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Mitochondrial gene marker identification for a targeted hornbill eDNA assay

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ABSTRACT

The effectiveness of traditional hornbill monitoring through visual and acoustic surveys is often hampered by their cryptic behavior and habitat preference in dense rainforests. The challenges in hornbill detection have led to insufficient population data, posing a significant challenge to hornbill conservation efforts. Environmental DNA (eDNA) offers a complementary approach that enables non-invasive detection of species from trace genetic material shed into the surrounding environments. Targeted eDNA assay using real-time PCR (qPCR) allows the detection and quantification of low amount of target DNA in complex environmental samples. The design of primers defines the region of target DNA to be amplified from the complex DNA sequences present in environmental samples. Gene marker selection is critical to ensure assay specificity by identifying regions that are conserved enough within target species but variable enough between target and non-target species, used for design of primers. This study identifies the mitochondrial gene markers for the development of a targeted eDNA assay, using Anthracoceros albirostris as a proof-of-concept study. We compiled mitochondrial DNA (mtDNA) sequences of target and non-target, closely related species from the GenBank nucleotide database. Due to the lack of hornbills' sequence data, we performed Sanger sequencing to generate mtDNA sequences targeting cytochrome c oxidase subunit 1 (COI), NADH dehydrogenase subunit 2 (ND2), and displacement loop (d-loop) genes using feather samples

from four hornbill individuals. Analysis of sequence alignment revealed a high level of sequence similarity (>90%) among the three mtDNA genes. Despite the high similarity, specific regions within ND2 genes exhibited a sufficient level of sequence variation. This study presents the preliminary development of targeted hornbill eDNA assay, utilizing ND2 as gene marker for primer design. It serves as a foundational step towards the broader application of molecular tools to protect the iconic hornbills in Borneo.

INTRODUCTION

Hornbills play ecological importance roles as seed dispersal and forest health indicator (Sherub & Tshering, 2019). Asian hornbills are especially threaten by habitat loss due to deforestation and land clearing, and poaching for their body parts (Dasgupta et al., 2022; Chakravorty et al., 2011). To preserve the pristine hornbill population, ongoing conservation efforts across the globe through habitat protection, law enforcement, community involvement and research and awareness programs (BirdLife International, 2025). However, the lack of fine-scale hornbill distribution data is one of the factors that impede effective hornbill conservation (Aik & Perumal, 2017). This could be contributed by the difficulty in hornbill detection due to their elusive nature and preference for dense forests (Kurniawan et al., 2025). Environmental DNA (eDNA) can address the limitations of traditional visual-based surveys, providing a rapid, non-invasive tool to detect their presence in vast, dense forests.

Environmental DNA is a molecular detection technique that increasingly being used for biodiversity monitoring (Beng & Richard, 2020). This method analyse genetic fragments shed by organisms into their surrounding habitats by collecting environmental samples, such as soil, water, and air (Newton et al., 2025; van der Heyde et al., 2020). Environmental DNA offers advantages, in terms of non-invasiveness, high sensitivity, and high efficiency, for species identification, complementing traditional monitoring methods that typically involved direct observations and species traps (Leempoel et al., 2020; Keck et al., 2022). Despite the application of eDNA across broad taxonomic taxa, there is no dedicated eDNA research has been validated for hornbills.

Environmental DNA method involves series of workflows from sample collection, DNA extraction, polymerase chain reaction (PCR), and/or sequencing (Langlois et al., 2020). PCR or real time PCR (qPCR) is applied for eDNA studies that target specific species and quantify target DNA in complex environmental samples (Langlois et al., 2020). Primer design is one of the key factors influencing detection success (Wu et al., 2022). Primers define the region of DNA to be amplified from the complex DNA sequences present in environmental samples (Wu et al., 2022). For targeted eDNA assay, researchers design specific primer that have significant mismatches to all non-target species (Langlois et al., 2020). Primer design start with gene marker selection, followed by sequence alignment, computational primer design, and specificity validation (Harper et al., 2018).

Mitochondrial DNA (mtDNA) is often preferred because of its higher abundance than nuclear DNA, which increase the chance of detecting fragmented and degrading genetic materials in environmental samples (Langlois et al., 2020). The limited availability of mitochondrial reference sequence for hornbills represent one of the key challenges for primer design. This study identifies the suitable mitochondrial gene markers for the development of a targeted eDNA assay, using Anthracoceros albirostris as a proof-of-concept study. Challenges in gene marker identification for targeted hornbill detection were also discussed to guide future research scope. The findings lay the groundwork for eDNA application in hornbill monitoring.

Future research can leverage the gene markers identified for real-world eDNA assay testing. The study focused on hornbill mitochondrial gene analysis, which contributed to their limited genetic data. The data can have wider application in environmental conservation strategies, supporting the ICBS sustainability goals for protecting endangered species in Borneo.

MATERIALS AND METHODS

Assessment of hornbill reference sequence availability in public database

Hornbill reference sequences were retrieved from the National Center for Biotechnology Information (NCBI) GenBank database. Hornbill mitochondrial gene regions that are available in the existing nucleotide database were identified.

Generation of local barcode sequences

Barcodes were generated from genomic DNA extracted from freshly collected molted feathers of two A. albirostris individuals (Bird A and Bird B), one Anthracoceros malayanus individual (Bird C), and one Anorrhinus galeritus (Bird D). A. malayanus and A. galeritus represent non-target, closely related species that cooccur with A. albirostris. Based on the availability of hornbill reference sequence in NCBI database, four mtDNA gene regions (i.e. COI, cyt b, d-loop, ND2) were amplified from feather DNA extracts using established universal bird primers (Table 1). The resulting PCR products were purified and sent out to Apical Scientific Sdn Bhd for DNA sequencing.

Sequence alignment and comparison

The barcodes generated were subjected to BLASTn analysis (https://blast.ncbi.nlm.nih.gov/) against the NCBI GenBank nucleotide database to verify taxonomic identity. Sequences were searched against the NCBI non-redundant nucleotide collection (nr/nt) database. The search was limited to the Bucerotidae family to increase the relevance of identified gene markers. Highly similar sequence (Megablast) algoritm was employed and all other parameter, including the E-value threshold and Match/Mismatch Scores, were maintained at the default settings. Sequence alignment across the local barcodes was also performed to assess variable regions. Sequence regions that exhibit considerable variations were identified as potential primer sites for eDNA markers.

Table 1: Primers used to obtain barcode sequences of hornbills.

General Bird Primer	Target Gene	Amplicon length (bp)	Ref
COIBuceF COIBuceR	COI	746	(Jarulis et al., 2018)
BirdF1 COIBirdR2	COI	746	(Kerr et al., 2009)
L5216 H6313	ND2	1041	(Sorenson et al., 1999)
DL700F DL700R	d-loop	700	(Jarulis et al., 2018)
DLBuceF DLBuceR	d-loop	788	-
L149963 H156463	cyt b	633	(Sorenson et al., 1999)

RESULTS

Assessment of hornbill reference sequence availability in public database

Efforts to retrieve reference gene sequences for target and non-target, closely related hornbill species indicated the limited availability of hornbill mtDNA sequences in nucleotide database. Assessment of the hornbill DNA sequences in NCBI GenBank database revealed several well-represented mtDNA gene regions, including COI, cyt b, d-loop, and ND2 (Table 2).

Generation of local barcode sequences

The COI and ND2 barcodes were successfully amplified using established universal primers from feather gDNA of four hornbill individuals. Two sets of universal bird primer were used to amplify d-loop region, but only one set showed successful amplification, except A. malayanus. Besides, the universal bird primer targeting cyt b region showed non-amplification. The

amplified PCR products were subjected to sequencing and the barcodes were analyzed using BLASTn against the reference sequence in Genbank database.

Table 2: Hornbills' reference gene sequence available in the NCBI Genbank database.

Hornbill	mtDNA gene region			
	COI	cyt b	d-loop	ND2
Antracoceros albirostris	O	O	O	O
Anthracoceros malayanus	O	O	O	X
Anorrhinus galeritus	X	O	O	X
Berenicornis comatus	X	O	O	X
Buceros rhinoceros	O	O	O	O
Buceros bicornis	O	O	O	O
Rhinoplax vigil	X	O	O	X
Rhabdotorrhinus corrugatus	O	O	O	O
Rhyticeros undulatus	O	O	O	O
Rhyticeros subruficollis	X	O	O	X

O – Available; X – Not available

Sequence alignment and comparison

The BLASTn results was summarized in Table 3, including the top species matches based on the highest query cover and percent identity achieved between local barcodes and the reference sequence in NCBI nucleotide database. The top match of COI barcodes from two A. albirostris individuals consistently corresponded to Anthracoceros coronatus reference sequence. The COI barcode of A. malayanus has the top hit to the intended reference sequence. As there is no reference COI sequence for A. galeritus in the Genbank database, mismatches occurred with the closest available match was found to be A. coronatus and Panelopides panini. Despite the top match, the sequence similarity was relatively low, which was found to be less than 90% match identity from BLASTn analysis. Overall, COI sequence similarity across hornbill species was found to be high, especially within similar genus hornbill species (Anthracoceros). This suggests the highly conserved nature of mitochondrial COI gene.

The ND2 and d-loop barcodes of A. albirostris has the top hit to the intended reference sequence. However, the barcodes also exhibited a high degree of sequence similarity to A. coronatus. The ND2 barcode of A. malayanus and A. galeritus showed sequence mismatches due to the absence of reference gene sequences in the existing nucleotide databases (Table 2).

Sequence alignment across the barcodes generated showed high sequence similarity of d-loop gene across the hornbill species (Table 4). The COI and ND2 barcodes exhibited lower sequence similarity across the different hornbill species compared to d-loop gene.

Table 4: Alignment of barcode sequences by BLASTn (Query Cover/Percent Identity).

		Bird A	Bird B	Bird C	Bird D
		d-loop			
Bird A	COI	-	97/99.12	N/Aa	97/99.12
Bird B	COI	95/99.14	-	N/Aa	98/100.00
Bird C	COI	76/86.56	70/90.55	-	N/Aa
Bird D	COI	84/88.71	87/89.22	NSSb	-
Bird A	ND2	-	-	-	-
Bird B	ND2	71/95.39	-	-	-
Bird C	ND2	61/85.92	71/86.18	-	-
Bird D	ND2	NSSb	83/86.11	97/84.78	-

[a] No amplification

[b] No significant similarity

DISCUSSION

The BLASTn analysis consistently resulted in top match sequence with a single reference sequence (NC_038152.1) available in the NCBI database. All the barcodes exhibited high degree of sequence similarity to a reference sequence from A. coronatus. This finding is inconclusive due to the severely limited dataset within the GenBank repository. The lack of COI reference gene sequences for hornbill was identified, despite that the COI gene has been the standard for DNA barcoding with extensive COI reference sequences in public nucleotide databases (Antil et al., 2023).

The BLASTn results reported high COI

Table 3: BLASTn analysis of barcode sequences.

Species	Gene	BLASTn Result			
		Species Match	Query %	Identity %	Accession
Anthracoceros albirostris (Bird A)	COI	*Anthracoceros coronatus* *Anthracoceros albirostris*	98 98	98.43 90.54	NC_038152.1 MT941468.1
	ND2	*Anthracoceros albirostris* *Anthracoceros coronatus*	72 72	95.09 95.09	KJ455322.1 NC_038152.1
	d-loop	*Anthracoceros coronatus* *Anthracoceros albirostris* *Anthracoceros convexus*	98 98 99	98.24 98.09 97.51	NC_038152.1 KM503107.1 KP123627.1
Anthracoceros albirostris (Bird B)	COI	*Anthracoceros coronatus* *Anthracoceros albirostris*	98 98	98.87 90.00	NC_038152.1 MT941468.1
	ND2	*Anthracoceros albirostris* *Anthracoceros coronatus*	97 93	97.88 98.75	KJ455322.1 NC_038152.1
	d-loop	*Anthracoceros coronatus* *Anthracoceros albirostris* *Anthracoceros convexus*	98 98 98	98.23 97.95 97.80	NC_038152.1 KM503107.1 KP123627.1
Anthracoceros malayanus (Bird C)	COI	*Anthracoceros malayanus* *Anthracoceros coronatus*	81 78	91.01 90.76	MT941460.1 NC_038152.1
	ND2	*Anthracoceros coronatus* *Anthracoceros albirostris*	98 97	86.32 86.31	NC_038152.1 KJ455322.1
Anorrhinus galeritus (Bird D)	COI	*Anthracoceros coronatus* *Panelopides panini*	98 98	89.57 89.42	NC_038152.1 NC_015087.1
	ND2	*Anthracoceros coronatus* *Anthracoceros albirostris* *Panelopides panini*	95 94 95	85.97 85.88 85.07	NC_038152.1 KJ455322.1 NC_015087.1
	d-loop	*Anthracoceros albirostris* *Anthracoceros coronatus* *Anthracoceros convexus*	98 98 98	98.23 97.95 97.80	KM503107.1 NC_038152.1 KP123627.1

sequence similarity between A. albirostris and A. coronatus with the percent identity ≥95%. Alignment between barcodes of A. albirostris and A. malayanus reported high degree of sequence similarity with percent identity 86% - 91%. This indicates that the hornbill COI gene lacks the genetic resolution needed to distinguish between closely related species within the same genus. Particularly, the COI gene is one of the widely used mitochondrial gene markers in eDNA metabarcoding studies (Wang et al., 2023). It has conserved priming regions that allow binding to a vast range of species for broad community characterization (Andújar et al., 2018). Meanwhile, the conserved regions within the COI gene that flank a variable core provide researchers with species-level resolution, enabling them to differentiate individual species (Andújar et al., 2018; Othman et al., 2021). The COI gene could be an effective marker for genus-level identification, but it might not be a suitable marker for species-level identification.

The ND2 marker is commonly used in phylogenetic studies, particularly for vertebrates (Dimcheff et al., 2002). The ND2 gene exhibits a greater degree of intraspecific variation than the COI gene, rendering the ND2 gene effective in resolving fine-scale genetic differences (Dimcheff et al., 2002). The BLASTn analysis of ND2 gene revealed that the reference sequence of A. coronatus exhibited a high degree of sequence identity (95-98%) to A. albirostris, but a much lower sequence identity (86%) to A. malayanus. Alignment of barcodes between two species of the same genus (A. albirostris and A. malayanus) revealed considerable variation within the ND2 marker. Furthermore, sequence alignment of barcodes revealed considerable genetic variation in ND2 region, which is a key finding for species-level

identification. This demonstrates that the ND2 marker is capable of resolving species within genus, but with varying degrees of success. It is important to note that the ND2 reference gene sequence for hornbill is less comprehensive (Table 2), where the available reference sequence could not representative of the full genetic diversity of the species. Despite the unrepresentative reference sequence, our findings suggest the potential of ND2 marker for species-level identification. A broader sequencing effort is necessary to validate its effectiveness.

The d-loop gene is known to be a hypervariable region (Nikbakhsh et al., 2023). While universal primers targeting d-loop are designed to target conserved flanking regions, the specific hypervariable regions within the d-loop can make universal amplification across taxa challenging. This may explain the failure to amplify A. malayanus sequence using universal d-loop bird primers, despite the success amplification with other individuals. The significant sequence divergence within d-loop region can indeed be useful for differentiation between closely related species that might be morphologically similar (Jarulis et al., 2020; Prakas et al., 2025). However, the BLASTn analysis of d-loop barcodes showed an unexpectedly high degree of sequence similarity across different species (Table 4). Furthermore, the d-loop barcode for A. galeritus warrant careful interpretation due to the top BLAST match of the barcode to reference sequence of A. albirostris. This is likely due to contamination based on the barcode sequence alignment that showed≥97% sequence identity (Table 4).

The local barcodes from four hornbill individuals and the available hornbill reference sequences in the NCBI database, albeit limited, were used to identify suitable gene regions. Our data suggested ND2 as the suitable molecular marker for hornbill identification at the species level. The incomprehensive hornbill sequence data in public nucleotide database is the key challenge in identifying suitable gene marker, which can impede the subsequent design of specific primers for targeted eDNA assay. While the small sample size provided valuable initial data, the limited hornbill nucleotide data restricts the ability to generalize sequence similarity across the full genetic diversity. For example, the high ND2 sequence similarity between A. albirostris and A. coronatus was based on a limited number of individuals. Incorporating DNA barcodes from a larger sample population would be helpful to verify genetic similarity and capture potential intraspecific variation (Bergsten et al., 2012). Future research should incorporate DNA barcodes from a more diverse array of individuals, with geographic representation for all species. Representative gene sequences can facilitate a more robust analysis of genetic diversity and the development of reliable species-specific eDNA assays.

Table 5: Comparison of the four mtDNA markers for hornbill detection

mtDNA gene marker	Amplification success	Sequence identity	Taxonomic resolution	Reference sequence availability
COI	Yes	High similarity	Low, suitable for	Moderate
ND2	Yes	Similar, exhibits sequence variation for species-level identification	Moderate to High	Low
cyt b	No	-	-	High
d-loop	Yes	High similarity	Low	High

CONCLUSION

This study performed the initial investigation of molecular markers for targeted hornbill eDNA assay. The mitochondrial ND2 showed promise for species-level identification, although more comprehensive sequencing is needed for full validation. The limited reference genetic data for hornbill species is the primary challenges for gene marker selection. The limited number

of available reference sequences hindered the BLASTn analysis for sequence comparison and distinguishing between species-level differences. Future work is needed to address the lack of comprehensive and high-quality reference genetic data for hornbill species. This would include sequencing multiple mitochondrial genes and individuals from a wider geographic range to capture intraspecific variation. This study contributes novel gene data from several hornbill species in the Sarawak region. The research provides a proof-of-concept for the identification of a potential species-specific marker for targeted hornbill detection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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