	MT806033.1	32	24	28	16	1545	30	25	32	13	684	31	27	28	14	784
14	*Trachypithecus germaini*	NC_019580.1	32	24	28	16	1545	30	25	31	14	684	31	27	28	14	784
15	*Trachypithecus mauritius*	MT806032.1	32	24	28	16	1545	31	24	32	13	684	32	27	28	14	784
16	*Trachypithecus mauritius*	NC_056325.1	32	24	28	16	1545	31	24	32	13	684	32	27	28	14	784
17	*Trachypithecus pileatus*	NC_024529.1	32	24	28	16	1545	29	26	31	14	684	31	27	28	14	784
18	*Trachypithecus pileatus*	KF680163.1	32	24	28	16	1545	29	26	31	14	684	31	27	28	14	784
19	*Trachypithecus obscurus*	NC_006900.1	32	24	28	16	1545	31	24	32	14	684	32	27	28	14	784
20	*Trachypithecus obscurus*	MF872234.1	32	24	28	16	1545	30	25	31	13	684	32	27	28	14	784
21	*Trachypithecus francoisi*	NC_023970.1	32	25	27	16	1545	30	25	32	13	684	32	26	28	13	784
22	*Trachypithecus francoisi*	KJ174502.1	32	25	27	16	1545	30	25	32	13	684	32	26	28	13	784
23	*Trachypithecus hatinhensis*	NC_019579.1	32	25	28	16	1545	30	25	31	14	684	32	26	28	13	784
24	*Trachypithecus hatinhensis*	MT806069.1	32	25	28	16	1545	30	25	32	13	684	32	27	28	13	784
25	*Trachypithecus shortridgei*	NC_019581.1	32	25	28	16	1545	29	26	31	13	684	31	27	28	14	784
26	*Trachypithecus shortridgei*	MF872227.1	32	25	28	16	1545	29	26	31	14	684	31	27	28	14	784
27	*Trachypithecus cristatus*	NC_023971.1	32	25	28	16	1545	30	25	32	14	684	32	27	28	14	784
28	*Trachypithecus cristatus*	KJ174503.1	32	25	28	16	1545	30	25	32	14	684	32	27	28	14	784
29	*Presbytis femoralis*	MN496092.1	33	24	28	16	1554	29	24	32	14	684	32	27	27	14	784
30	*Presbytis femoralis*	MN496088.1	33	24	28	16	1554	29	24	32	14	684	32	27	27	14	784



No.	Species	Accession Number	COI					COII					COIII				
			T	C	A	G	Total	T	C	A	G	Total	T	C	A	G	Total
31	*Presbytis melalophos*	NC_008217.1	33	23	28	16	1554	31	23	32	14	684	30	28	28	14	784
32	*Presbytis melalophos*	DQ355299.1	33	23	28	16	1554	31	23	32	14	684	30	28	28	14	784
33	*Presbytis percura*	MN496096.1	33	23	27	16	1554	29	25	33	13	684	32	27	28	14	784
34	*Presbytis percura*	MN496095.1	33	24	27	16	1554	29	25	33	13	683	32	27	28	14	784
35	*Semnopithecus schistaceus*	NC_057099.1	32	25	27	16	1545	28	27	29	16	684	32	26	28	14	784
36	*Semnopithecus schistaceus*	MN163131.1	32	25	27	16	1545	28	27	29	16	684	32	26	28	14	784
37	*Semnopithecus entellus*	NC_008215.1	32	25	27	16	1545	28	27	30	15	684	32	26	28	14	784
38	*Semnopithecus entellus*	DQ355297.1	32	25	27	16	1545	28	27	30	15	684	32	26	28	14	784
39	*Semnopithecus johnii*	NC_019583.1	33	24	27	16	1545	29	26	31	15	684	32	27	28	14	784
40	*Semnopithecus johnii*	HQ149050.1	33	24	27	16	1545	29	26	31	15	684	32	27	28	14	784
41	*Semnopithecus vetulus*	NC_019582.1	32	25	27	16	1545	29	26	31	15	684	31	27	27	15	784
42	*Semnopithecus vetulus*	KY117599.1	33	24	27	16	1545	28	26	31	15	684	32	27	28	14	784
43	*Pygathrix cinerea*	NC_088556.1	32	24	27	16	1545	30	24	31	14	684	31	27	28	14	784
44	*Pygathrix cinerea*	PP623106.1	32	24	27	16	1545	30	24	31	14	684	31	27	28	14	784
45	*Pygathrix nigripes*	NC_018061.1	32	24	27	17	1545	30	25	32	13	684	32	27	28	14	784
46	*Pygathrix nigripes*	JQ821840.1	32	24	27	17	1545	30	25	32	13	684	32	27	28	14	784
47	*Pygathrix nemaeus*	NC_008220.1	32	24	27	16	1545	31	24	31	14	684	31	27	28	14	784
48	*Pygathrix nemaeus*	DQ355302.1	32	24	27	16	1545	31	24	31	14	684	31	27	28	14	784
49	*Rhinopithecus bieti*	NC_015486.1	33	24	28	16	1545	30	25	31	14	684	33	26	28	13	784
50	*Rhinopithecus bieti*	HM125579.1	33	24	28	16	1545	30	25	31	14	684	33	26	28	13	784
51	*Rhinopithecus avunculus*	NC_015485.1	33	24	27	16	1545	29	26	32	13	684	32	26	28	14	784
52	*Rhinopithecus avunculus*	HM125578.1	33	24	27	16	1545	29	26	32	13	684	32	26	28	14	784
53	*Rhinopithecus roxellana*	NC_008218.1	34	23	27	16	1545	30	25	31	14	684	33	26	28	14	784
54	*Rhinopithecus roxellana*	KM504390.1	34	23	27	16	1545	30	25	31	14	684	33	26	28	14	784
55	*Rhinopithecus brelichi*	NC_018057.1	34	23	27	16	1545	30	25	31	14	684	33	25	28	14	784
56	*Rhinopithecus brelichi*	JN540032.1	34	23	27	16	1545	30	25	31	14	684	33	25	28	14	784
57	*Rhinopithecus strykeri*	NC_018059.1	34	23	27	16	1545	30	26	31	14	684	33	26	28	13	784
58	*Rhinopithecus strykeri*	JQ821838.1	34	23	27	16	1545	30	26	31	14	684	33	26	28	13	784
59	*Simias concolor*	NC_020667.1	32	25	27	16	1545	30	26	31	14	684	31	27	28	14	784
60	*Simias concolor*	JF293095.1	32	25	27	16	1545	30	26	31	14	684	31	27	28	14	784
61	*Colobus guereza*	NC_006901.1	31	26	27	16	1545	28	26	32	14	684	30	29	27	14	784



No.	**Species**	**Accession Number**	**COI**					**COII**					**COIII**				
			T	**C**	**A**	**G**	**Total**	**T**	**C**	**A**	**G**	**Total**	**T**	**C**	**A**	**G**	**Total**
62	*Colobus guereza*	AY863427.1	31	26	27	16	1545	28	26	32	14	684	30	29	27	14	784
63	*Piliocolobus badius*	NC_008219.1	33	25	27	16	1545	29	26	32	14	684	32	27	27	14	784
64	*Piliocolobus badius*	DQ355301.1	33	25	27	16	1545	29	26	32	14	684	32	27	27	14	784
65	*Procolobus verus*	NC_020666.1	32	25	26	16	1545	29	26	31	14	684	29	29	28	14	784
66	*Procolobus verus*	JF293092.1	32	25	26	16	1545	29	26	31	14	684	29	29	28	14	784
67	*Macaca fuscata*	NC_025513.1	29	28	26	16	1569	25	29	31	15	684	27	32	28	13	784
68	*Macaca fuscata*	KM401548.1	29	28	26	16	1569	25	29	31	15	684	27	32	28	13	784
69	*Papio papio*	NC_020009.2	29	28	26	17	1569	25	29	32	14	684	28	32	27	14	784
70	*Papio papio*	JX946203.2	29	28	26	17	1569	25	29	32	14	684	28	32	27	14	784

Genetic Distance

Triangular heatmaps (Figure 1) represent pairwise genetic distances among colobine primates, including multiple sequences of *N. larvatus* (KM889667.1; NC_008216.1; DQ355298.1; JF293094.1). Color gradients range from blue (low genetic distance) to red (high genetic distance), indicating increasing levels of sequence divergence. Across all markers, *N. larvatus* forms a compact low-distance cluster, indicating high intraspecific consistency. However, marker dependent differences in phylogenetic resolution are evident. COI shows a relatively conserved pattern with limited contrast among closely related genera, whereas COII and COIII display sharper gradients and clearer clustering boundaries, more effectively separating *Nasalis larvatus* from Presbytis, Trachypithecus, and Semnopithecus. This contrast underscores the higher intergeneric resolving power of COII and COIII relative to COI.

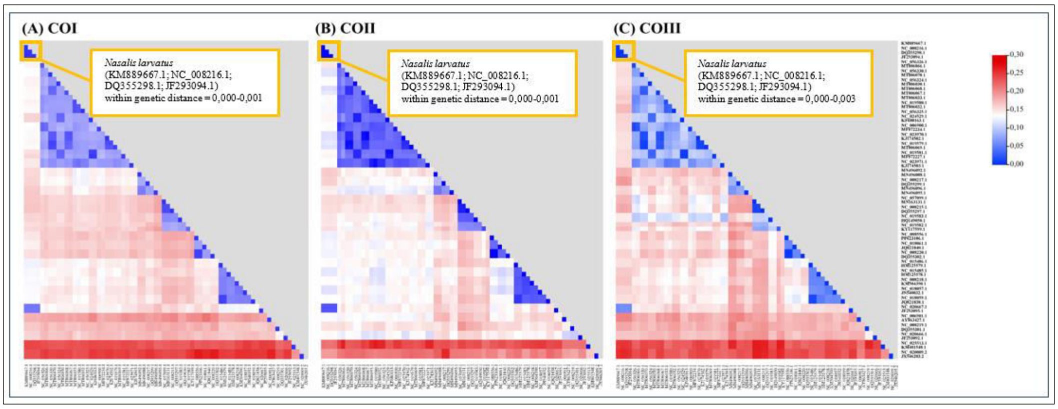


Figure 1. Comparative Heatmaps of Genetic Distance Matrices for COI, COII, and COIII.

These patterns are consistent with broader mitochondrial and genomic evidence indicating that colobine evolution has been shaped by interacting forces of geographic isolation, ecological specialization, and dietary adaptation. Mitochondrial phylogeographic data reveal strong population structuring driven by geographic barriers, as exemplified by long-term divergence across the Rufiji River in *Colobus angolensis* (McDonald et al., 2022). Complementary genomic studies further demonstrate adaptive signatures linked to folivory, including lineage-specific accelerated regions involved in metabolite detoxification (Bi et al., 2023) and hindgut microbiome specialization for fiber digestion (Liu et al., 2022), as well as selection on energy metabolism and neurohormonal pathways associated with ecological adaptation (Qi et al., 2023). Together, these findings provide a mechanistic framework for the divergence patterns observed here, while also highlighting persistent gaps in genomic coverage that continue to limit resolution of relationships within several colobine lineages (Roos & Zinner, 2022).

Phylogenetic Reconstruction

Phylogenetic reconstruction based on three mitochondrial protein coding genes (COI, COII, and COIII) revealed marker dependent topological variation, with COI resolving five clusters, COII four, and COIII three (Figure 2). This gradient in clustering capacity reflects inherent differences in phylogenetic signal among mitochondrial loci and aligns with growing evidence that single mitochondrial genes vary substantially in their ability to recover species relationships. For example, Main et al. (2024) study on Bradypodion phylogeny demonstrated that while some mitochondrial genes yield topologies broadly consistent with complete mitogenomes, others produce discordant reconstructions. Similarly, Finnegan et al. (2025) demonstrated that individual mitochondrial genes, including COX loci, fail to reliably recapitulate genome-scale phylogenies in Platyrrhini, with accuracy contingent on divergence depth and evolutionary context.

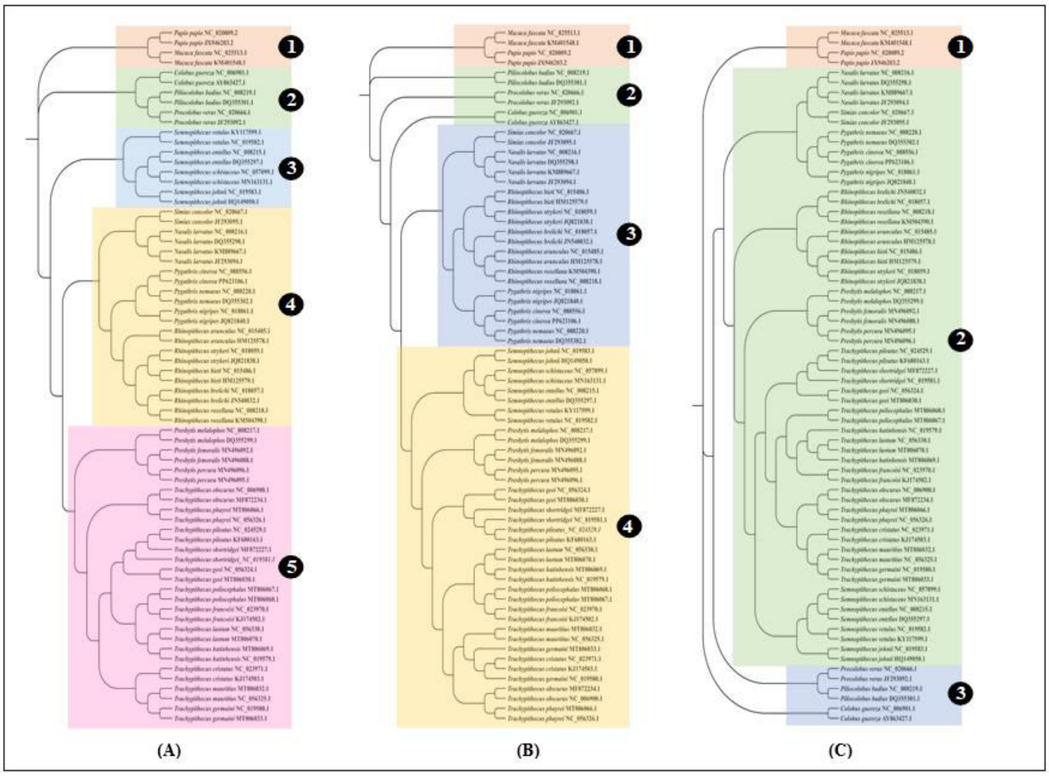


Figure 2. Comparison of Phylogenetic Tree Topologies Reconstructed Using Three Mitochondrial Genes: COI (A), COII (B), and COIII (C).

Despite these differences, a robust signal emerges: *Nasalis larvatus* consistently clusters with *Simias concolor* across all gene trees, despite shifts in its relative position (COI: fourth; COII: third; COIII: second). This stable affinity strongly supports the monophyly of the odd-nosed monkey clade. The grouping is consistent with Arenson. (2024) odd-nosed monkeys review and genome scale evidence from Qi et al. (2023) colobinae phylogenomics, which resolve Asian colobines into two primary lineages: odd-nosed monkeys and classic langurs.

The observed variation in cluster number is mechanistically consistent with differences in substitution rates and the distribution of phylogenetically informative sites among genes. COI, characterized by elevated nucleotide variability, particularly at third codon positions, exhibits greater discriminatory power, whereas COIII appears comparatively conserved. This pattern mirrors findings from Yu et al. (2025), felidae mitochondrial diversity study, where gene specific variation strongly influenced topological resolution, and robust inference required nearcomplete mitogenomic datasets. Likewise, Licuanan & Matias (2022) echinoderm DNA barcoding study demonstrated that COI alone may lack sufficient resolution to capture higher level diversity, reinforcing the need for multi-marker approaches. Topological discordance among markers likely reflects the complex evolutionary history of Asian colobines, including rapid radiation and incomplete lineage sorting (ILS). Rapid diversification compresses internal branch lengths, reducing the consistency of phylogenetic signal across loci. As noted by Roos & Zinner (2022), colobine phylogeny review, relationships within Colobinae remain partially unresolved due to limited molecular data and complex evolutionary dynamics. Comparable patterns have been documented in Macaca, where radiation genomics identified extensive ILS and historical hybridization as major sources of gene tree discordance (Tan et al., 2023). Genome-scale analyses, such as those in Kuang et al. 2023 Rhinopithecus phylogeography, highlight the importance of integrating multilocus and genomic data to resolve these complexities.

Our findings support a key idea in molecular phylogenetics: using only one gene to make evolutionary trees is limited and can lead to different results. In contrast, using multiple genes or complete mitochondrial genomes gives more reliable and consistent results. According to Doyle et al. (2021), mitochondrial genomes should be seen as a single unit, and combining them is a valid method for studying organelle evolution. However, it is still important to include nuclear genomic data. Vanderpool et al. (2020) showed that in primates, there are many differences in genealogical data due to gene mixing and complex speciation.

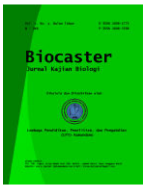
CONCLUSION

This study demonstrates that mitochondrial markers (COI, COII, and COIII) provide valuable yet variable phylogenetic signals in resolving relationships within Colobinae. Despite marker-dependent differences in topology and clustering resolution, a consistent evolutionary pattern is observed, where *Nasalis larvatus* is closely associated with *Simias concolor*, supporting the monophyly of the odd-nosed monkey lineage.

The findings confirm that COII and COIII possess higher discriminatory power compared to COI, particularly for intergeneric differentiation. However, the observed topological discordance highlights the inherent limitations of single-gene analyses, likely influenced by substitution rate variation, rapid radiation, and incomplete lineage sorting. Therefore, robust phylogenetic inference in primates requires integration of multiple mitochondrial markers and, ideally, genome-scale data. This study provides a critical baseline for future evolutionary and conservation genetics research on *Nasalis larvatus*, particularly in the context of its declining habitat and conservation urgency.

SUGGESTION

Future research should integrate complete mitochondrial genomes and nuclear genomic data to enhance phylogenetic resolution and address incomplete lineage sorting and hybridization, while expanding geographic sampling of *Nasalis larvatus* to better resolve population structure and haplotype diversity. Methodologically, multi-locus approaches combined with coalescent based



frameworks and phylogeographic analyses are essential to accurately reconstruct evolutionary history, particularly under rapid radiation. These advances should be directly translated into conservation practice by incorporating genetic evidence into habitat management, prioritizing genetically distinct populations, and integrating molecular data with ecological assessments to develop more effective and targeted conservation strategies.

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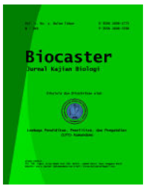

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