Journal of Mammalogy, 2025, **00**, 000–000
https://doi.org/10.1093/jmammal/gyaf072
Research Article

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First comprehensive approach to the systematics of *Myotis barquezi* using morphology, molecules, and acoustics

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Associate Editor: Michael McGowen

Abstract

The genus *Myotis* comprises a diverse group of vespertilionid bats distributed almost worldwide. In Argentina, 15 species have been recorded, with *Myotis barquezi* one of the most recently described, and reported only from the Southern Andean Yungas ecoregion. We present the first dataset integrating morphological, molecular, and acoustic information for *M. barquezi*, aimed at improving species delimitation and clarifying its phylogenetic placement within the *ruber* species group. Additionally, we report a second geographic record for *M. barquezi*, which significantly extends its known range (ca. 930 km SE). Specimens of *Myotis* were collected in Corrientes province, and their characteristics are coincident with those described for *M. barquezi*. The acoustic parameters were consistent with those reported for the genus *Myotis* worldwide, characterized by wide broadband frequency-modulated (FM) pulses, with minimal constant frequency. Phylogenetic analyses based on the cytochrome *b* (*Cytb*) and cytochrome C oxidase I (*COI*) genes, employing both single-locus and multilocus approaches, identified a well-supported monophyletic clade distinct from other species within the *ruber* species group. These integrative findings provide critical insights into the systematics and distribution of *M. barquezi* and underscore the need for further research on this species.

Key words: Corrientes, echolocation, Iberá Wetlands, integrative taxonomy, mitochondrial DNA, morphology

Primer abordaje integral de la sistemática de *Myotis barquezi* utilizando morfología, moléculas y acústica

Resumen

El género *Myotis* comprende un grupo diverso de murciélagos vespertiliónidos con una distribución casi global. En Argentina, se han registrado 15 especies, siendo *Myotis barquezi* una de las más recientemente descritas, únicamente reportada para la ecorregión de las Yungas Andinas del Sur. En este estudio se presenta el primer conjunto de datos que integra información morfológica, molecular y acústica para *M. barquezi*, con el objetivo de mejorar la delimitación de la especie y aclarar su ubicación filogenética dentro del grupo *ruber*. Además, se agregar un nuevo registro geográfico para la especie, que amplía significativamente su área de distribución conocida (app. 930 km SE). Los especímenes de *Myotis*, colectados en la provincia de Corrientes, presenta características coincidentes con las que identifican a *M. barquezi*. Los parámetros acústicos fueron como los típicos del género *Myotis*, caracterizados por pulsos de banda ancha de frecuencia modulada (FM) con una frecuencia constante mínima. Además, los análisis filogenéticos basados en los genes Citocromo b (*CytB*) y citocromo C oxidasa I (*COI*), empleando enfoques de un solo locus y multilocus, identificaron un clado monofilético bien sustentado y distinto de otras especies dentro del grupo *ruber*. Estos resultados integradores aportan una visión crítica de la sistemática y distribución de *M. barquezi* y reflejan la importancia de continuar realizando investigaciones sobre ella.

Palabras clave: ADN mitocondrial, Corrientes, ecolocalización, Esteros del Iberá, morfología, taxonomía integrativa

The genus *Myotis* Kaup, 1829 is a diverse group of vespertilionid bats with a nearly worldwide distribution, absent only from polar regions and some oceanic islands (Simmons 2005). This extensive range reflects their exceptional adaptability to diverse ecosystems, from tropical forests to arid and mountainous regions (Simmons 2005; Novaes et al. 2022a). *Myotis* is the most speciose genus of bats and

Received: April 01, 2025; Accepted: September 15, 2025

the second largest genus of mammals, with 140 extant species (Díaz et al. 2025; MDD 2025; Simmons and Cirranello 2025). This remarkable species diversity, however, contrasts with the reduced phenotypic variation and conserved morphology observed in the genus, which often fails to reflect the true extent of species-level diversity and complicates taxonomic analyses and species delimitation (Gunnell et al. 2017; Moratelli et al. 2019).

Consequently, delimiting species within the genus *Myotis* based solely on morphology represents significant challenges because many species exhibit high levels of morphological conservatism and overlap in key diagnostic traits, often leading to underestimation of species diversity and increased likelihood of cryptic species remaining undetected. To address this issue, it is strongly recommended that researchers integrate morphological, molecular, and acoustic techniques across the entire distribution of the genus to improve systematic classifications (Stadelmann et al. 2007; Moratelli et al. 2013, 2019; Novaes et al. 2021, 2022b, 2022c; Carrión Bonilla et al. 2024).

Currently, 47 species of *Myotis* are recognized in the Neotropics (Díaz et al. 2021, 2025; Novaes et al. 2022a, 2022b, 2022c, 2025; Carrión Bonilla et al. 2024; MDD 2025). However, previous revisions have suggested that at least 20% of Neotropical *Myotis* diversity consists of undescribed species (Novaes et al. 2018, 2021, 2022a, 2022b, 2022c). In Argentina, 15 species have been reported (*M. albescens*, *M. barquezi*, *M. chiloensis*, *M. dinellii*, *M. guarani*, *M. izecksohni*, *M. keaysi*, *M. lavali*, *M. levis*, *M. nigricans*, *M. oxyotus*, *M. pampa*, *M. riparius*, *M. ruber*, and *M. simus*; Barquez and Díaz 2020; Novaes et al. 2022b; Argoitia et al. 2024; Novaes et al. 2025). However, despite recent systematic revisions of Neotropical *Myotis*, a significant portion of species in Argentina lack adequate taxonomic knowledge. Limited distribution records and the absence of comprehensive studies incorporating multiple lines of evidence make the accurate identification of species even more difficult (Novaes et al. 2018, 2022a).

Myotis barquezi was recently described from 2 specimens and is known only from its type locality in Salta Province in northwestern Argentina, where it inhabits lowland tropical forests within the Southern Andean Yungas ecoregion (Novaes et al. 2022b). These specimens were initially identified as *M. lavali* by Barquez et al. (2017), but a subsequent revision determined that they corresponded to the new species *M. barquezi* (Novaes et al. 2022b).

New specimens of *M. barquezi* have recently been collected in northeastern Argentina significantly expanding its known range. We present the first dataset integrating morphological, molecular, and acoustic information for the species and provide valuable insights into systematics and distribution of the species.

Methods

Study area and sample collection

The study was conducted in the AICOM (Area of Importance for Bat Conservation) Don Luis Natural Reserve, -27.8573 latitude, -56.9058 longitude (Collett and Argoitia 2022), located within the Portal Cambyretá of the Parque Nacional Iberá, in Corrientes, Argentina (Fig. 1). Don Luis Natural Reserve covers an area of 1600 ha and belongs to the Iberá Wetlands ecoregion (Burkart et al. 1999) and the Paranaense phytogeographical province (Cabrera 1976). The Ibera Wetlands ecoregion is one of the largest and most important wetlands in South America (Neiff 2004). The site consists of 2 woodlands characterized by relatively low trees.

Research on bats in Iberá Park has been conducted since 2014, with authorization granted by the Parks and Reserves authorities of Corrientes Province. Surveys occurred during March to April, and September to December 2014 to 2024. Bats were primarily captured using Faunatech Harp Traps and Forest Filter Harp Traps, with mist nets used to a lesser extent. Harp traps were placed within the woodland at sunset and checked every 30 minutes, while mist nets (9 m × 2.4 m) were deployed 20 minutes after sunset and inspected

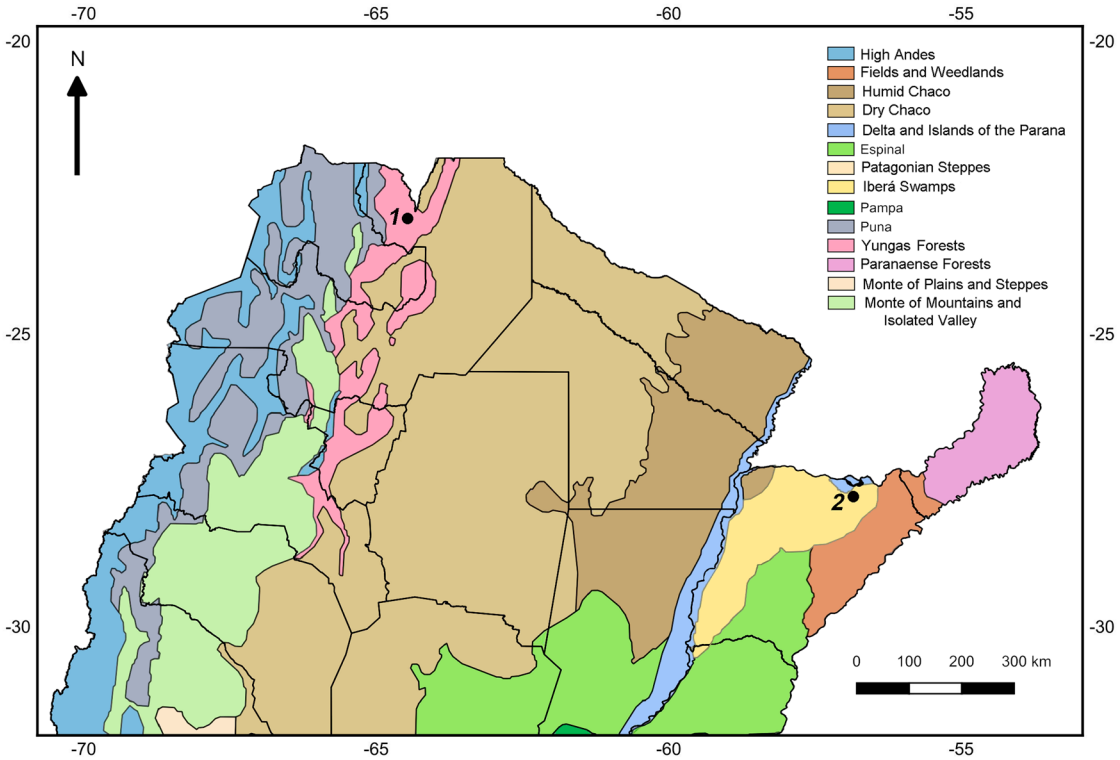

Fig. 1. Geographic distribution of *Myotis barquezi* in Argentina. The number 1 indicates the previously reported locality (type locality: Finca Alto Verde, approximately 20 km SW Orán, Orán Department, Salta Province), while the number 2 represents the new site reported in this study (Reserva Natural Don Luis, Portal Cambyretá, Iberá Park, Ituzaingó department, Corrientes Province). Colors correspond to the different ecoregions of Argentina.

every 15 minutes to minimize stress. Captured bats were carefully extracted, placed in cotton bags, and transported to the processing area, where they were handled in the order of capture. Forearm measurements (in mm) and mass (in g) were taken and we followed Barquez et al. (1999) and Barquez and Díaz (2020) to identify species. Captured animals were subsequently released following the protocol of Mitchell-Jones and McLeish (2004).

Morphological data

We compared morphological information of the collected specimens to other species in Argentina. Skin and membrane coloration, forearm measurements, and other external characteristics were examined in particular for comparison with species of similar coloration including *M. barquezi*, *M. ruber*, and *M. simus* (Appendix I).

Acoustic data

Acoustic recordings were conducted upon the release of specimens using a Wildlife Acoustics Echo Meter Touch microphone connected to an Apple iPhone via the Echo Meter Touch application (www.wildlifeacoustics.com). The microphone was positioned 1 to 2 m from the bat at a 45-degree angle pointing upwards at the moment of release. Recording parameters were set as follows: sample rate = 256 kHz, frequency range 8 to 129 kHz, 2 to 500 ms min/max length of detected pulses, full spectrum, trigger sensitivity = medium, and maximum trigger length = 10 s. Bats were released in cluttered conditions, which affected the quality and accuracy of the recordings. Recordings were stored in WAV files and later analyzed using Wildlife Acoustics Kaleidoscope software version 5.6.8. This software identifies target signals based on predefined acoustic parameters (duration; inter-pulse interval; minimal, maximum, and peak frequency; and time between calls), enabling the detection and classification of bat echolocation calls. Echolocation call parameters were subsequently compared to those of other South American species of *Myotis*.

Molecular data

Hair and fecal samples were collected from the 2 specimens, preserved in 96% ethyl alcohol at -20°C, and stored in the sample bank of the Centro de Bioinvestigaciones (Pergamino, Argentina) until processing.

DNA was extracted from hair samples using a phenol-chloroform protocol (Sambrook and Russell 2006) and eluted in 50 µL of Tris-EDTA buffer, while fecal samples were processed using the PuriPrep Soil Kit (Inbio Highway) following the manufacturer's instructions. Extraction efficiency and quality of the genomic DNA were assessed using a 1% (w/v) agarose gel stained with ethidium bromide (10 mg/mL) and visualized under UV light. Extracted genomic DNA was stored at -20°C under sterile conditions.

For genetic characterization, 2 mitochondrial molecular markers were employed: cytochrome *b* (*Cytb*) and cytochrome *c* oxidase subunit 1 (*COI*). For *Cytb*, a 755 bp fragment was amplified using conventional PCR with L15162 5′-GCAAGCTTCTACCATGAGGACAAATATC-3′ and reverse H15915 -5′ AACTGCAGTCATCTCCGGTTTACAAGAC-3 primers (Irwin et al. 1991). For *COI*, a 654 bp fragment was amplified using a touchdown PCR approach with primers L5310 5′-CCTACTCRGCCAT-3′and R6056R 5′-ACTTCTGGGTGTC-3′, following the protocol described by Clare et al. (2007). PCR reactions for both markers were prepared in a final volume of 20 µL containing 50 ng of template DNA, 2 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each dNTP, 1X reaction buffer with anti-inhibitors, 1 U of Taq T-Plus DNA polymerase, and ultrapure sterile water to reach the final volume.

Thermocycling conditions for *Cytb* amplification included an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 35 sec at 95 °C, 35 sec at 54 °C, and 1 min at 72 °C, with a final extension at 72 °C for 10 min. For *COI*, thermocycling conditions consisted of an initial denaturation at 94 °C for 5 min, 5 cycles of 30 sec at 94 °C, 40 sec at 50 °C, and 1 min at 72 °C, followed by 35 cycles of 30 sec at 94 °C, 40 sec at 55 °C, and 1 min at 72 °C, with a final extension at 72 °C for 10 min. All amplifications were performed alongside a negative control (distilled water). Amplification of DNA fragments was confirmed by electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide and visualized under UV light. DNA concentration was subsequently quantified by capturing high-resolution images of the agarose gel using GeneSys V1.4.6.0 software (Syngene), which were then analyzed with ImageJ software (Abràmoff et al. 2004). The PCR products were purified and sequenced by Macrogen (South Korea).

Sequences were visualized and manually edited using BioEdit version 7.2 (https://bioedit.software.informer.com/). In both cases, we scrutinized the coding sequences for premature stop codons to exclude potential pseudogene amplification.

A preliminary analysis was conducted to confirm that the bat genus under investigation was *Myotis* by assessing the identity of each sequence and its statistical significance by conducting a homology search using the nBLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the GenBank nucleotide database.

Haplotype and nucleotide diversities as well as number of polymorphic sites were calculated making use of DnaSP 6.12.03 (Rozas et al. 2017). Haplotype sequences were uploaded to the nucleic acid sequence database GenBank (http://www.ncbi.nlm.nih.gov/genbank) under accession numbers PV105698 and PV105699 for *Cytb* and, PV196852 and PV196853 for *COI*.

For analysis of phylogenetic and haplotype relationships among Neotropical *Myotis* species, the sequences generated in this study were combined with sequences obtained from the GenBank nucleotide database ($n = 76$), representing 23 species (Supplementary Data SD1). The outgroup species used were *Eptesicus fuscus* for *Cytb* and *COI*, and *Miniopterus* spp. exclusively for *Cytb*.

We performed both single-locus and multilocus phylogenetic analyses. The Farris test was initially conducted in PAUP*, employing parsimony inference, to evaluate the feasibility of gene concatenation. Subsequently, sequences were concatenated using the Mesquite program (Farris et al. 1994; Swofford and Sullivan 2003; Maddison and Maddison 2021). When data from the same voucher specimens were available, sequences from different genes were concatenated; otherwise, sequences from different specimens were used.

A multiple alignment was performed for the complete set of sequences using the ClustalW algorithm in MEGA v.11 software (Tamura et al. 2021). Subsequently, we used this alignment to construct phylogenetic trees using 3 methods of statistical inference: Bayesian Inference; Maximum Likelihood (ML); and Neighbor-Joining (NJ).

For the NJ phylogeny, the confidence degree for nodes was assigned by bootstrapping with 1,000 replicates using the Kimura 2-parameter model in MEGA v.11 (Felsenstein 1985; Tamura et al. 2021). Evolutionary distances were computed with the Maximum Composite Likelihood model, assuming gamma-distributed rates across sites (Gamma parameter = 1.00) and a homogeneous pattern among lineages.

Maximum Likelihood (ML) trees were inferred using IQ-TREE (Nguyen et al. 2015) with 1,000 nonparametric bootstrap replicates. Branch support was further assessed using single-branch tests (Trifinopoulos et al. 2016). For both single-locus (*Cytb* and *COI*) and multilocus (*Cytb* + *COI*) analyses, a partitioned approach by codon position was applied. The best-fit substitution model for each codon position was determined using ModelFinder, implemented in IQ-TREE. For *Cytb*, the selected models were HKY + I for the first and second codon positions, and F81 + I for the third codon position; for *COI*, the models were HKY for the first and second codon positions, and F81 + G for the third codon position. For the multilocus dataset, partitioned analyses were conducted using the same substitution models selected for each partition in the single-locus analyses.

For the Bayesian phylogenetic tree, the mutational model that best fit the dataset was determined using JModelTest software v2.1.4 (Hasegawa et al. 1985; Darriba et al. 2012). The models identified for the individual genes were HKY + G for *Cytb*, and HKI + I+G for *COI*. For the multilocus analysis, a substitution model was inferred for each codon position (first, second, and third). In *Cytb*, the models used were HKI + I+G for the first codon position, HKI + G for the second, and F81+I for the third; whereas for *COI*, the models were HKI + I+G, HKI + G, and F81+G, respectively.

We converted the data into BEAST XML format using BEAUti 2.6.3 (Bouckaert et al. 2014). For tree reconstruction, the following settings were applied: the species tree prior was set to the Yule process; a strict clock was used as the molecular clock rate variation model; and 100,000,000 generations of MCMC (Monte Carlo Markov Chain) were run, with sampling every 1,000 generations. Other priors were set to default values. All calculations were performed in BEAST2 (Bouckaert et al. 2014). The first 25% of the sampled trees and estimated parameters were discarded as burn-in using TreeAnnotator v2.6.3 (Bouckaert et al. 2014). To verify if the runs had reached convergence, the BEAST log files were examined in Tracer v1.6 (Rambaut 2012). FigTree v1.4.0 was employed to visualize the phylogenetic tree (Rambaut 2012). For phylogenetic tree resolution, we considered both the posterior probability and bootstrap values, where posterior probabilities <0.95, <0.75, and <0.55, along with bootstrap values <95, <75, and <55, were interpreted as high, medium, and low node support, respectively. Values lower than these thresholds were not considered.

Using *Cytb* only (due to higher representation), we constructed a haplotype network using sequences obtained in this study as well as other species of *Myotis* from the Neotropics using the Median-Joining algorithm through PopART v1.7 software (Bandelt et al. 1999; Leigh and Bryant 2015). Pairwise genetic distances, measured as p-distances, and the number of nucleotide differences were calculated using MEGA v.11 software (Tamura et al. 2021) for both the individual mitochondrial genes (*Cytb* and *COI*) and the concatenated data.

Results

Morphological data

In Don Luis Natural Reserve (Corrientes Province), one of the authors (MJC) observed individuals of the genus *Myotis* with reddish/orange coloration, whose external measurements did not match those of other species of the genus (Fig. 2). Between 2017 and 2024, a total of 46 unidentified specimens of *Myotis* with this characteristic fur were captured using harp traps and mist nets. The 2 specimens that we collected in March and April 2024 were adult females, with forearm lengths of 33.5 and 35.6 mm and body masses of 6 and 8 g, respectively. The dorsal and ventral coloration was strongly bicolor, with dorsal hairs having dark brown bases and light-brown tips, and ventral hairs having dark brown bases and bright orangish tips. The reddish dorsum contrasted with the orange venter and the membranes were dark brown, the tragus was long and slender with a narrow spear-shaped terminal half and a rounded tip, the uropatagium was furred with hairs extending beyond the knees, and the plagiopatagium was attached to the foot at the level of the toes. These characters coincide with those described for *M. barquezi* both in size (forearm: 35.1 to 35.2 mm; mass 6 to 8 g) and coloration. Although 1 of the new specimens was smaller than the original species description, this was based on only 2 specimens, so there may be more variability given that the sample size was small.

The collected specimens were similar in coloration to other reddish species of *Myotis* but were smaller (*M. ruber* forearm = 35.8 to 40.5 mm and *M. simus* forearm = 38 to 41.3 mm; Table 1). Other differences between *M. barquezi* and *M. ruber* are the strong bicolor dorsal pelage (vs. barely darker hair bases) and the extension of fur on the uropatagium and knees (vs. uropatagium naked). Differences with *M. simus* include its bicolor dorsal pelage (vs. unicolor), and plagiopatagium attached to the toes (vs. attached to the ankle). On the other hand, species that are phylogenetically closer to the *M. barquezi* specimens, such as *M. elegans*, tend to be smaller (forearm < 35 mm), while *M. riparius* differs in having bicolor dorsal coloration (vs. tricolor) and fur extending on the uropatagium and knees (vs. a naked uropatagium). Finally, *M. pampa*, a species that was recently documented in the area, has a tricolor dorsal pelage and the forearm >37.5 mm.

Acoustic data

Echolocation calls were typical of the genus *Myotis*, characterized by wide broadband frequency-modulated (FM) pulses with minimal constant frequency (CF). Initial findings revealed that the minimum and peak frequencies of calls from these specimens of *M. barquezi* upon release were lower compared to other species of *Myotis* recorded at Don the Luis Natural Reserve. One pass and around 12 pulses were analyzed—average minimum frequency (F_{min}) was approximately 47 kHz, average maximum frequency (F_{max}) was approximately 86 kHz (although attenuation may reduce the true maximum frequency),

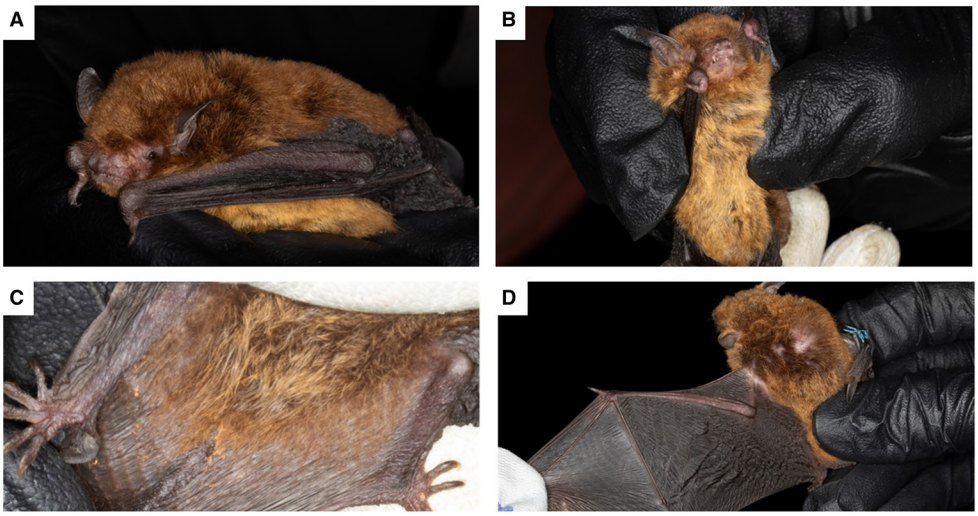

Fig. 2. Specimens of *Myotis barquezi* from which hair and feces were collected in this study. A) Frontal view of a specimen. B) Bicolored ventral pelage and detail of the elongated tragus. C) Uropatagium with fur extending beyond the knees. D) Bicolored dorsal pelage.

Table 1. Echolocation call parameters and body size characteristics of *Myotis barquezi* compared to other *Myotis* species.

Species	Forearm (mm)	Mass (g)	F_{min} (kHz)	F_{max} (kHz)	D (ms)	IPI (ms)	Citation Country
Myotis barquezi	33.5-35.6	6-8	47	86	2.3	72	This study
Myotis dinellii	34-38	4-8	40.0 ± 2.1 (35.5-45.3)	67.4 ± 8.0 (50.8-78.9)	3.4 ± 0.8 (2.4-4.5)	91.1 ± 28.9 (60.0-185.0)	González-Noschese et al. (2024) Argentina
Myotis keaysi	40-42	10	40.2 ± 0.8 41.4 ± 0.8	70.3 ± 10.1 85.1 ± 4.7	5.7 ± 0.9 3.3 ± 0.5	102.1 ± 27.5 65.8 ± 9.0	González-Noschese et al. (2025) Argentina[a]
Myotis elegans	34.2	3-5	65.59 ± 1.3 (64.0-76.2)	77.7 ± 10.3 (65.0-119.4)	1.9 ± 0.7 (1.0-4.8)		Miller (2003) Belize
Myotis cf. ruber	37-39	6-8	58	65	5		Fenton et al. (1999) Brazil

Mean ± SD and the minimum–maximum ranges of the parameters (in parentheses) are indicated. F_{min}, minimum frequency; F_{max}, maximum frequency; D, call duration; IPI, inter-pulse interval.
[a]Two methods were used for recording (hand release and flying cage).

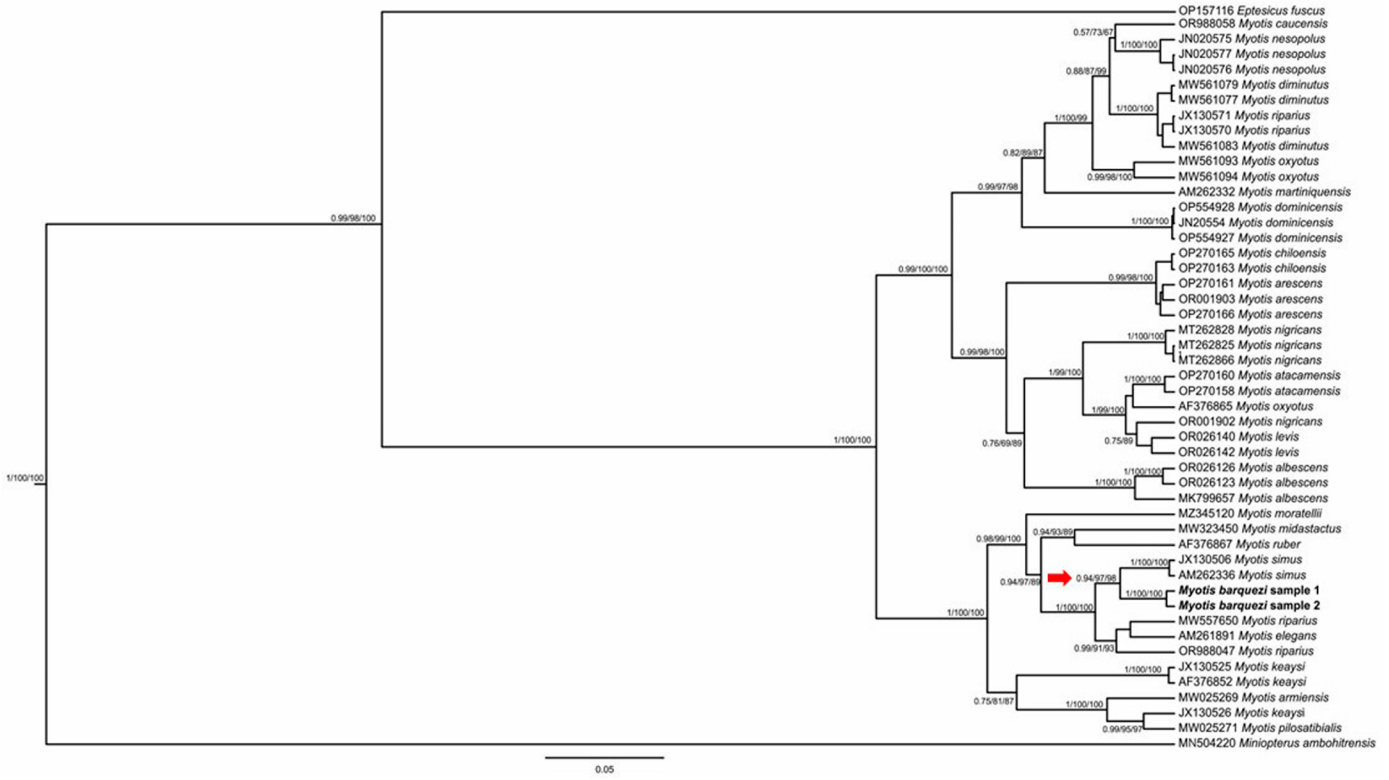

Fig. 3. Phylogenetic tree of *Myotis* species from the Neotropics based on the *Cytb* marker. Topological concordance was observed across Bayesian, ML, and NJ phylogenetic reconstructions. Node support is indicated by posterior probabilities for Bayesian, followed by bootstrap values for ML and NJ analyses, respectively. Only posterior probabilities >0.5 and bootstrap values >50 are shown. Sequences from this study are highlighted in black (Sample 1, hair; Sample 2, fecal), with the arrow indicating the strongly supported node confirming their monophyly.

average peak frequency (F_{peak}) was around 51 kHz, average duration was 2.3 ms, and average inter-pulse interval (IPI) was 72 ms (Table 1). These results differ significantly from those reported for other *Myotis*, especially when compared to closely related species from South America.

Molecular data

Amplification of *Cytb* and *COI* genes was successful from hair and fecal samples. For *Cytb*, a 637 bp fragment was obtained for both sequenced individuals. Both samples corresponded to different mitochondrial haplotypes with 5 variable sites (S) (Supplementary Data SD2), a haplotype diversity (Hd) of 1.000 ± 0.500, and nucleotide diversity per site (π) of 0.00787 ± 0.00394. For *COI*, a 573 bp fragment was obtained for both sequenced individuals. The 2 samples corresponded to distinct mitochondrial haplotypes with 3 variable sites (S; Supplementary Data SD2), a haplotype diversity (Hd) of 1.000 ± 0.500, and nucleotide diversity per site (π) of 0.00524 ± 0.00262.

The nBLAST searches for *Cytb* and *COI* markers were conducted against the comprehensive GenBank nucleotide database to assess sequence similarity. The *Cytb* sequence exhibited a maximum identity of 95.6%, with full (100%) query coverage and an e-value of 0.0, confirming assignment to the genus *Myotis*. The *COI* marker showed similar results, with 95.46% identity, 100% coverage, and an e-value of 0.0, supporting the genus-level identification.

Bayesian, ML, and NJ trees constructed from single-locus and multilocus data yielded identical topologies. Therefore, values at the corresponding nodes are presented for the 3 methods. The phylogenetic tree based on the *Cytb* gene is shown, as it offers broader species representation across the Neotropics (Fig. 3; other trees in Supplementary Data SD3 and SD4). The 3 generated trees consistently show that the *Myotis* spp. samples from this study form a monophyletic clade, distinct from other previously reported species, with strong statistical support as evidenced by both bootstrap values and posterior probabilities. Notably, the *Myotis* samples are most closely related

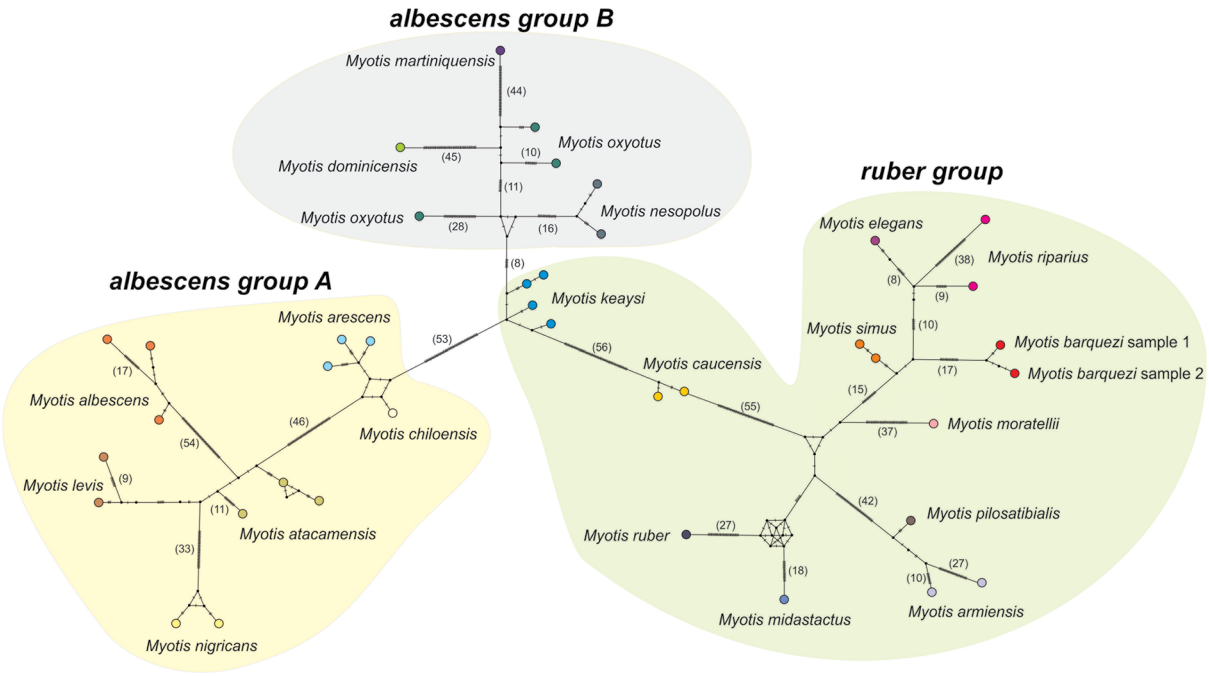

Fig. 4. Median-Joining haplotype network based on the *Cytb* marker for *Myotis* species in the Neotropics. Colored circles represent distinct species, with red indicating samples from this study (Sample 1, hair; Sample 2, fecal). Lines between circles show the number of mutations separating haplotypes, with numbers in parentheses for distances greater than 8 mutations. Black dots represent median vectors.

to *M. simus*, followed by *M. riparius* and *M. elegans* (Fig. 3; Supplementary Data SD3 and SD4).

The Median-Joining haplotype network, which visually represents the mutational differences between species within the genus, reinforces the monophyletic grouping observed in the phylogenetic trees (Fig. 4); these results further support the classification of *M. barquezi* as a distinct species. Additionally, the network reveals the close phylogenetic relationship with the previously mentioned species *M. riparus*, *M. simus*, and *M. elegans*.

Our results were further validated through pairwise genetic distance analysis, both for individual genes and the concatenated dataset. This analysis showed that genetic distances between the new samples and other species fall within the range of interspecific distances typically observed in *Myotis* (Supplementary Data SD5–SD7). Notably, the species from this study cluster closely with members of the *ruber* species group, as defined by Carrión Bonilla et al. (2024), which includes *M. armiensis*, *M. elegans*, *M. keaysi*, *M. midastactus*, *M. moratellii*, *M. pampa*, *M. pilosatibialis*, *M. riparius*, *M. ruber*, and *M. simus*. The concatenated dataset reveals that the closest genetic distance was between the sequences from this study and *M. elegans* (7.35%), while the most distant was with *M. albescens* (17.65%) (Supplementary Data SD5). For the *Cytb* gene, where we have a broader representation of Neotropical species, the data obtained previously in this research are consistent. The sequences generated in this study show the smallest distance with *M. simus* (4.43%), while the greatest distances were observed with *M. atacamensis* (19.77%) and *M. levis* (19.56%; Supplementary Data SD6).

Discussion

Myotis barquezi resembles species allocated to the *ruber* species group, both in external (e.g., fur texture) and cranial morphology (Novaes et al. 2022b). Our study provides a significant advance in the systematics of this species by integrating multiple lines of evidence (morphological, molecular, and acoustic) to clarify its taxonomic identity and phylogenetic placement within Neotropical *Myotis*. The collection locality represents the second known record for this species, so it is important to mention that this new locality corresponds to a protected natural reserve, which is also an AICOM (Important Area for the Conservation of Bats) recognized by RELCOM (Latin American and Caribbean Network for the Conservation of Bats).

The only other species of *Myotis* whose echolocation call characteristics have been published in Argentina are *M. dinellii* and *M. keaysi* (González-Noschese et al. 2024, 2025). In the first species, the call is characterized by a single harmonic with downward modulated frequency followed by a downward quasi-constant frequency emitted with a minimum frequency of 39.9 kHz and an average duration of 3.5 ms. Calls of *M. barquezi* had a F_{min} higher than *M. dinellii* and *M. keaysi*, but with shorter average duration (Table 1), which is in accordance with the negative relationship between frequency and animal size (Jones 1999). The other species is closely related to *M. barquezi* and *M. elegans*, is one of the smallest species of the genus, and has echolocation calls with an average F_{min} that is higher in frequency and shorter in duration—calls of short duration are typical of *Myotis* (Miller 2003). The only acoustic data available on *M. ruber* are from Brazil (Fenton et al. 1999), where species identification is doubtful. However, it appears that their calls are longer in duration and the F_{min} is higher compared to *M. barquezi*.

Myotis species generally spread the energy of their calls over a wide range of frequencies, and *M. barquezi* was not an exception. The primary reason proposed for this pattern is that *Myotis* species often hunt in cluttered environments such as forests and require a wide bandwidth for both successful insect hunting and safe navigation (Russ 2012). It is important to note that upon release a bat is typically stressed, and as a result, the frequencies tend to be higher, with shorter durations and IPI compared to normal search-phase calls (Russ 2012). Further work is needed to expand the database of recordings and establish more definitive guidelines to facilitate acoustic identification of this new species in the future. As a subsequent step, we released captured bats in an uncluttered environment and tracked them using a fluorescent adhesive, which detaches after few minutes but enables extended tracking with a flashlight.

Regarding molecular data, the first key aspect to highlight is the efficiency of non-invasive techniques, such as DNA extraction from feces and hair. These techniques represent a viable alternative to traditional methods such as tissue sampling through wing membrane biopsies, muscle tissue extraction, or blood collection (e.g., Clare et al. 2007, 2011; Stadelmann et al. 2007; Gunnell et al. 2017; Carrión Bonilla et al. 2024). Those methods not only cause high levels of stress in the bats but also, in the case of muscle tissue extraction, require sacrificing the bat. In contrast, analysis of non-invasive samples enables efficient genetic data retrieval without causing any negative impact on bats.

Mitochondrial genes are widely used as a first step in biodiversity characterization due to the rapid evolution of the mitochondrial genome in mammals, its limited exposure to recombination, the absence of introns, and its high copy number (Galtier et al. 2009). However, the limitations of single-locus analyses are well-documented and include gene-tree discordance in phylogenetic inference and species delimitation due to homoplasy, selection, introgression, incomplete lineage sorting, or interspecific variation in mutation rates (Toews and Brelsford 2012; Dávalos and Russell 2014; Caraballo et al. 2020). Despite these limitations, mitochondrial DNA remains a valuable initial approach for assessing genetic diversity, particularly in understudied taxa from poorly sampled geographic regions. Following this approach, the 2 mitochondrial markers employed in this study are widely used in *Myotis* genetic research (e.g., Clare et al. 2007, 2011; Stadelmann et al. 2007; Caraballo et al. 2020; Carrión Bonilla et al. 2024).

The specimens collected in this study were distinct from all previously reported species, supported by both single-locus (*Cytb* and *COI*) and multilocus mitochondrial analyses including phylogenetic trees and haplotype networks (Figs 3 and 4). These analyses indicate that the specimens form a monophyletic group, clustering most closely with *M. simus*, *M. riparius*, *M. elegans*, *M. ruber*, and *M. midastactus*, placing them within the "*ruber* species group" (Novaes et al. 2022b; Carrión Bonilla et al. 2024). This placement is further supported by genetic distance matrices (Supplementary Data SD5–SD7). These molecular findings align with morphological analyses, which also position the specimens near these species. Therefore, our results confirm that the specimens belong to *M. barquezi*, providing the first molecular records for this species.

Our findings represent the first record of *M. barquezi* in the Iberá Wetlands ecoregion, significantly extending its known distribution beyond its originally described range in the Southern Andean Yungas. Our records also add a new province in eastern Argentina (Corrientes) to the distribution of the species, extending the distribution some 930 km SE of the previous record in the province of Salta (Novaes et al. 2022b). These ecoregions differ markedly in their environmental characteristics. While the Southern Andean Yungas are a montane forest ecosystem with high humidity and dense vegetation, the Iberá Wetlands comprise vast wetlands and open grasslands at lower elevations (Burkart et al. 1999). The presence of *M. barquezi* in such distinct habitats suggests that the species might exhibit a broader ecological tolerance than previously assumed. Finally, the presence of this species in an AICOM confirms the importance of preserving this area.

These new records raise important questions about habitat use and adaptability of *M. barquezi*. Given that other species of *Myotis* are known to occupy diverse environments, the presence of *M. barquezi* in multiple ecoregions may indicate a more flexible ecological strategy than previously assumed (Carrión Bonilla et al. 2024). Further studies integrating ecological niche modeling and long-term surveys will be essential to elucidate factors driving their distribution pattern.

Our findings not only provide key information on the taxonomy and distribution of *M. barquezi* but also highlight the need for further research into understudied regions. In line with previous studies on *Myotis*, we consider the integration of morphological, acoustic, and genetic analyses essential for expanding existing databases and improving species identification accuracy, a crucial aspect for strengthening conservation strategies.

Acknowledgments

We sincerely thank the Collett Trust for Endangered Species and Fundación Cambyretá para la Naturaleza for their generous support. Special thanks to Leonardo Giorgadis and Molly Watson for help with bat research and to Susan Loeb for proofreading the English. Finally, the acoustic recordings were conducted based on collaboration with Bruce W. Miller.

Author contributions

Diana B. Acosta (Conceptualization, Formal analysis, Methodology, Writing—original draft, Writing—review & editing), Miranda J. Collett (Methodology, Resources, Writing—review & editing), and M. Mónica Díaz (Conceptualization, Methodology, Resources, Writing—review & editing)

Supplementary data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1. Sequences from the GenBank nucleotide database.

Supplementary Data SD2. Polymorphic sites found in the *Cytb* and *CO1* genes from the samples analyzed in this study (Sample 1: hair; Sample 2: fecal).

Supplementary Data SD3. Phylogenetic tree constructed based on the concatenated *Cytb* and *CO1* gene markers for species of the *Myotis* genus in the Neotropics. Nodes show posterior probabilities (Bayesian) >0.5 and bootstrap (ML and NJ) values >50. Sequences obtained in this study are highlighted in black (Sample 1: hair; Sample 2: fecal).

Supplementary Data SD4. Phylogenetic tree constructed based on the *CO1* marker for species of the *Myotis* genus in the Neotropics. Nodes show posterior probabilities (Bayesian) >0.5 and bootstrap (ML and NJ) values >50. Sequences obtained in this study are highlighted in black (Sample 1: hair; Sample 2: fecal).

Supplementary Data SD5. Interspecific uncorrected pairwise genetic distances between all lineages included in the concatenated dataset (*Cytb* and *CO1*).

Supplementary Data SD6. Interspecific uncorrected pairwise genetic distances between all lineages included in the dataset of *Cytb*.

Supplementary Data SD7. Interspecific uncorrected pairwise genetic distances between all lineages included in the dataset of *CO1*.

Funding

This work was supported by the Collett Trust for Endangered Species and Fundación Cambyretá para la Naturaleza.

Conflict of interest

The authors declare no conflict of interest.

Data availability

The GenBank accession numbers are PV105698 and PV105699 for *Cytb*, and PV196852 and PV196853 for *COI*.

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APPENDIX I
SPECIMENS EXAMINED

For each species, the localities are listed alphabetically by province; within each province they are listed by the department followed by specific locality, and geographic coordinates; for each locality the number of individuals examined is indicated, followed by the collection code and collection number between parenthesis. Abbreviations used in the text are: CML (Colección Mamíferos Lillo, Tucumán, Argentina); MLP (Museo de La Plata, Buenos Aires, Argentina), MUSM (Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Perú).

Myotis barquezi (4). Argentina, Corrientes, Ituzaingó department, Don Luis Natural Reserve, Portal Cambyretá of the Parque Nacional Iberá, 27° 51' 26.28'' S, 56° 54' 20.82'' W, 72 m, 2 (released). Salta, Orán department: Finca Alto Verde, approximately 20 km SW Orán, 23° 13' S, 64° 32' W, 670 m, 2 (CML 7622 paratype, CML 7623 holotype).

Myotis ruber (3). Argentina, Buenos Aires, partido de Ensenada, Reserva Natural Punta Lara, 34° 47' 26'' S, 57° 59' 56'' W, 70 m, 1 (MLP 14.XI.08.13); Corrientes, Santo Tomé department: Laguna Galarza y Lago de Luna, 28° 6' 40.94'' S, 56° 43' 36.47'' W, 70 m, 1 (CML 3698); Entre Ríos, Islas de Ibicuy department, Quinta La Chilena, arroyo Brazo Chico, a 2 km de la desembocadura en el río Uruguay, 33° 45' 56'' S, 58° 32' 02'' W, 1 (MLP 1924); Misiones, Guaraní department: Jct. Hwy 21 and Arroyo Oveja Negra, approx. 2 km W Parque Provincial Moconá, 27° 8' S, 53° 54' W, 1 (CML 3877).

Myotis simus (5). Argentina, Formosa, Patiño Department: Río Porteño, km 64, 5 km al S de Estancia Santa Catalina, 24° 52' 13.48'' S, 59° 15' 52.99'' W, 103 m, 1 (CML 2051). Pilcomayo department: Parque Nacional Pilcomayo, Paso Pomelo, 25° 0' 45.21'' S, 58° 8' 2.02'' W, 78 m, 1 (CML 4680). Bolivia, Beni: Río Mamoré, 23 km al E de San Javier, 1 (CML 2261). Perú, Loreto, Maynas Province: Asentamiento Héroes de Cenepa, Fundo Gallo Giro, 3° 47.244' S 73° 17.178' W, 1 (CML 13529); Moena Caño, 3° 46.728' S 73° 13.495' W, 1 (MUSM 30022).