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Molecular identification of the main raptor bird species from the Arequipa-Lima region, Peru, using sanger sequencing

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ABSTRACT. A total of 14 feather samples were collected from the following birds of prey: *Buteo polyosoma, Geranoaetus melanoleucus, Bubo magellanicus, Athene cunicularia, Falco sparverius, Vultur gryphus, Spizaetus ornatus, Falco rufigularis, Morphnus guianensis, Oroaetus isidori, Spizaetus melanoleucus, Spizaetus tyrannus, Harpyhaliaetus solitarius,* and *Accipiter bicolor*. The taxonomic identity of 12 birds of prey species from two regions was inferred, with similarity percentages ranging from 99-100% compared to the database sequences. While the amplification of D-Loop/12S was unsuccessful due to the specific primers for *Vultur gryphus*, the COI and NAD2 regions showed high efficacy in the molecular identification of the analyzed samples. The analysis of the COI and NAD2 sequences revealed a marked intraspecific genetic divergence, with divergence percentages of up to 6-8% in some Neotropical birds of prey from Arequipa compared to the reference sequences. This finding suggests more complex patterns of variability and differences in regional sequence homology in these species than in North American birds. DNA barcodes based on mitochondrial sequences, particularly COI and NAD2, have proven to be accurate and non-invasive tools for the taxonomic identification of these birds of prey using feather samples as a source of genetic material.

Keywords: genome; amplification; mitochondrial DNA; COI; NAD2.

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Introduction

Taxonomic identification of species using traditional morphological methods can be challenging for immature individuals or cryptic specimens that lack distinctive phenotypic characteristics (Hebert et al., 2004; Aliabadian et al., 2009). The DNA barcoding technique, based on the analysis of standardized gene sequences such as cytochrome c oxidase subunit I (COI), has emerged as an effective tool to overcome these limitations (Borisenko et al., 2008; Hebert et al., 2003; Hebert & Gregory, 2005).

DNA barcodes allow rapid and accurate identification of species, regardless of their ontogenetic stage or cryptic morphology. This approach is particularly useful for biodiversity surveys, forensic studies of threatened species, and conservation programs (Hajibabaei et al., 2007; Kerr et al., 2007; Clare et al., 2007). This methodology is based on the amplification by PCR (Polymerase Chain Reaction) and sequencing of standardized gene regions, followed by sequence homology analysis in databases (NCBI).

The COI gene is one of the most widely used mitochondrial markers for DNA barcoding in animals, because of its ability to discriminate between closely related species (Hebert et al., 2003; Tavares & Baker, 2008). Conversely, other regions, such as ND2, allow for more robust analysis of intraspecific genetic variability (Lohman et al., 2009). Sanger sequencing has been demonstrated to be a reliable technique for obtaining DNA barcode sequences (Jinbo et al., 2011).

In megadiverse countries such as Peru, where knowledge of their genetic diversity and significant evolutionary units remains limited due to geographical barriers (Pacheco et al., 2009; Särkinen et al 2012), the use of molecular techniques, such as DNA barcoding, is of paramount importance for the accurate identification of threatened species and combating problems such as illegal wildlife trade (Kirkpatrick & Emerton, 2010).

Feathers represent a non-invasive source of DNA for genetic studies in birds (Horváth et al., 2005), providing a viable alternative to more invasive and stressful methods (Taberlet & Luikart, 1999; Beja-Pereira et al., 2009; Horváth et al., 2005), particularly useful for threatened or rare species.

In the present study, Sanger sequencing of the COI and ND2 mitochondrial regions was used as a DNA barcode for molecular identification of the main species of birds of prey from the Arequipa region, Peru, using feather samples as a source of genetic material.

Material and methods

In this study, molecular identification of the main species of birds of prey from the Arequipa-Lima region, Peru, was carried out by sequencing the mitochondrial DNA regions using the Sanger method.

Sample collection

The biological material consisted of feathers that were randomly collected from birds of prey inhabiting the "Zoo Mundo" Zoo, Paucarpata district, Arequipa (16°25'12.7" S 71°28'31.6" W; Figure 1), and from the "El Huayco" breeding facility in Huachipa, Lima (12°00'30.0" S 76°55'23.9 "W; Figure 2). A total of 25 feathers were processed, 19 of which were samples of interest, while the remaining 6 were used as DNA extraction controls. The feathers were obtained from the abdomen of the specimens or collected from the floor of their cages and stored in ziplock bags for transportation to the laboratory.

Figure 1. Map of the location of the Arequipa Zoo (Zoo Mundo) 16°25'12.7" S 71°28'31.6" W (Source: Google Maps).

Figure 2. Map of the location of the Huachipa-Lima breeding facility (El Huayco) 12°00'30.0" S 76°55'23.9" W (Source: Google Maps).

DNA extraction

The basal calami of the feathers, including the internal membrane containing nucleated cells, were cut (Figure 3). The samples were then digested with proteinase K at 56°C in TLB overnight. Subsequently, DNA was extracted using the High Pure PCR Template Preparation Kit (Roche) following the manufacturer's instructions. The DNA concentration was subsequently measured using spectrophotometry (NanoDrop, Thermo Fisher, USA).

Figure 3. Extraction of DNA from feather calamus. Source: Own

PCR amplification of the D-Loop/12S region

The mitochondrial control region and 12S ribosomal subunit were amplified using the AmpliTaq Gold 360 PCR Master Mix and 0.8 µM of the L798 and H1455 primers (see Table 1) in a final volume of 25 µL. The thermal cycling conditions were as follows: 94°C for 2 min., followed by 40 cycles of 94°C for 1 min., 51°C for 1 min, 72°C for 1 min., and a final extension at 72°C for 5 min.

Table 1. Species of birds of prey living at the "Zoo Mundo" Zoo in Arequipa, Peru, donors of feather samples for molecular analysis in the present study.

Sample	COI Gene	% Identity (COI)	NAD2 Gene	% Identity (NAD2)	% Identity L5143 chain	% Identity H6313 chain
M01	*B. polyosoma*	100	*B. polyosoma*	99.8	-	-
M02	*G. melanoleucus*	100	*G. melanoleucus*	-	100	99
M03	*B. magellanicus*	99.5	*B. scandiacus*	-	91	97
M04	*G. melanoleucus*	99.6	*G. melanoleucus*	-	100	99
M05	*B. polyosoma*	100	*B. p. polysoma*	99.9	-	-
M06	*B. polyosoma*	100	*B. p. polysoma*	99.9	-	-
M07	*A. cunicularia*	99.8	*A. cunicularia*	-	95	98
M08	*F. sparverius*	98.8	*F. sparverius*	95.4	95	-
M09	*V. gryphus*	100	*V. gryphus*	100	100	-
M10	*S. ornatus*	98.7	*S. ornatus*	-	98	99

COI gene

The mitochondrial COI gene was amplified using the same PCR mix and 0.8 µM of the Bird F1 and Bird R1 primers (Table 1) in a volume of 25 µL. The conditions were: 94°C for 2 min., 5 cycles of 94°C for 1 min., 45°C for 1.5 min., 72°C for 1.5 min., then 30 cycles of 94°C for 1 min., 51°C for 1.5 min., 72°C for 1.5 min., and 72°C for 5 min.

NAD2 gene

The same PCR mix was used with 0.8 µM of L5143 and H6313 primers (Table 1) in 25 µL. The thermal cycling program was: 94°C for 2 min., 40 cycles of 94°C for 1 min., 53°C for 1 min., 72°C for 1 min., and 72°C for 5 min.

The amplification of the NAD2 and COI genes resulted in products of the expected size, as shown in Figures 4, 5, and 6. However, in the case of the NAD2 gene, a non-specific band of smaller size (~400bp; Figure 7, lanes 1 and 6) was observed.

Electrophoresis

PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. A 100 bp marker was used to estimate the size of the obtained bands.

Sequencing

A volume of 50 µL of each PCR product was sent to Psomagen Inc. (Rockville, MD, USA) for purification and bidirectional sequencing using the same primers used for amplification.

Bioinformatic analysis

Consensus sequences were assembled from forward and reverse primer readings using Geneious Prime software (USA). The identification of species was performed by comparing sequence homology levels using BLAST analysis.

Results

Nineteen feathers of birds of prey were obtained for DNA extraction and the mitochondrial COI and ND2 regions were successfully amplified and sequenced. The analysis of these sequences enabled the identification of 14 different raptor species in the Arequipa and Lima regions.

DNA extraction

The concentrations of the extracted DNA ranged from 2 to 185 ng μL^{-1} (Table 1).

PCR amplification

D-loop:

No amplification of the expected ~600 bp fragment corresponding to this region was obtained (Figure. 2), probably because of the specificity of the primers for *Vultur gryphus*. As illustrated in Figure 2, an amplification product of 600 bp was expected (lane 9), yet it was not detected in most samples or a non-specific product of larger size was observed (lanes 10; 12-16) (Figure 4).

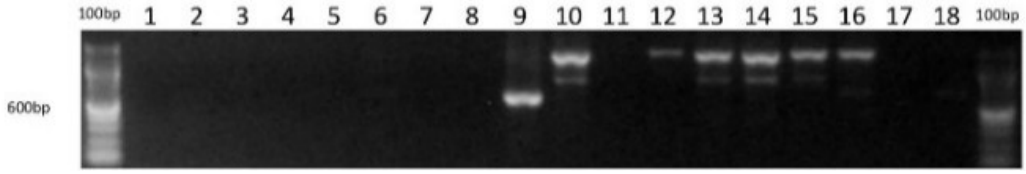

Figure 4. Amplification of the D Loop–12S region. Lanes 1–18: Amplification products of the mitochondrial D Loop–12S region, samples 1–18. An amplification product of approximately 600 bp was expected as observed in lane 9 (DNA sample extracted from *Vultur gryphus* feather) (Source: Own).

NAD2**:** 85% of the samples (16/19) showed a main band of ~1100 bp and a non-specific band of ~400 bp (Figures 5 and 6).

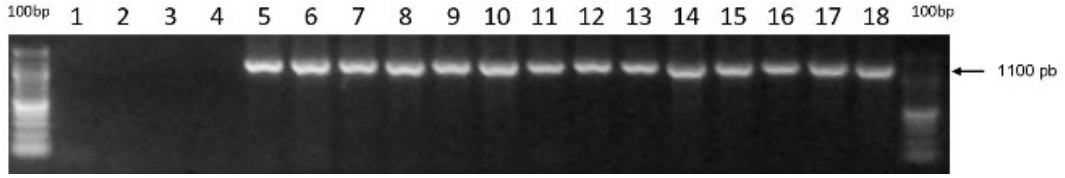

Figure 5. Amplification of the D Loop - 12S and NAD2 regions. Lanes 1–4: Amplification products of the mitochondrial D Loop–12S region, samples 19–22. Lanes 5–18: Amplification products of the mitochondrial NADH Dehydrogenase Subunit (NAD2) gene region (samples 1–14) (Source: Own).

COI: The expected product of ~700 bp was successfully amplified in 95% of the samples (18/19) (Figures 6 and 7).

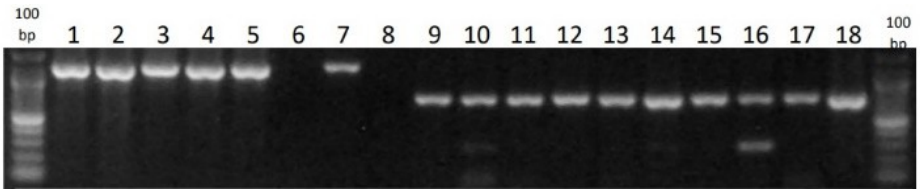

Figure 6. Amplification of the NAD2 and COI gene regions. Lanes 1–7: Amplification products of the mitochondrial NADH Dehydrogenase Subunit (NAD2) gene region, samples 15–22 (expected product 110 bp). Lanes 9–18: Amplification products of the mitochondrial Cytochrome Oxidase Subunit I (COI) gene region, samples 1–9 (expected product 700 bp) (Source: Own).

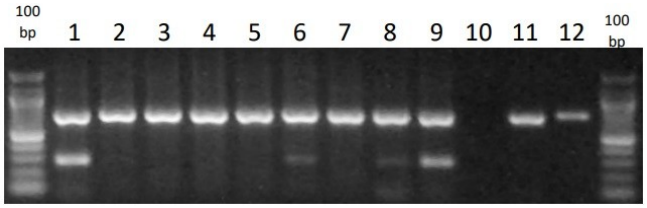

Figure 7. Amplification of the COI gene region. Lanes 1–12: Amplification products of the mitochondrial Cytochrome Oxidase Subunit I (COI) gene region, samples 10–22 (expected product 700 bp) (Source: Own).

Sequencing and BLAST analysis

PCR products from the 19 samples were sequenced. The consensus sequences were assembled from the forward and reverse reads using Geneious Prime, with the exception of ND2, where the short reverse fragments did not allow proper assembly and were analyzed separately.

BLAST analysis of both the forward and reverse sequences yielded matches with the same assigned species in all cases (Table 2). However, some discrepancies are observed:

Table 2. Concentration of nucleic acids obtained from bird feather samples.

Sample	Concentration (ng μL^{-1})	Sample	Concentration (ng μL^{-1})
M01	11.6	M11	15.7
M02	21.7	M12	2.2
M03	11.9	M13	22.9
M04	9.1	M14	113.0
M05	13.6	M15	25.7
M06	11.0	M16	79.1
M07	11.5	M17	185.0
M08	6.7	M18	4.3
M09	7.5	M19	11.4
M10	57.7	-	-

i) For *Morphnus guianensis* (AV-12), There was no COI sequence in GenBank, reflecting 90% identity compared to almost 100% identity for ND2;

ii) No COI sequences were identified for *Oroaetus isidori* (AV-13) and *Harpyhaliaetus solitarius* (AV-16);

iii) No ND2 entry was found for *Bubo magellanicus* (AV-03) in the database (Figure 8).

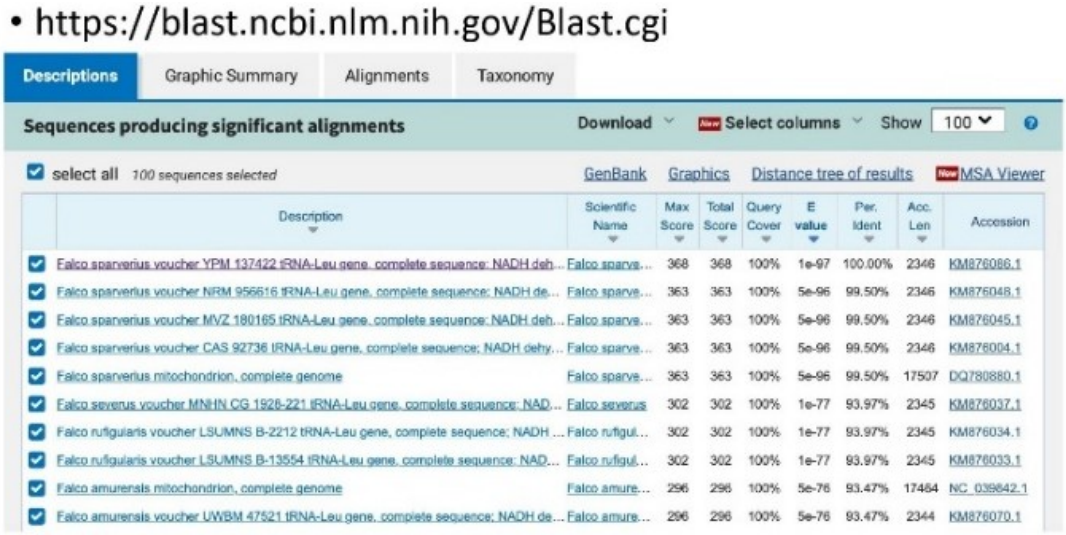

Figure 8. Comparison of the obtained COI gene sequences with those in the NCBI database (Source: Own work).

Species identification

The COI sequences enabled the unambiguous identification of 14 raptor species, exhibiting identity percentages of 99-100% compared to the reference sequences (Table 1).

Discussion

High-quality DNA was successfully extracted from feathers of the following birds of prey: (*Buteo polyosoma, Geranoaetus melanoleucus, Bubo magellanicus, Athene cunicularia, Falco sparverius, Vultur gryphus, Spizaetus ornatus, Falco rufigularis, Morphnus guianensis, Oroaetus isidori, Spizaetus melanoleucus, Spizaetus tyrannus, Harpyhaliaetus solitarius,* and *Accipiter bicolor*), specifically from the basal calamus, achieving concentrations ranging from 2 to 185 ng µL-1 (Table 2).

The use of feathers as a DNA source represents a non-invasive and economically viable alternative for genetic studies in birds, avoiding risky blood sampling methods (Dai et al., 2015; Horváth et al., 2005; Rudnick et al., 2008). Several authors have reported the successful use of feather DNA for genetic analysis (Rudnick et al., 2007; Griffiths & Tiwari, 1995; Segelbacher et al., 2003; Bayard De Volo, 2008; Seki, 2006).

However, environmental factors such as humidity can affect the integrity of DNA in feathers by causing physical damage that allows the entry of degrading microorganisms (Horváth et al., 2005). This may have influenced the lack of amplification of the D-Loop region in most of the samples (Horváth et al., 2005). Conversely, the mitochondrial markers COI and ND2 exhibited high amplification efficiency (95% and 85%, respectively), enabling the identification of 12 species of birds of prey from Arequipa with 99-100% accuracy compared to reference sequences. (Kerr et al., 2007; Chaves et al., 2015; Aliabadian et al., 2009).

These findings are consistent with those of previous studies on South American birds, which demonstrated high accuracy (93-98%) of the COI gene for species-level identification (Kerr et al., 2007; Chaves et al., 2015; Milá et al., 2012). However, a marked intraspecific divergence of up to 6-8% was observed in some of the obtained COI and ND2 sequences compared to the reference sequences. This divergence suggests intricate patterns of regional genetic variability in these Neotropical birds of prey, in contrast to the lower variability documented in North American birds (Tavares et al., 2011; Milá et al., 2012; Barrowclough et al., 1999). This substantial divergence could be attributed to biogeographic factors, such as geographic and ecological barriers, that promote the divergence and isolation of populations in the Neotropical region (Kerr et al., 2007; Milá et al., 2012).

The complex patterns of genetic variability observed in these Neotropical birds of prey highlight the need to re-evaluate current taxonomic classifications, which may obscure cryptic diversity at the species level (Martín-Gálvez et al., 2018; Moritz, 1994; Arguello & García, 2014). Future investigations incorporating a greater number of samples from multiple localities as well as the use of next-generation genomic markers would allow for more precise delineation of subspecific evolutionary units or cryptic species in these taxa (Moritz, 1994). A comprehensive understanding of these evolutionary processes is paramount for effective conservation strategies, as the underestimation of diversity may result in the unintended loss of unique lineages. Therefore, it is imperative to deepen these studies to ensure effective management and robust conservation strategies to preserve the exceptional evolutionary legacy of Neotropical birds of prey (Frankham et al., 2004; Griffiths & Tiwari, 1995).

Conclusion

Nucleic acid concentrations were successfully extracted from the feather samples of the studied birds of prey, thereby facilitating the extraction of mitochondrial DNA. According to the variability provided by the data analysis results from the sequencing of birds of prey specimens, identity ranged from 395/400 (99%) to 641/641 (100%). To ensure the validity of the molecular identification of the bird of prey specimens, their respective similarity percentages were confirmed by BLAST analysis for comparison with the database. This study demonstrated the usefulness of DNA extracted from feathers for molecular identification of regional birds of prey species. Moreover, it revealed an underestimation of genetic diversity and complex evolutionary patterns in these Neotropical species, challenging the current taxonomic classifications. These findings lay the foundation for future investigations that employ comprehensive genomic approaches to elucidate the cryptic evolutionary richness of these important top predators. A deeper understanding of their diversity is crucial for developing effective conservation strategies that preserve the unique evolutionary legacy of birds of prey in this megadiverse region.

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