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Research paper

Molecular tools for identifying the geographic origin and sex of military macaws (*Ara militaris*) from wildlife rescue centers

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Abstract

Molecular tools for identifying the geographic origin and sex of military macaws (Ara militaris*) from wildlife rescue centers*. The green macaw (*Ara militaris*), a Neotropical species threatened by illegal trade and habitat loss, is often held in rescue centers where its geographic origin and sex are frequently unknown. In this study, molecular tools (COI gene and sex markers) were used on 70 individuals from five centers in Mexico. Phylogenetic and haplotype analyses identified three main lineages and five regionally structured haplotypes, consistent with previously defined evolutionary units. Sex was determined in 75.7% of individuals, with greater success using blood samples. These results emphasize the importance of identifying origin and sex to prevent hybridization, improve captive breeding efforts, and support genetically informed conservation and reintroduction strategies.

Key words: Illegal trade, Psittacidae, Conservation *ex situ*, Wildlife management units, COI

Introduction

The Psittacidae, which includes parrots, parakeets, and macaws, is one of the most vulnerable bird groups in their natural habitats. About 31% of neotropical species within this family are listed as endangered (IUCN 2013). This trend is also evident in Mexico, where this bird group faces a high level of threat. Of the 21 species recorded there, 10 are classified as endangered according to NOM-059-2010 (SEMARNAT 2010). The main threats to psittacine populations include pressure from the illegal pet trade and, more importantly, rapid habitat loss caused by deforestation in temperate and tropical ecosystems (Escalante et al 1993, Cantú et al 2015, Berkunsky et al 2017). These conditions are especially concerning because they may limit gene flow, decrease genetic diversity, and promote inbreeding, which can threaten the long-term survival of populations (Fahrig 2003).

Among Neotropical Psittacidae, the military macaw (*Ara militaris*) faces a particularly urgent situation (Rivera-Ortíz et al 2016, Berkunsky et al 2017, BirdLife International 2020). It is among the five most traded species in this group (Cantú et al 2015). In Mexico, its geographic range is broad but fragmented into four main regions: North Pacific, Central Pacific, Upper Balsas Basin, and Gulf of Mexico (fig. 1) (Monterrubio-Rico et al 2016, Rivera-Ortíz et al 2017). This patchy distribution suggests the existence of distinct evolutionary units, possibly adapted to local ecological conditions. These differences likely resulted from historical isolation between populations, caused by both geographical barriers and the green macaw's responses to climatic changes during the Quaternary period (Howell and Webb 1995, Iñigo 2000, Rivera-Ortíz et al 2016, Navarro-Sigüenza et al 2018).

In this context, *ex situ* conservation programs such as wildlife rescue centers, zoos, and Wildlife Management

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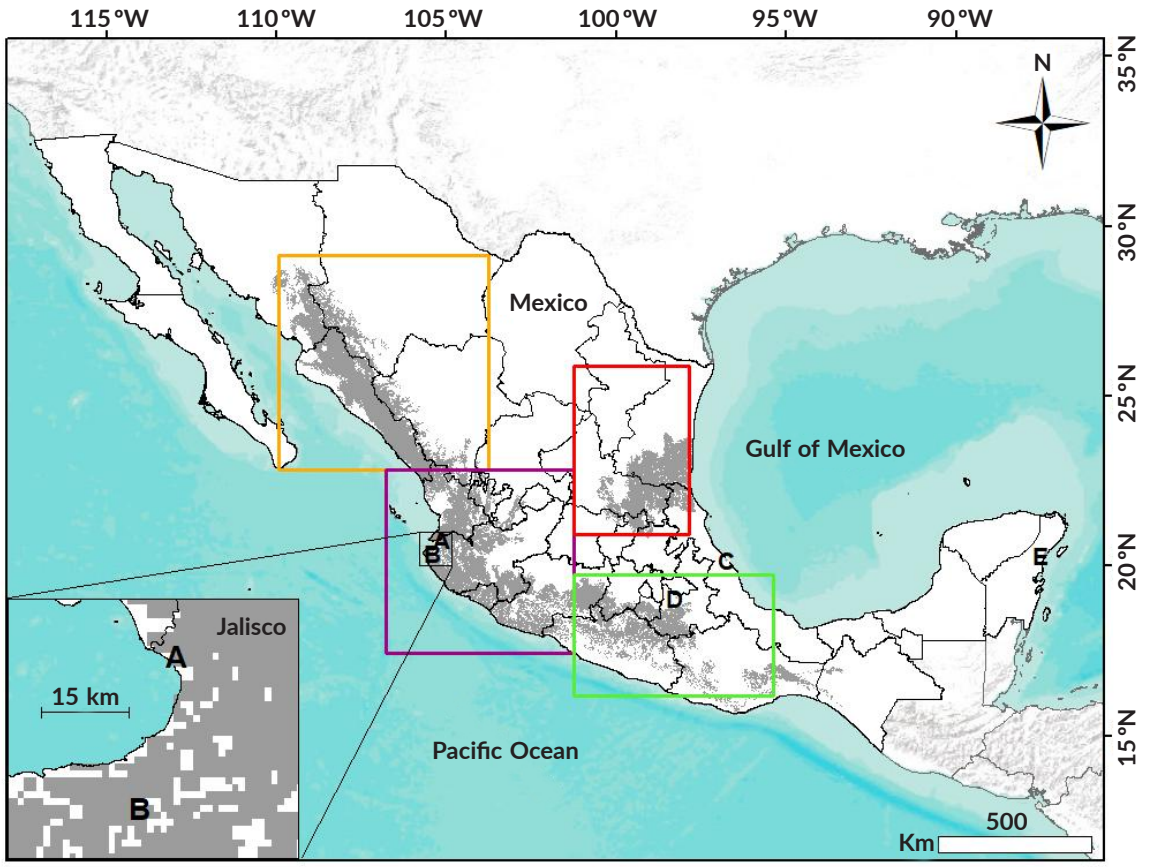


Fig. 1. Geographic distribution of *Ara militaris*. The colors represent the four regions where the military macaw is found in Mexico, as proposed by Monterrubio-Rico et al (2021). Yellow indicates the North Pacific, purple shows the Central Pacific, green represents the Upper Balsas Basin, and red depicts the Gulf of Mexico. Each letter corresponds to a rescue center: A, CEMBAB; B, Sanctuary; C, Istirncha; D, Amazon; and E, AMSRA.

Units (Unidades de Manejo Ambiental, UMAs) are crucial for protecting endangered species, including *A. militaris* (Witzenberger and Hochkirch 2011). UMAs are legally recognized institutions supported by SEMARNAT, the federal agency responsible for their regulation and oversight under the General Wildlife Law, published in the Official Journal of the Federation on December 30, 2010 (SEMARNAT 2010). These facilities not only protect individuals confiscated from illegal trafficking but also promote their rehabilitation, breeding, and eventual reintroduction into the wild (Frankham et al 2002).

However, several studies have shown that releasing individuals without considering their genetic background –whether due to escapes, institutional decisions, or accidental releases– can lead to harmful effects, such as genetic introgression, loss of local adaptations, and decreased population viability (Ralls et al 2017, Rivera-Ortíz et al 2023). This issue is further complicated because most individuals held in conservation centers have unknown origins and are often kept together, which increases the risk of hybridization and diminishes adaptive genetic diversity, ultimately impairing the success of reintroduction programs focused on species conservation (Lynch 1991, Pertoldi et al 2007, Frankham 2010).

The use of molecular tools has become a vital part of conservation efforts, enabling researchers to assess genetic diversity, identify the geographic origin of individuals, and avoid unwanted crosses between populations (Godoy 2009, Allendorf et al 2012). Mitochondrial DNA, especially, has proven effective in distinguishing populations and is highly valuable for assigning geographic origin in Neotropical bird species with fragmented ranges (Tavares et al 2011, Mendoza et al 2016). Of these markers, the cytochrome c oxidase subunit I (COI) gene is widely used for its ability to differentiate population haplotypes and aid decision-making in conservation research (Avise 2000, Hebert et al 2003a, 2003b, Rocha et al 2014). These tools have already been successfully used in other macaw species, including *Anodorhynchus hyacinthinus*, and *Ara ararauna*, helping to develop management strategies and reintroduction efforts (Fernandes and Caparroz 2012, Eberhard et al 2015, Presti et al 2015).

Despite these advances, few studies have thoroughly explored the population genetics of the military macaw (*A. militaris*). The research done so far has used mitochondrial markers like *cyt b*, ND2, and RC, revealing a clear phylogeographic structure and identifying at least two main populations in Mexico –one in the Sierra Madre

Occidental/Sierra Madre del Sur and another in the Sierra Madre Oriental. These studies also show high genetic diversity and signs of population bottlenecks followed by recent expansions (Eberhard et al 2015, Rivera-Ortíz et al 2017, 2023). In a more applied context, a recent study used eight microsatellites to determine the possible geographic origin of a captive *A. militaris* individual of unknown provenance, comparing it with previously genetically characterized specimens. They found that the captive bird could be assigned to the Sierra Madre Oriental genetic group with a probability of 71.2 %-82.4 % (Rivera-Ortíz et al 2021).

Furthermore, sex identification through molecular techniques is especially important for species like *A. militaris*, where there are no obvious sexual dimorphisms (Camacho et al 2009, Del Puerto et al 2017). Knowing the sex of individuals is vital for planning captive breeding programs because it supports evidence-based management in conservation centers and helps create balanced breeding pairs, which can improve reproductive success and reduce the risk of incompatible pairings (Pereira et al 2021, Savitri et al 2021).

Nevertheless, in Mexico, the use of molecular techniques in conservation centers remains limited. This study uses molecular tools to analyze genetic diversity, identify potential geographic origin, and determine the sex of *A. militaris* individuals from wildlife rescue centers. This information is essential for supporting genetically informed conservation and reintroduction efforts.

Materials and methods

Location of individuals for sample collection

The samples analyzed in this study were obtained from individuals under human care and housed in various facilities dedicated to wildlife conservation and management. Specifically, Akumal Monkey Sanctuary and Rescued Animals (AMSRA) and Istirincha, located in the states of Quintana Roo and Veracruz, respectively, harbor a variety of vertebrate species, including the military macaw (*A. militaris*). In contrast, the Conservación de las Especies Maravillosas de Bahía de Banderas (CEMBAM), the Santuario of the Guacamaya, and Amazonia - Fundación Alas Verdes, located in the states of Nayarit, Jalisco, and Puebla, focus exclusively on the rehabilitation and management of this species. Most of these organizations serve as reception centers for individuals seized by Mexican environmental authorities such as the Procuraduría Federal de Protección al Ambiente (PROFEPA) and have implemented management programs aimed at reintroducing individuals into their natural habitat.

Handling, collection, and storage of *A. militaris* samples

A total of 70 samples, including blood and feathers, were collected from *A. militaris* individuals. Of these, a subset (n = 7) with documented geographic origin was used as reference controls in the genetic analyses. To collect blood, the birds were carefully restrained, and the puncture was made at the basilic vein. Peripheral blood was collected using heparinized microcapillaries, following the recommendations of Albino-Miranda et al (2024). The volume taken was minimal, in accordance with the safety limits established by Campbell (2015), to prevent any physiological risks to the birds. In some cases, feathers were collected as a less invasive alternative. Two to three feathers were plucked from each bird from the area between the cloaca and the base of the tail, aiming to recover epithelial cells and a small clot of adhered blood (González and Arenas-Castro 2017). The feathers were trimmed at the lower part of the calamus and stored in 2 mL microtubes. All collected material was kept at -80 °C to preserve genetic integrity.

DNA Extraction from *A. militaris* Samples

Genomic DNA was extracted from blood samples using the DNeasy Blood and Tissue Kit (QIAGEN), following the manufacturer's protocol. For feather samples, the protocol proposed by Aljanabi and Martinez (1997) was optimized. Incubation times with 20 µL of proteinase K were extended to at least 24 hours to ensure efficient lysis and removal of protein excess (due to the high keratin content). Additionally, 20 µL of RNase was added to maximize the yield and purity of the extracted DNA. The concentration of the extracted genetic material was then measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, model One C).

Amplification of COI fragments was performed using Polymerase Chain Reaction (PCR). Our research team designed a set of primers: Fw_PP_COIML (5'-CGATGATTATTCTCAACCAACC-3') and Rv_PP_COIML (5'-GCTGTGATTAGGACGGATC-3'), based on the reference genome (NC_027839.1) available in NCBI. The targeted region corresponds to a highly variable nucleotide segment linked to geographic origin (Hebert et al 2004), approximately 565 base pairs in length. Primer design and *in silico* validation were carried out using SnapGene version 8.1 and PearlPrimer version 1.1.21.

The final PCR reaction volume was 50 µL, consisting of 25 µL of 5'BIO brand mastermix, 16 µL of nuclease-free H_2O, 1 µL of each primer, and 7 µL of DNA. The PCR protocol conditions included an initial denaturation at 95 °C for five minutes, followed by 38 cycles of denaturation at 95 °C for 30 seconds, annealing at 53 °C for 33 seconds, and extension at 72 °C for 34 seconds. A final elongation at 72 °C for five minutes was performed, then the reaction was held indefinitely at 4 °C.

PCR products were separated by electrophoresis on a 2% agarose gel stained with SYBR Green (Applied Biosystems). Electrophoresis was performed at 100 V for 40 minutes, and DNA fragments were visualized under UV light using a transilluminator. Subsequently, the target PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The purified products were then sent to Macrogen in South Korea for Sanger sequencing.

For sex determination, two primer pairs were used: P8/P2 (Griffiths et al 1998) and 2550F/2718R (Fridolfsson and Ellegren 1999), following the optimized conditions for each primer pair as described by Albino-Miranda et al (2024). Sex identification was based on banding patterns observed on 3% agarose gels. This method allows sex differentiation, as males exhibit a single band corresponding to the CHD1-Z chromosome, while females display two bands corresponding to the CHD1-W and CHD1-Z

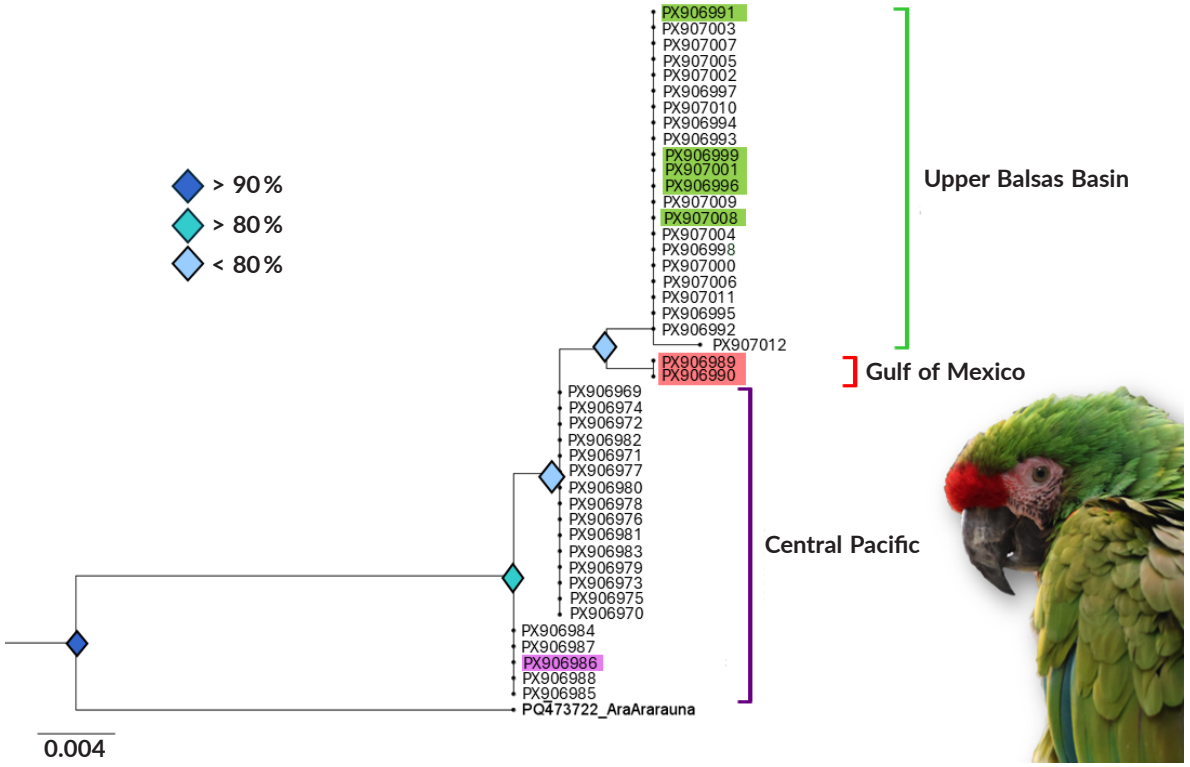


Fig. 2. Phylogenetic tree constructed using the Maximum Likelihood method from a fragment of the mitochondrial COI gene (410 bp) of *Ara militaris*. The diamonds indicate bootstrap values. The shaded letters represent sequences of known origin.

genes. Band lengths vary depending on the primer set: with P8/P2, bands of 420 and 380 bp are expected, and with 2550F/2718R, bands of 630 and 400 bp correspond to CHD1-W and CHD1-Z chromosomes, respectively (Griffiths et al 1998, Fridolfsson and Ellegren 1999).

The obtained COI sequences were edited and aligned using the MUSCLE algorithm and visually inspected with Geneious Prime (https://www.geneious.com/) and MEGA X (Kumar et al 2018). Additionally, to ensure the accuracy of our analyses, only sequences with a clearly documented origin were selected, and only those that showed adequate alignment with the amplified fragment were used. Three sequences available in the GenBank database (NCBI) were incorporated into this process: two from the Gulf of Mexico (NC_027839.1, KM611466. 1) and one from the Central Pacific (JX524613.1), as well as a sequence from *A. ararauna* (PQ473722.1), used as an outgroup in the phylogenetic analysis. In total, 45 different sequences were used for the bioinformatic analyses.

To reconstruct phylogenetic relationships among the analyzed individuals, a Maximum Likelihood (ML) phylogenetic tree was built using the K2P molecular evolution model and 1,000 ultrafast bootstrap replicates, performed on the IQ-TREE web server. Additionally, pairwise uncorrected genetic distances (*p*-distances) between clades were calculated using MEGA X (Kumar et al 2018).

To visualize genetic relationships, the sequences were grouped based on geographic origin, and a haplotype network was created using the TCS criterion, which applies statistical parsimony to reconstruct networks that reflect complex evolutionary relationships. This method allows for the identification of mutational steps and genetic connections among haplotypes with high accuracy, as described by Paradis (2018). The network was constructed using DnaSP version 6 (Rozas et al 2017) and PopArt version 1.7 (Leigh et al 2015). Additionally, haplotype diversity was measured for each rescue center.

Results

A total of 45 COI sequences, each 410 base pairs long and of optimal quality, were analyzed after assembly and trimming in Geneious Prime. Phylogenetic analysis identified three main clades (fig. 2): one representing individuals from the Upper Balsas Basin, another grouping those from the Gulf of Mexico, and a third corresponding to the Central Pacific population. The observed intra-population genetic distances were 0.005 % (table 1).

Additionally, five distinct haplotypes were identified from mitochondrial sequence analysis of *A. militaris* (fig. 3). The haplotype network revealed clear genetic structuring among the analyzed populations, with unique haplotypes linked to each geographic region. The most common haplotype in the Balsas Basin was Hap_4, while Hap_1 was predominant in the Central Pacific. The Gulf of Mexico was characterized by a unique haplotype (Hap_3).

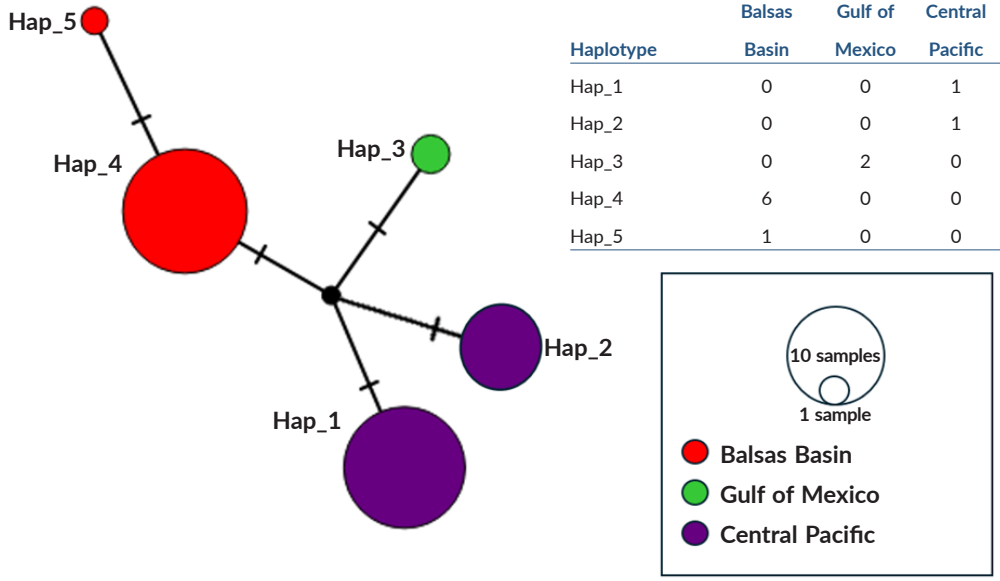
When examining the relationship between haplotypes and the origin of samples based on the rescue centers

Table 1. Genetic distances (p) between different populations of *Ara militaris* from Mexico.

	A. ararauna	Central Pacific	Gulf of Mexico	Upper Balsas Basin
A. ararauna				
Central Pacific	0.04			
Gulf of Mexico	0.05	0.005		
Upper Balsas Basin	0.05	0.005	0.004	

Table 2. Haplotype clustering in *Ara militaris* based on the time each haplotype was distributed in each conservation center.

	Hap 1	Hap 2	Hap 3	Hap 4	Hap 5
CEMBAB	X	X		X	X
Istirincha				X	
Amazonia	X			X	
AMSRA	X			X	
Sanctuary		X			



Haplotype	Balsas Basin	Gulf of Mexico	Central Pacific	Unknown
Hap_1	0	0	1	14
Hap_2	0	0	1	5
Hap_3	0	2	0	0
Hap_4	6	0	0	15
Hap_5	1	0	0	0

Fig. 3. Haplotype network created from a 410 bp segment of the mitochondrial COI gene from *Ara militaris* samples. Each circle represents a unique haplotype; the circle's size corresponds to the number of individuals who share it. The table indicates the number of known and unknown halpotypes used in thehaplotype network.

where they were collected, Hap_4 was found in individuals from CEMBAB, Amazonia, AMSRA, and Istirincha. In contrast, Haplotypes 2, 5, and, to a lesser extent, Hap_4, were mainly composed of individuals from CEMBAB. Notably, Hap_3 was exclusive to individuals classified as originating from the Gulf of Mexico. Of the five haplotypes observed, four were present in at least one rescue center. CEMBAB showed the highest diversity, with four haplotypes, followed by AMSRA and Amazonia, each with two. In contrast, Istirincha and the Guacamaya Sanctuary each had only a single haplotype (table 2).

On the other hand, the sex of 53 out of 70 individuals analyzed was determined (75.7%). Feather samples experienced greater amplification difficulties than blood samples. Conversely, all 10 blood-derived samples produced successful results. Among those whose sex was identified, most conservation centers had a balanced ratio of males and females, except for AMSRA, which had only males (table 3).

Discussion

Our results confirmed the existence of distinct intraspecific genetic lineages of *A. militaris* in Mexico. Furthermore, we observed that confiscated individuals housed in CEMBAB, AMSRA, and Amazonia originated from multiple genetic and geographic groups. In contrast, Istirincha and Santuario of the Guacamayas contained individuals from only one geographic lineage.

In the context of identifying the geographic origin of seized individuals, there was a clear link to three regions previously proposed as critical evolutionary units (Rivera-Ortiz et al 2016, Monterrubio-Rico et al 2016, Rivera-Ortiz et al 2021). Although the divergence was minimal, the mitochondrial patterns recovered (4% and 5%) exhibited sufficient structure to support regional origin identification. However, additional research using nuclear markers will be needed to confirm these as evolutionary units.

Table 3. Shows the summary of *Ara militaris* individuals analyzed at different conservation centers, including the type of sample obtained (blood/feather), the number of COI sequences generated, and the sex ratio.

Rescue center	Individuals	Sample type (Blood/Feather)	COI sequences obtained	Sex (♀/♂)	No sexing
CEMBAB	44	0/44	27	13/19	12
Amazonia	11	0/11	5	5/3	3
Sanctuary	5	0/4	1	1/2	2
AMSRA	5	5/0	4	5/0	-
Istirincha	5	5/0	4	3/2	-

Four out of five rescue centers housed individuals from the Upper Balsas Basin. Meanwhile, the clade representing the Central Pacific region mainly included individuals at CEMBAB and the Guacamaya Sanctuary, which makes sense given their geographic proximity. However, individuals from this same clade were also found in AMSRA and Amazonia. Notably, no rescue centers had individuals from the Gulf of Mexico clade.

This pattern shows that illegal wildlife trade does not occur randomly but is focused in specific areas. This trend is likely related to the nesting habits of birds in different regions. For example, Gulf of Mexico populations usually nest in crevices along cliff walls, canyons, or deep rock cavities, which may offer better protection (Rivera-Ortíz et al 2008, Rodríguez-Ruiz 2022). In contrast, populations from the Balsas Basin and Central Pacific typically nest in trees, making them more vulnerable to poaching (Bonilla et al 2007, Marín-Togo et al 2012, Rivera-Ortíz et al 2013, Monterrubio-Rico et al 2016). Supporting this idea of vulnerability, earlier studies on the illegal bird trade have shown that the states of Jalisco, Nayarit, Guerrero, and Puebla, areas with many tree-nesting populations, are active hotspots for Psittacidae removal and trafficking (Cantú et al 2015).

Additionally, sex identification using DNA extracted from feathers faced challenges due to the low quality and quantity of genetic material collected. Although feather-based sexing remains a useful non-invasive method often employed in bird studies (Fridolfsson and Ellegren 1999), our results indicate that blood samples are generally more reliable, especially when trained staff are available to collect them at facilities where birds are kept under human care (Franco-Gutiérrez et al 2017, Ramos et al 2021).

The results presented here offer valuable insights for developing responsible management strategies in conservation centers and reintroduction programs for *A. militaris*. Identifying possible regional genetic patterns within the species' distribution highlights the importance of considering the geographical origin of individuals when planning management actions, especially if there are differences related to local adaptations (Frankham et al 2010). In this context, incorporating provenance information serves as a useful resource for guiding management decisions and promoting future research to more accurately assess the level of population differentiation and the potential benefits or risks of hybridization in conservation efforts.

Furthermore, knowing the sex of individuals is vital for planning reproductive pairings and allows for customized nutritional and health management, especially in reproductively active females. When a facility only has one individual of the species, like AMSRA, it might be beneficial to consider exchange programs with other rescue centers to establish reproductive pairs (Del Puerto et al 2017, Pereira et al 2021, Savitri et al 2021, Albino-Miranda et al 2024). This method has been successfully implemented in other programs, such as the scarlet macaw (*Ara macao*) conservation project at Xcaret Park (Quintana Roo, Mexico), where careful selection of breeding pairs, sex determination, and parental rearing contributed to successful reintroductions (Figueras 2013).

Despite the relevance of our findings using a COI fragment, it is essential to acknowledge certain methodological limitations. One limitation is the geographic coverage of the reference samples, which did not include sequences from regions such as the Northern Pacific. Additionally, although not the primary objective of this study, future research could incorporate genomic markers such as single nucleotide polymorphisms (SNPs), which would increase genetic resolution and enable the detection of processes such as hybridization, gene flow, or direct kinship all of which are critical for reproductive program planning (Lee et al 2013, Mahler 2018, Olah et al 2021, Garcia-Raventós et al 2023). Similarly, the development of faster and more sensitive molecular protocols, such as qPCR or dPCR-based diagnostic panels, would be highly beneficial for future studies (Coghlan et al 2012, Mazzoleni et al 2021, Petrou et al 2024). Another problem identified was the difficulty in obtaining genetic material from birds using feathers. Although this sampling method is recommended to avoid causing stress during the handling of individuals (Harvey et al 2006), the DNA obtained is usually low in quantity and quality, which favors its rapid degradation (Cerit and Avanus 2007). In contrast, bird blood contains nucleated erythrocytes, so a small sample is sufficient to obtain large amounts of DNA (Bosnjak et al 2013, Del Puerto et al 2017).

This study highlights the importance of molecular tools in determining the geographic origin and sex of *A. militaris* individuals in rescue centers. Genetic evidence indicates the presence of intraspecific differentiation in Mexico and shows that some centers harbor individuals from various regions, underscoring the need for informed, evidence-based genetic management. While the match

between the detected haplotypes and previously proposed significant evolutionary units supports the value of mitochondrial analysis, additional data will be needed to understand the species' population structure better. Furthermore, non-random patterns in illegal trafficking were observed, possibly linked to differences in nesting habits, providing helpful information for prevention and surveillance efforts. Ultimately, increasing collaboration between rescue centers and scientific institutions could enhance reintroduction programs and support the conservation of the genetic diversity in wild macaw populations.

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N Serio-Covarrubias: experimental work, formal analysis, manuscript writing. **C Flores de los Ángeles**: experimental work, methodology. **S Albino-Miranda**: conceptualization, methodological supervision, experimental design, formal analysis, manuscript writing. **JC Serio-Silva**: conceptualization, supervision, review, and editing.

Data Availability Statement
The sequences generated in this study were deposited in GenBank (NCBI) under accession numbers PX906969–PX907012.

Conflicts of interest
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