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In sillico Analysis of Mitochondrial DNA and Phylogenetic Relationships Among Genus Presbytis (*Presbytis comata, Presbytis fredericae, Presbytis mitrata, and Presbytis melalophos*)

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ABSTRACT

This study investigates the phylogenetic relationships among four species of Presbytis—*Presbytis comata, Presbytis fredericae, Presbytis mitrata, and Presbytis melalophos*—based on the mitochondrial cytochrome b gene sequences. These species, collectively known as leaf monkeys, share similar morphological features, which complicates species identification based solely on physical traits. To address this challenge, an in silico approach was employed, utilizing mitochondrial DNA sequences retrieved from the NCBI GenBank database. The sequences were analyzed using BLAST to ensure accuracy in alignment, followed by phylogenetic tree construction with MEGA software. The phylogenetic analysis revealed clear genetic distinctions between the species, despite their morphological similarities, with each species forming distinct clusters on the phylogenetic tree. The results demonstrate the utility of mitochondrial markers like cytochrome b in resolving evolutionary relationships in closely related species. These findings provide valuable insights into the genetic diversity and evolutionary history of Presbytis species, contributing to a better understanding of their taxonomy and evolutionary divergence. Furthermore, the study underscores the importance of genetic analysis in primate conservation efforts, as the genetic distinctions observed may inform targeted conservation strategies. This research highlights the critical role of molecular tools in understanding biodiversity and evolution, especially for species that are difficult to differentiate morphologically.

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INTRODUCTION

Indonesia is renowned for its rich biodiversity, including a diverse range of endemic primates. Among these are *Presbytis comata*, *Presbytis fredericae*, *Presbytis mitrata*, and *Presbytis melalophos*, which are collectively known as leaf monkeys. These primates are not only ecologically important

but also hold significant cultural and scientific value(Wang et al., 2024). The genus *Presbytis* comprises a group of arboreal primates found predominantly in Southeast Asia. These monkeys are known for their slim bodies, long tails, and varied fur coloration, which often includes contrasting patterns of black, gray, or brown. They inhabit tropical rainforests, ranging from primary to secondary forests, and are highly adapted to life in the trees. Their diet primarily consists of leaves, fruits, and seeds, which they digest with the help of a specialized, multi-chambered stomach designed to break down tough plant material. Socially, *Presbytis* species typically form small to medium-sized groups led by a dominant male, and they display diurnal activity patterns, spending most of their time in the forest canopy (Populasi et al., 2018). However, habitat fragmentation, deforestation, and human conflict have posed significant threats to many species within this genus. Conservation efforts are critical, as several species in *Presbytis*, including *Presbytis comata*, *Presbytis fredericae*, *Presbytis mitrata*, and *Presbytis melalophos*, are listed on the IUCN Red List as Vulnerable, Endangered, or Critically Endangered. This classification highlights the urgent need for measures to protect their populations and habitats. These threats, coupled with their already limited distribution as endemic species in Indonesia, underscore the importance of understanding their ecology, genetic diversity, and evolutionary relationships to inform conservation strategies effectively (Ang et al., 2024).

The four Presbytis species—*Presbytis comata, Presbytis fredericae, Presbytis mitrata*, and *Presbytis melalophos*—are morphologically similar, making it challenging to distinguish them based solely on physical traits. These similarities, including body size, fur coloration, and other external features, often reflect their adaptation to similar ecological niches within their overlapping habitats in Indonesia(Avenzora et al., 2024; Populasi et al., 2018). However, despite these outward resemblances, underlying genetic differences may provide critical insights into their evolutionary divergence and adaptations. To uncover these differences and clarify their evolutionary relationships, the use of mitochondrial DNA (mtDNA), particularly the cytochrome b gene, proves highly effective(Chen et al., 2022).

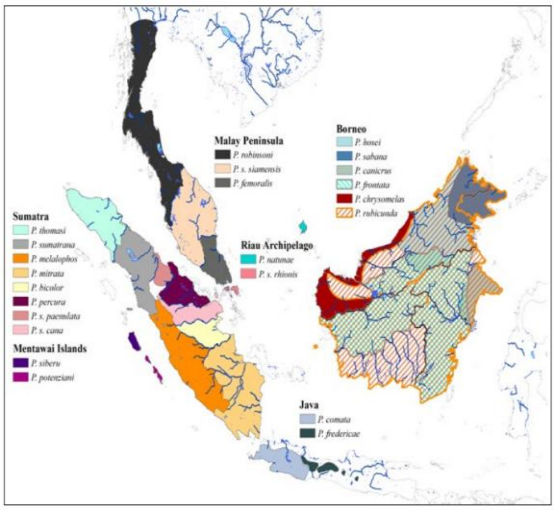


Figure 1. Presbytis Distribution

Mitochondrial DNA is widely regarded as an invaluable tool in phylogenetic studies due to its unique characteristics. Unlike nuclear DNA, mtDNA has a high mutation rate, which enables detailed resolution in studies of closely related species(Li et al., 2022). Furthermore, mtDNA is maternally inherited and does not undergo recombination, providing a clear and unambiguous genetic lineage that simplifies evolutionary analysis. Among the regions of mtDNA, the cytochrome b gene stands out as a powerful molecular marker. It combines conserved sequences, crucial for aligning distantly related taxa, with variable regions that allow for the differentiation of closely related species(Christinaki et al., 2022).

This balance makes cytochrome b ideal for resolving phylogenetic relationships at multiple taxonomic levels. By analyzing the cytochrome b sequences of these Presbytis species, we can identify genetic distinctions that are not evident through morphology alone and construct a detailed phylogenetic tree(Phromnoi et al., 2022). Such molecular analyses provide a deeper understanding of their evolutionary histories and relationships. Moreover, this approach is particularly relevant for these primates, as they are endemic to Indonesia and face significant

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threats such as habitat loss and hunting. Understanding their genetic and evolutionary diversity is critical for developing conservation strategies tailored to their unique ecological and genetic needs. By integrating morphological observations with molecular data, this study aims to contribute to the broader effort of preserving Indonesia's biodiversity and addressing the conservation challenges facinsg these threatened species(Seddigh & Darabi, 2018).

METHOD

This research employs an in silico approach to analyze the phylogenetic relationships among *Presbytis comata*, *Presbytis fredericae*, *Presbytis mitrata*, and *Presbytis melalophos(Budiarsa et al., 2022)*. The study begins with the retrieval of mitochondrial DNA (mtDNA) sequences, specifically targeting the cytochrome b gene, from the NCBI GenBank database.

These sequences serve as the primary data source for subsequent analyses. The retrieved sequences are first aligned using the BLAST tool to confirm their identity and ensure accurate comparison. Following this, the sequences are processed and aligned using bioinformatics software to identify conserved and variable regions. MEGA (Molecular Evolutionary Genetics Analysis) software is then utilized to construct phylogenetic trees using appropriate methods such as Maximum Likelihood or Neighbor-Joining. These trees help elucidate the evolutionary relationships and genetic divergence among the four *Presbytis* species.

RESULT AND DISCUSSION

The phylogenetic analysis of *Presbytis comata*, *Presbytis fredericae*, *Presbytis mitrata*, and *Presbytis melalophos* was based on cytochrome b gene sequences, which provided insights into the genetic relationships among these species. Despite their similar morphologies, the analysis revealed significant genetic distinctions, particularly when comparing their cytochrome b gene sequences. This gene is a well-established molecular marker for studying evolutionary relationships in primates, offering valuable insights into the divergence and speciation processes of the Presbytis species. To ensure the accuracy and robustness of the phylogenetic tree, sequences were retrieved from the NCBI GenBank database and aligned using the BLASTn tool with the following parameters(Anwar et al., 2022):

- Word size (28): This word size strikes a balance between sensitivity and speed, allowing for effective detection of similar sequences while maintaining computational efficiency.
- Expect value (E-value) of 0.05: The E-value determines the statistical significance of sequence matches. By using a threshold of 0.05, the analysis minimized the risk of false positives, ensuring that only highly significant alignments were considered for further analysis.
- Hitlist size (100): This parameter specified that the top 100 hits should be returned. This was important for obtaining a broad range of sequence data for comparison, enhancing the reliability of the alignment.
- Match/Mismatch scores (1, -2): The scoring system of +1 for matches and -2 for mismatches is commonly used in nucleotide sequence alignments. This scoring choice reflects the cost of mismatches relative to matches and ensures a balanced alignment.
- Gap costs (0, 2.5): These gap penalties allowed for the introduction of gaps into the sequences during alignment, with a lower penalty for opening gaps and a higher penalty for extending them. This approach accommodates insertions and deletions in the sequences while maintaining their biological relevance.
- Low Complexity Filter (Yes): The low complexity filter helped remove regions with simple, repetitive sequences that might otherwise introduce bias or noise into the alignment.

Table 1. Parameters on BLASTn tool NCBI

Search Parameters	
Program	blastn
Word size	28
Expect value	0.05
Hitlist size	100
Match/Mismatch scores	1,-2
Gapcosts	0,2.5
Low Complexity Filter	Yes
Filter string	L;m;
Genetic Code	1

Database	
Posted date	Nov 27, 2024 12:29 AM
Number of letters	2,534,369,448,025
Number of sequences	111,121,377
Entrez query	None

Karlin-Altschul statistics		
Lambda	1.33271	1.28
K	0.620991	0.46
H	1.12409	0.85

Results Statistics	
Length adjustment	36
Effective length of query	111
Effective length of database	2530369078453
Effective search space	280870967708283
Effective search space used	280870967708283

With a database comprising over 2.5 trillion base pairs and 111 million sequences, the analysis was conducted over an effective search space of approximately 1.2 quadrillion base pairs. This large-scale search space ensured that the sequences were compared against a broad and comprehensive set of genomic data, maximizing the chances of identifying relevant and closely related sequences from other species. By using this extensive database, the BLAST search ensured that the retrieved sequences were representative of the genetic diversity within the Presbytis species and were accurately aligned for subsequent phylogenetic tree construction. The successful alignment and identification of significant genetic markers based on these search parameters provided a solid foundation for understanding the genetic relationships between the species(Ang et al., 2020). As a result, the phylogenetic tree constructed based on these sequences revealed clear genetic distinctions between *Presbytis comata*, *Presbytis fredericae*, *Presbytis mitrata*, and *Presbytis melalophos*, suggesting that these species, while morphologically similar, have distinct evolutionary histories and adaptations.

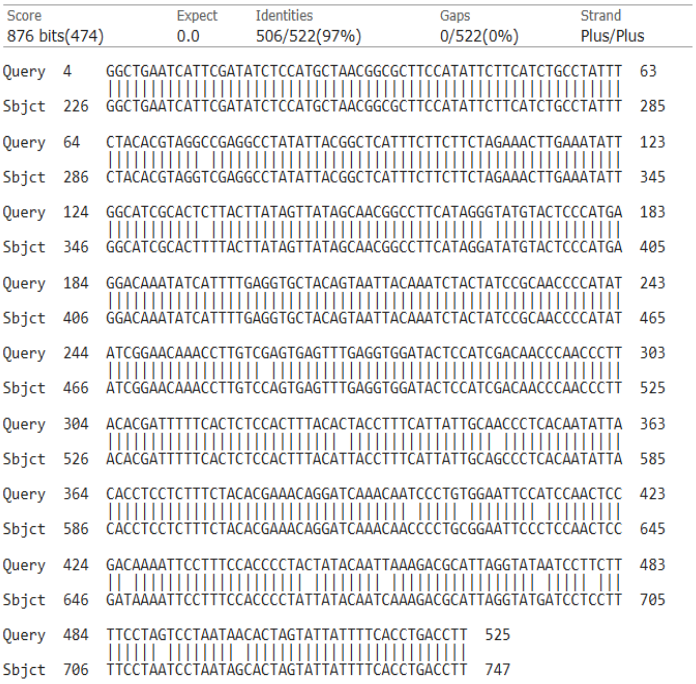


Figure 1. The nucleotide sequence of the cytochrome b gene in the mitochondria of Presbytis comata



Figure 2. The nucleotide sequence of the cytochrome b gene in the mitochondria of Presbytis fredericae

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Score	Expect	Identities	Gaps	Strand
843 bits(456)	0.0	500/522(96%)	0/522(0%)	Plus/Plus

```
Query  4    GGCTGAATCATTCGATATCTCCATGCTAACGGCGCTTCCATATTCTTCATCTGCCTATTT  63
            ||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  226  GGCTGAATTATTCGATACCTCCATGCTAACGGCGCTTCCATATTCTTCATCTGCCTATTT  285

Query  64   CTACACGTAGGCCGAGGCCTATATTACGGCTCATTTCTTCTTCTAGAAACTTGAAATATT  123
            ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  286  CTACACGTAGGCCGAGGTCTATATTACGGCTCATTTCTTCTTCTAGAAACTTGAAATATT  345

Query  124  GGCATCGCACTCTTACTTATAGTTATAGCAACGGCCTTCATAGGGTATGTACTCCCATGA  183
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  346  GGCATCGCACTTTTACTTATAGTTATAGCAACAGCCTTCATAGGGTATGTACTCCCATGA  405

Query  184  GGACAAATATCATTTTGAGGTGCTACAGTAATTACAAATCTACTATCCGCAACCCCATAT  243
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  406  GGACAAATATCATTTTGAGGTGCTACAGTAATTACAAACCTACTATCCGCAACCCCATAT  465

Query  244  ATCGGAACAAACCTTGTCGAGTGAGTTTGAGGTGGATACTCCATCGACAACCCAACCCTT  303
            |||||||||||||||| | ||||||||||||||||||||||||||||||||||||||||||
Sbjct  466  ATCGGAACAAACCTTGTCCAATGAGTTTGAGGTGGATACTCCATCGACAACCCAACCCTT  525

Query  304  ACACGATTTTTCACTCTCCACTTTACACTACCTTTCATTATTGCAACCCTCACAATATTA  363
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  526  ACACGATTTTTCACTCTCCACTTTACACTACCTTTCATTATCGCAGCCCTCACAATATTA  585

Query  364  CACCTCCTCTTTCTACACGAAACAGGATCAAACAATCCCTGTGGAATTCCATCCAACTCC  423
            |||||||||||||||||||||||||||||||||||| ||||| |||||| || |||||||||
Sbjct  586  CACCTCCTCTTTCTACACGAAACAGGATCAAACAACCCCTGCGGAATCCCCTCCAACTCC  645

Query  424  GACAAAATTCCTTTCCACCCCTACTATACAATTAAAGACGCATTAGGTATAATCCTTCTT  483
            || ||||| |||||||||| || |||||||| ||||||||||||||||||||||||||||
Sbjct  646  GATAAAATCCCTTTCCACCCTTATTATACAATCAAAGACGCATTAGGTATAATCCTTCTT  705

Query  484  TTCCTAGTCCTAATAACACTAGTATTATTTTCACCTGACCTT  525
            ||||||||| |||||  |||||||||||||||||||||||||
Sbjct  706  TTCCTAGTCATAATAGTACTAGTATTATTTTCACCTGACCTT  747
```

Figure 3. The nucleotide sequence of the cytochrome b gene in the mitochondria of Presbytis mitrata

Score	Expect	Identities	Gaps	Strand
826 bits(447)	0.0	497/522(95%)	0/522(0%)	Plus/Plus

```
Query  4    GGCTGAATCATTCGATATCTCCATGCTAACGGCGCTTCCATATTCTTCATCTGCCTATTT  63
            ||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  226  GGCTGAATTATTCGATATCTCCATGCTAACGGCGCTTCCATATTCTTCATCTGCCTATTT  285

Query  64   CTACACGTAGGCCGAGGCCTATATTACGGCTCATTTCTTCTTCTAGAAACTTGAAATATT  123
            ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  286  CTACACGTAGGCCGAGGCTTATATTACGGCTCATTTCTTCTTCTAGAAACTTGAAATATT  345

Query  124  GGCATCGCACTCTTACTTATAGTTATAGCAACGGCCTTCATAGGGTATGTACTCCCATGA  183
            || |||||||| |||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  346  GGTATCGCACTTTTACTTATAGTTATAGCAACGGCCTTCATAGGCTATGTACTCCCATGA  405

Query  184  GGACAAATATCATTTTGAGGTGCTACAGTAATTACAAATCTACTATCCGCAACCCCATAT  243
            |||||||||||||||||||||||||||||||||||| || |||||||| |||||||||||
Sbjct  406  GGACAAATATCATTTTGAGGTGCTACAGTAATTACAAATTTATTATCCGCAATCCCATAT  465

Query  244  ATCGGAACAAACCTTGTCGAGTGAGTTTGAGGTGGATACTCCATCGACAACCCAACCCTT  303
            || |||||||||||||| | |||||||||||||||||||||| || ||||||||||||||||
Sbjct  466  ATTGGAACAAACCTTGTCCAATGAGTTTGAGGTGGATACTCCATTGATAACCCAACCCTT  525

Query  304  ACACGATTTTTCACTCTCCACTTTACACTACCTTTCATTATTGCAACCCTCACAATATTA  363
            |||||||| |||||||||||||||||||||||||||||||| |||||||| ||||||||||
Sbjct  526  ACACGATTCTTCACTCTCCACTTTACACTACCTTTTATTATTGCAGCCCTCACAATATTA  585

Query  364  CACCTCCTCTTTCTACACGAAACAGGATCAAACAATCCCTGTGGAATTCCATCCAACTCC  423
            |||||||||||||||||||||||||||||||||||| ||||||||| |||||||||||||
Sbjct  586  CACCTCCTCTTTCTACACGAAACAGGATCAAACAATCCCTGCGGAATTCCCTCCAACTCC  645

Query  424  GACAAAATTCCTTTCCACCCCTACTATACAATTAAAGACGCATTAGGTATAATCCTTCTT  483
            || |||||||||||||||||| ||||||| |||||||||||||| |||||||||||||||
Sbjct  646  GATAAAATTCCTTTCCACCCCTATTATACAACCAAAGACGCATTAGGCATAATCCTTCTT  705

Query  484  TTCCTAGTCCTAATAACACTAGTATTATTTTCACCTGACCTT  525
            |||||  |||||||||||||||||||||||||||||||||||
Sbjct  706  TTCCTCATCCTAATAACACTAGTATTATTTTCACCTGACCTT  747
```

Figure 4. The nucleotide sequence of the cytochrome b gene in the mitochondria of Presbytis melalophos

Based on the results of selecting 4 sequences from a total of 100 sequences, the BLAST analysis indicates that the distribution levels between one sequence and another are relatively similar. The Graphic Summary shows that the alignment score for each sequence is greater than or equal to 200 (Figure 6).

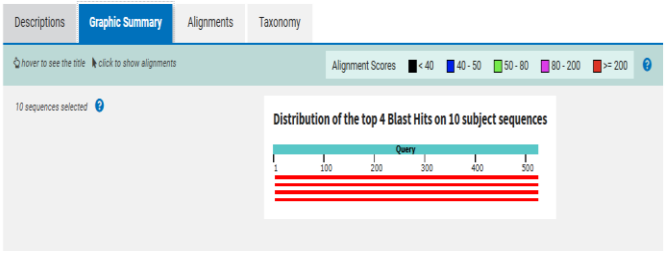


Figure 5. Graphic Summary

The phylogenetic tree illustrates the evolutionary relationships among four species of Presbytis: *Presbytis melalophos* mitrata, *Presbytis melalophos* melalophos, *Presbytis comata* fredericae, and *Presbytis comata* comata (Fig. 7). This analysis is based on the mitochondrial cytochrome b gene, a powerful molecular marker for resolving evolutionary relationships. The tree clearly separates the species into two major clades: one comprising P. melalophos mitrata and P. melalophos melalophos, and another comprising P. comata fredericae and P. comata comata. The close grouping of P. melalophos mitrata and P. melalophos melalophos is supported by a high bootstrap value of 90, indicating strong statistical confidence in their genetic similarity and evolutionary proximity. Similarly, the P. comata species form a distinct cluster, separate from the P. melalophos group, highlighting significant evolutionary divergence between these two lineages(Chen et al., 2022).

Despite their morphological similarities, which can make distinguishing these species challenging, the genetic analysis reveals clear differences that help clarify their evolutionary history. The clustering of P. melalophos and P. comata groups reflects

their adaptation to different ecological niches and potential geographic isolation, which may have driven their genetic divergence(Karyanto et al., 2024). The use of mitochondrial DNA, particularly the cytochrome b gene, provides high resolution for understanding these relationships due to its maternal inheritance, lack of recombination, and high mutation rate. This molecular approach is essential for uncovering evolutionary patterns that are not evident through physical traits alone. These findings are critical for conservation strategies, as they emphasize the genetic diversity within the genus Presbytis and the need to protect these species, many of which are threatened by habitat loss and other anthropogenic pressures. Understanding their genetic and evolutionary relationships contributes to informed efforts to preserve Indonesia's rich biodiversity(Widiana et al., 2018).

(subspecies P. melalophos mitrata and P. melalophos melalophos) and another consisting of P. comata species (subspecies P. comata fredericae and P. comata comata). These clusters reflect significant genetic differentiation that would be difficult to detect through morphology alone, highlighting the importance of molecular analysis for understanding evolutionary relationships(Vun et al., 2011).

The separation of P. melalophos into two closely related subspecies supports the hypothesis that these populations have undergone recent divergence, likely influenced by geographic isolation or ecological specialization within their overlapping habitats.

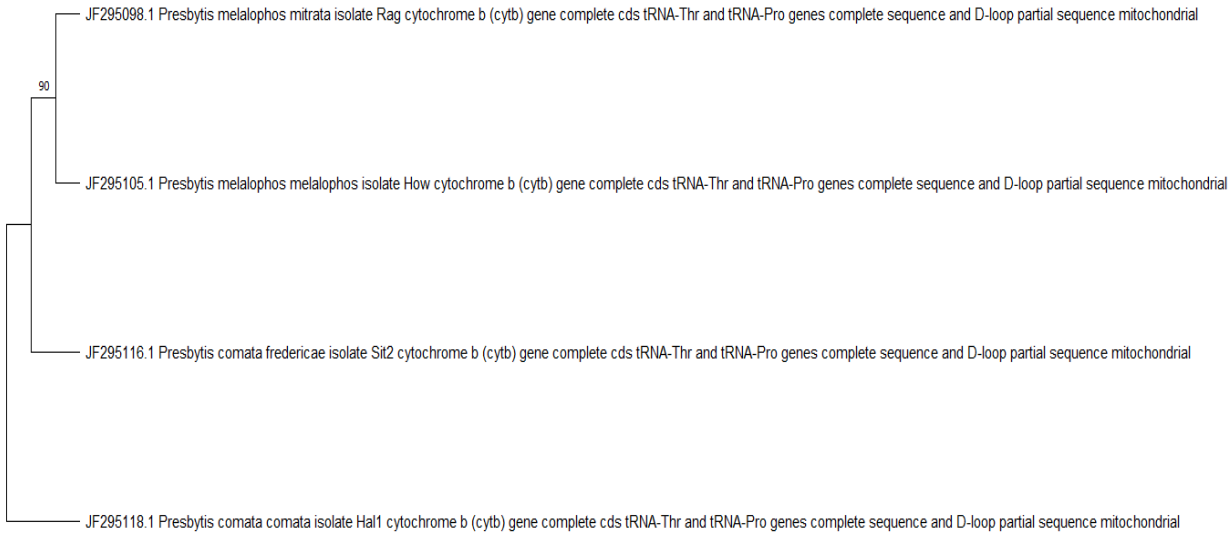


Table 2. Parameters on BLASTn tool NCBI

Genetic Divergence and Phylogenetic Relationships Among Presbytis Species:

The phylogenetic tree constructed from the cytochrome b gene sequences clearly illustrates the genetic divergence between the Presbytis species, despite their morphological similarities. The analysis revealed two major clusters: one containing *Presbytis melalophos*

This genetic proximity, with a bootstrap value of 90, suggests that P. melalophos mitrata and P. melalophos melalophos have retained a high degree of genetic similarity, indicating they might share a common recent ancestor or have adapted similarly to their environments(Devi et al., 2021). This finding challenges traditional morphological-based classification and suggests that subtle genetic

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differences may play a significant role in species differentiation within Presbytis. Furthermore, the distinct separation of P. comata species from P. melalophos reinforces the idea of a deeper evolutionary divergence between these two groups, which may be driven by ecological factors such as habitat preferences, dietary specializations, or behavioral differences(Lane & Bird, 2020).

This analysis provides valuable insights into the evolutionary history of these primates and suggests that genetic data, particularly from regions like the cytochrome b gene, can offer a clearer picture of the speciation processes in Presbytis. The molecular approach not only helps resolve phylogenetic relationships at finer taxonomic levels but also uncovers previously overlooked genetic distinctions that might have been masked by shared morphological traits. As Presbytis species are ecologically and genetically unique, understanding these evolutionary relationships is crucial for future conservation efforts, as it ensures that each species' specific genetic makeup is recognized and preserved.

Conservation Implications and the Role of Genetic Divergence in Presbytis Species

The genetic divergence observed among Presbytis species has significant implications for their conservation, particularly in the context of their IUCN Red List status. The fact that these species are genetically distinct, as revealed by the phylogenetic tree based on cytochrome b gene sequences, underlines the importance of addressing each species' unique evolutionary and ecological needs in conservation planning. Despite their similar morphological features, the genetic analysis shows clear differences that could reflect their adaptations to specific habitats and ecological niches, which may be at risk due to human-induced pressures such as habitat fragmentation, deforestation, and hunting.

As noted, several Presbytis species, including *Presbytis comata*, *Presbytis fredericae*, *Presbytis mitrata*, and *Presbytis melalophos*, are currently listed on the IUCN Red List as vulnerable, endangered, or critically endangered. These classifications are not only a result of their limited geographic distribution but also reflect the ongoing threats to their habitats and population sizes. The genetic differences uncovered in this study emphasize that conservation efforts should be tailored to preserve not only the species themselves but also their unique genetic diversity, which is essential for the long-term survival and adaptability of the genus. The identification of evolutionary lineages within Presbytis means that conservation actions should recognize the distinct genetic makeup of each species or subspecies, ensuring that these evolutionary lineages are maintained in the wild. The findings suggest that conservation strategies should not only focus on habitat protection but also incorporate genetic monitoring to preserve the diversity within the Presbytis genus. Genetic data could be instrumental in identifying priority populations for protection and could help design conservation breeding programs if necessary. Moreover, understanding the genetic structure of these species could also guide reintroduction programs, where maintaining the integrity of genetic lineages is crucial. Overall, the study emphasizes the need for a genetic-based approach to conservation, particularly for species like Presbytis that are threatened by both environmental and anthropogenic factors(Saputra et al., 2023).

Table 3. IUCN Redlist status(IUCN, 2022)

Species	IUCN Red List Status
Presbytis comata	Endangered
Presbytis fredericae	Vulnerable
Presbytis mitrata	Vulnerable
Presbytis melalophos	Vulnerable

CONCLUSION

The phylogenetic analysis of *Presbytis comata*, *Presbytis fredericae*, *Presbytis mitrata*, and *Presbytis melalophos* based on cytochrome b gene sequences revealed significant genetic differentiation among these species, despite their morphological similarities. The in silico approach using sequences retrieved from the NCBI GenBank database, coupled with a detailed alignment process using BLASTn, ensured that the genetic data used for the analysis was both

comprehensive and reliable. The parameters set during the BLAST search, including word size, E-value threshold, gap costs, and the large search space, facilitated the identification of accurate and biologically relevant alignments, which were essential for the phylogenetic tree construction.

These findings underscore the utility of molecular markers like cytochrome b in revealing evolutionary relationships, and highlight the potential of using genomic data to refine the taxonomic understanding of species that exhibit similar morphological traits. The clear genetic distinctions observed in this study provide a deeper understanding of the evolutionary processes shaping the diversity of the *Presbytis* genus and contribute to the broader field of primate evolutionary biology.

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