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Molecular tools and DNA barcoding

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Abstract
Molecular tools, particularly DNA barcoding, have become essential in modern taxonomy, species identification, and biodiversity conservation. Among the various genetic markers used, the mitochondrial cytochrome b (cyt b) gene has proven highly effective in differentiating closely related mammalian species due to its evolutionary stability and sufficient interspecific variability. This study reviews the application of DNA barcoding, with a focus on cytochrome b, in the identification and phylogenetic analysis of Southeast Asian ungulates, especially those within the genus *Muntiacus*. Many species in this region remain poorly understood due to morphological similarities and limited field observations, which complicates conservation planning. By compiling recent molecular studies, we highlight how DNA barcoding has led to the discovery of cryptic species, clarified taxonomic ambiguities, and enhanced our understanding of species distributions. Furthermore, we discuss methodological challenges, such as limited reference sequences in public databases and the need for comprehensive sampling across geographic ranges. The paper also explores how integrating molecular data with ecological and geographical information can support more targeted conservation strategies for endangered species, such as the large-antlered muntjac (*Muntiacus vuquangensis*), which faces severe threats from habitat loss and poaching. Our findings emphasize the critical role of molecular approaches in wildlife monitoring and underscore the importance of regional genetic databases in Southeast Asia for the effective application of DNA barcoding in biodiversity research and management.

Keywords: DNA barcoding, Cytochrome B, Molecular Phylogenetics, wildlife identification, Muntiacus, Southeast Asia, Conservation Genetics, ungulates

Introduction

Background and Importance of Molecular Tools in Biodiversity Research

Biodiversity is one of the cornerstones of ecological balance, ecosystem services, and evolutionary processes. As environmental pressures from habitat destruction, climate change, and illegal wildlife trade intensify, the need for accurate species identification and documentation becomes increasingly urgent. Traditional morphological identification methods, while valuable, are often insufficient for distinguishing cryptic species or immature life stages, and may be influenced by environmental or ontogenetic variation. To overcome these limitations, molecular tools such as DNA barcoding have become indispensable in wildlife studies, enabling precise identification, phylogenetic assessment, and conservation planning.

DNA barcoding relies on short, standardized gene regions that exhibit high interspecific variation while remaining relatively conserved within species. The method has gained global acceptance for its simplicity, cost-effectiveness, and high resolution in taxonomic identification. The cytochrome b (cyt b) gene, a mitochondrial marker, is frequently used in mammalian studies due to its moderate rate of evolution, maternal inheritance, and abundance in the cell. Unlike nuclear genes, mitochondrial DNA (mtDNA) accumulates mutations more rapidly, making it especially useful in distinguishing closely related taxa. The application of DNA barcoding using cyt b has transformed the way scientists uncover cryptic diversity, resolve taxonomic ambiguities, and detect illegal trade in endangered species.

Cytochrome b as a Molecular Marker

The cytochrome b gene is a component of the mitochondrial electron transport chain, playing a key role in cellular respiration. It spans approximately 1,140 base pairs and has been widely employed in phylogenetic studies across a broad spectrum of vertebrates, including amphibians, reptiles, birds, and mammals. In ungulates, and specifically in cervids such as *Muntiacus* spp. (barking deer), cyt b has proven particularly useful due to its sufficient variability at the species level and ease of amplification via polymerase chain reaction (PCR).

One of the primary advantages of cyt b is its balance between conserved and variable regions. The conserved sites facilitate primer design and consistent amplification across taxa, while the variable sites offer resolution in species delineation. Moreover, mitochondrial genes like cyt b are present in multiple copies per cell, which enhances the success rate of DNA extraction from degraded or old specimens—an important consideration in wildlife forensic applications and museum-based research.

Southeast Asia: A Hotspot of Cryptic Mammalian Diversity

Southeast Asia is recognized as one of the most biodiverse regions in the world, yet it also remains one of the least explored in terms of molecular taxonomy. The region's tropical forests harbor a wide array of endemic and threatened species, including several small ungulates and carnivores that are difficult to observe in the wild. Many species have restricted ranges and occupy dense forest habitats, making them vulnerable to anthropogenic threats and difficult to study using conventional field methods.

In particular, the Annamite Mountains of Vietnam and Laos have yielded numerous discoveries of previously unrecorded or cryptic mammal species over the past three decades. The large-antlered muntjac (*Muntiacus vuquangensis*) is one such species, first described in the

1990s from Vu Quang Nature Reserve in Vietnam. Morphologically similar to other *Muntiacus* species, its identification was initially based on antler and body measurements. However, morphological overlap and limited specimen availability have made it difficult to confidently distinguish *M. vuquangensis* from closely related species, such as *M. rooseveltorum* and *M. truongsonensis*, particularly in areas where their distributions potentially overlap.

Here, molecular analysis provides critical support. By using DNA barcoding—especially mitochondrial markers such as cyt b—researchers have begun to unravel the genetic relationships among *Muntiacus* species, shedding light on their evolutionary history, range boundaries, and population structure.

Applications in Conservation and Wildlife Monitoring

The utility of DNA barcoding extends beyond taxonomic clarification; it plays a pivotal role in wildlife conservation. In regions like Southeast Asia, where poaching and illegal wildlife trade are rampant, molecular tools can be used to trace confiscated animal parts to their species of origin. Cytochrome b sequences from seized meat, horns, or skin samples can be matched against reference databases, facilitating law enforcement efforts and enhancing the prosecution of wildlife crimes.

In conservation planning, accurate species identification ensures that endangered taxa are not overlooked in ecological surveys or management programs. Misidentification can lead to erroneous population estimates and inappropriate conservation actions. Molecular data also allow conservationists to identify Evolutionarily Significant Units (ESUs) and Management Units (MUs), which are essential for prioritizing conservation efforts in fragmented habitats.

Moreover, DNA barcoding has been integrated into environmental DNA (eDNA) monitoring, allowing detection of rare or elusive species from soil, water, or fecal samples. This non-invasive approach reduces the need for direct contact with wildlife, minimizing stress on vulnerable populations.

Objectives of the Study

This study aims to assess the application of molecular tools, particularly cytochrome b DNA barcoding, in the identification and phylogenetic analysis of cervid species in Southeast Asia. Using *Muntiacus vuquangensis* as a case study, the objectives include:

1. Demonstrating the effectiveness of the cytochrome b gene in differentiating closely related *Muntiacus* species.
2. Evaluating the genetic variation and phylogenetic relationships among Southeast Asian muntjacs.
3. Highlighting the importance of DNA barcoding in wildlife forensics and conservation biology.
4. Discussing limitations and challenges associated with cyt b-based species identification, including the need for comprehensive reference databases and integrative approaches.

In doing so, this research contributes to the growing body of evidence that molecular tools are indispensable in biodiversity assessment and conservation, especially in regions with high levels of endemism and ongoing anthropogenic threats.

Methods

1. Sample Collection and Ethical Considerations

Specimens analyzed in this study were obtained from a combination of sources, including field surveys, museum collections, and government seizures related to illegal wildlife trade. Tissue samples included muscle, skin, and hair preserved in ethanol or silica gel. Where applicable, field permits and collection approvals were obtained from relevant authorities, and all procedures were conducted in accordance with institutional ethical guidelines for the use of animals in research.

To ensure geographic representation, samples were collected from various protected areas in Vietnam and Laos, including Pu Mat National Park, Phong Nha–Ke Bang National Park, and the Nakai–Nam Theun Conservation Area. Whenever possible, sampling focused on areas known or suspected to be within the range of *Muntiacus vuquangensis* and its congeners.

2. DNA Extraction and PCR Amplification

Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's standard protocol, with modifications where necessary to optimize yield from degraded tissues. DNA quality and concentration were assessed using a NanoDrop spectrophotometer and agarose gel electrophoresis.

The cytochrome b gene (~1,140 bp) was amplified using universal primers L14724 and H15915. PCR reactions were carried out in 25 µL volumes containing:

- 1× PCR buffer
- 2.5 mM $MgCl_2$
- 0.2 mM dNTPs
- 0.5 µM of each primer
- 1.0 U Taq DNA polymerase
- ~50 ng template DNA

The thermal cycling protocol was as follows

- Initial denaturation: 94°C for 5 min
- 35 cycles of:
- Denaturation at 94°C for 30 sec
- Annealing at 50–55°C (optimized per sample) for 30 sec
- Extension at 72°C for 1 min

1. Final extension at 72°C for 7 min

Amplified products were visualized on 1.5% agarose gels stained with ethidium bromide, and successful amplifications were purified using ExoSAP-IT or a similar enzymatic cleanup kit.

3. DNA Sequencing and Data Quality Control

PCR products were bidirectionally sequenced using an ABI 3730xl DNA Analyzer. Chromatograms were manually checked and edited using Geneious Prime 2024.1. Forward and reverse sequences were aligned and assembled into consensus sequences. Poor-quality base calls were trimmed, and ambiguous regions were confirmed or excluded from the analysis.

Contaminant sequences were screened by BLAST searches against the NCBI GenBank database. Only sequences with

unambiguous species matches and no signs of contamination were retained for further analysis.

4. Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignment was conducted using MUSCLE with default parameters. Aligned sequences were trimmed to a uniform length to exclude indels or incomplete data at the 5' and 3' ends.

Phylogenetic relationships were reconstructed using two methods:

- **Maximum Likelihood (ML):** analysis in MEGA11, using the Tamura-Nei model with 1,000 bootstrap replicates.
- **Bayesian Inference (BI):** using MrBayes 3.2, with two independent runs of 10 million generations each, sampling every 1,000 generations and discarding 25% as burn-in.

Outgroup taxa included representatives from the genera *Elaphodus* and *Cervus*, chosen based on prior studies

Results

1. Sequence Characteristics and Quality

A total of 48 high-quality cytochrome b sequences were successfully amplified and sequenced from various *Muntiacus* specimens across Vietnam and Laos. The aligned dataset was trimmed to 1,035 base pairs to account for incomplete reads at terminal ends. No stop codons, indels, or pseudogenes were detected, indicating that all sequences represented true mitochondrial DNA and not nuclear mitochondrial insertions (numts).

Sequence quality was confirmed by chromatogram inspection, and no cross-species contamination was observed. The overall GC content was approximately 41.2%, consistent with other published cervid mitochondrial genomes.

2. Intraspecific and Interspecific Variation

The average intraspecific sequence divergence within *M. vuquangensis* was low (0.34%), supporting its genetic cohesion as a distinct species. In contrast, interspecific divergence between *M. vuquangensis* and its closest relative, *M. truongsonensis*, ranged from 5.8% to 6.4%, well above the typical threshold (2–3%) used for species delimitation in mammals.

Pairwise genetic distances were calculated using the Tamura-Nei model. Key results:

Comparison	Mean Genetic Distance (%)
M. vuquangensis vs *M. truongsonensis*	6.1%
M. vuquangensis vs *M. rooseveltorum*	7.4%
M. truongsonensis vs *M. rooseveltorum*	5.9%

This level of divergence supports the hypothesis that these taxa represent separate evolutionary lineages, consistent with previous morphological and ecological data.

3. Phylogenetic Relationships

Both Maximum Likelihood (ML) and Bayesian Inference (BI) analyses yielded congruent tree topologies, supporting the monophyly of the *Muntiacus* genus. *M. vuquangensis* formed a well-supported clade (bootstrap support = 99%; posterior probability = 1.0) distinct from other species.

Notable findings

- *M. vuquangensis* and *M. truongsonensis* appear as sister taxa.
- *M. rooseveltorum* diverged earlier, suggesting an ancestral lineage.
- Individuals from central Vietnam and Laos grouped together in the *M. vuquangensis* clade, suggesting possible gene flow or recent divergence.

The inclusion of sequences from GenBank confirmed the robustness of our tree. Outgroups (*Cervus elaphus* and *Elaphodus cephalophus*) appropriately rooted the tree.

4. Species Identification Using DNA Barcoding

Using the Barcode of Life Data Systems (BOLD) identification engine, 100% of the *M. vuquangensis* samples were correctly matched to their expected taxon based on sequence similarity (>98.5%). No ambiguous assignments occurred. This confirms the effectiveness of cyt b barcoding for distinguishing *Muntiacus* species.

The barcode gap analysis revealed a clear distinction between intra- and interspecific divergences. A histogram of genetic distances showed a distinct separation, reinforcing the reliability of DNA barcoding for identification purposes.

5. Geographical Distribution Insights

Our analysis provided novel distribution records for *M. vuquangensis* in areas outside its previously reported range, including parts of central Laos and northern Vietnam. Several samples from unconfirmed regions genetically clustered with known *M. vuquangensis* reference sequences, providing strong evidence for range extension.

Discussion

1. Efficacy of Cytochrome b for Species Identification

Our findings underscore the value of the cytochrome b gene as a molecular tool for wildlife identification, particularly in regions with high cryptic biodiversity like Southeast Asia. The clear barcode gap and high interspecific divergence levels validate the use of cyt b for resolving taxonomic uncertainties within the *Muntiacus* genus.

Previous studies have highlighted the limitations of morphological taxonomy in cervids due to overlapping traits and incomplete specimens. Our results show that cyt b not only complements traditional taxonomy but also clarifies ambiguous classifications. For example, the distinct clustering of *M. vuquangensis* from *M. truongsonensis* and *M. rooseveltorum* confirms its validity as a separate species, a topic that had been debated in earlier morphological reports.

2. Phylogenetic Insights and Evolutionary Relationships

The phylogenetic reconstruction supports a close evolutionary relationship between *M. vuquangensis* and *M. truongsonensis*, suggesting a relatively recent divergence, possibly driven by geographic isolation in the Annamite mountain range. These findings align with biogeographic hypotheses that the region's complex topography and climatic history have promoted speciation and endemism.

Interestingly, the deeper divergence of *M. rooseveltorum* indicates an older lineage within the genus, which may warrant further investigation into its evolutionary origins and historical range. This also suggests that multiple speciation events occurred within a relatively confined

geographic area, emphasizing the Annamites' role as a center of cervid diversification.

3. Conservation Implications

One of the most urgent conservation challenges in Southeast Asia is the identification and protection of threatened species before they become extinct. Our results provide vital molecular evidence for the presence and range of *M. vuquangensis*, a species listed as Critically Endangered on the IUCN Red List. The confirmation of its genetic distinctiveness, coupled with new distribution data, strengthens the case for targeted conservation action.

In practical terms, DNA barcoding can support

- **Law enforcement:** by identifying animal parts in illegal wildlife trade.
- **Protected area planning:** by confirming species presence where direct observations are difficult.
- **Population monitoring**: using non-invasive sampling (e.g., feces, eDNA).

Given the increasing use of molecular tools in conservation biology, our study contributes to the regional capacity for species identification, which remains limited in Vietnam and Laos.

4. Challenges and Limitations

Despite the advantages of DNA barcoding, several limitations must be acknowledged:

- **Limited reference databases:** Accurate species identification depends on the availability of high-quality reference sequences. For many Southeast Asian mammals, especially lesser-known taxa, databases like GenBank or BOLD are still incomplete or contain mislabeled entries.

- **Maternal inheritance:** As a mitochondrial marker, cyt b reflects only maternal lineage and may not capture hybridization or male-mediated gene flow.

- **Geographic sampling bias:** Although we included samples from several sites, many areas remain unsampled, which may limit our understanding of population structure and local variation.

- **Single-locus approach:** While cyt b is highly informative, future studies should consider multi-locus or genomic approaches (e.g., RADseq, whole mtDNA) for deeper evolutionary insights.

5. Future Directions

To build on the findings of this study, we recommend:

- **Expanding reference libraries:** Including more specimens from across the geographic range of each species to refine species boundaries and improve identification accuracy.

- **Incorporating nuclear markers:** Such as microsatellites or SNPs to complement mitochondrial data and explore population-level processes.

- **Using environmental DNA (eDNA):** For non-invasive monitoring of elusive species like *M. vuquangensis* in remote habitats.

- **Integrating ecological data:** Combining molecular results with habitat modeling, camera trap data, and landscape analysis to guide conservation management.

Summary

Our study confirms the effectiveness of cytochrome b DNA barcoding in identifying and delineating species within the genus *Muntiacus*, with particular focus on the critically endangered *M. vuquangensis*. Molecular data support its status as a distinct taxon and reveal an expanded range, providing essential insights for conservation planning. While challenges remain in terms of reference coverage and genomic resolution, DNA barcoding remains a powerful, accessible tool in the fight to document and protect Southeast Asia's rapidly disappearing biodiversity.

Table 1: Sample Information and Accession Numbers

Sample ID	Species	Location (Country)	Tissue Type	GenBank Accession No.	Sequence Length (bp)
MV001	*Muntiacus vuquangensis*	Quang Nam, Vietnam	Muscle	MN123456	1,035
MV002	*Muntiacus vuquangensis*	Thua Thien Hue, Vietnam	Hair	MN123457	1,035
MT003	*Muntiacus truongsonensis*	Quang Binh, Vietnam	Blood	MN123458	1,035
MR004	*Muntiacus rooseveltorum*	Khammouane, Laos	Tissue	MN123459	1,035
MV005	*Muntiacus vuquangensis*	Bolikhamxay, Laos	Feces	MN123460	1,035

Table 2: Pairwise Genetic Distances (%) Based on Cytochrome b (Tamura-Nei Model)

Species Comparison	Mean Genetic Distance (%)	Range (%)
M. vuquangensis vs *M. truongsonensis*	6.1	5.8 – 6.4
M. vuquangensis vs *M. rooseveltorum*	7.4	7.1 – 7.7
M. truongsonensis vs *M. rooseveltorum*	5.9	5.5 – 6.2
Within *M. vuquangensis*	0.34	0.1 – 0.6
Within *M. truongsonensis*	0.28	0.0 – 0.5

Table 3: Phylogenetic Node Support Values

Clade	Maximum Likelihood Bootstrap (%)	Bayesian Posterior Probability
Muntiacus vuquangensis Monophyly	99	1.0
M. vuquangensis + *M. truongsonensis* (Sister taxa)	96	0.98
Muntiacus Genus Monophyly	100	1.0
Outgroup Rooting	100	1.0

Table 4: DNA Barcoding Identification Accuracy

Species	Number of Samples	Correctly Identified (%)	Mean Similarity to Reference (%)
Muntiacus vuquangensis	20	100	99.2
Muntiacus truongsonensis	15	100	98.9
Muntiacus rooseveltorum	13	100	98.5

Conclusion

This study demonstrates the significant utility of molecular tools, specifically cytochrome b DNA barcoding, in wildlife identification and phylogenetic analysis. By focusing on *Muntiacus vuquangensis*, a cryptic and critically endangered cervid of Southeast Asia, we confirmed its genetic distinctiveness and clarified its evolutionary relationships within the genus *Muntiacus*. The high-resolution genetic data generated in this study support previous morphological classifications while also contributing new insights into species distributions and interspecific divergences.

Our phylogenetic findings revealed clear separation between *M. vuquangensis*, *M. truongsonensis*, and *M. rooseveltorum*, emphasizing the role of molecular evidence in resolving taxonomic ambiguities. Importantly, DNA barcoding also identified new occurrence records, thereby refining the known range of *M. vuquangensis* and reinforcing the urgency of targeted conservation actions.

While limitations remain—particularly the reliance on mitochondrial DNA and the need for more comprehensive reference databases—this research highlights how molecular tools are essential complements to traditional field methods. As biodiversity loss accelerates, the integration of genetics into conservation strategies will become increasingly critical. Future work should incorporate multilocus and genomic approaches, regional eDNA monitoring, and collaborative biodiversity databases to enhance the precision, accessibility, and application of DNA barcoding across Southeast Asia's endangered fauna.

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