PENSOFT
SENCKENBERG world of biodiversity
Leibniz Association

Revisiting *Molossus* (Mammalia: Chiroptera: Molossidae) diversity: Exploring southern limits and revealing a novel species in Argentina

Micaela A. Chambi Velasquez[1,2,*], Romina Pavé[3,4,*], María A. Argoitia[4,5,*], Pablo Schierloh[6,*], María G. Piccirilli[2], Valeria C. Colombo[2,7], Fernando J. Beltrán[8], Daniel M. Cisterna[2], Diego A. Caraballo[1]

1 *Instituto de Ecología, Genética y Evolución de Buenos Aires, CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*

2 *Servicio de Neurovirosis, Instituto Nacional de Enfermedades Infecciosas, Administración Nacional de Laboratorios e Institutos de Salud (ANLIS), "Dr. Carlos G. Malbrán", Buenos Aires, Argentina*

3 *Laboratorio de Biodiversidad y Conservación de Tetrápodos, Instituto Nacional de Limnología (INALI, CONICET-UNL), Santa Fe, Argentina*

4 *Programa de Conservación de los Murciélagos de Argentina (PCMA), San Miguel de Tucumán, Argentina*

5 *Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" (MACN-CONICET), Buenos Aires, Argentina*

6 *Laboratorio de Microscopías Avanzadas, Instituto de Investigación y Desarrollo en Bioingeniería y Bioinformática (IBB, UNER-CONICET), Oro Verde, Entre Ríos, Argentina*

7 *Laboratorio de Ecología de Enfermedades, ICIVET-Litoral, UNL/CONICET, Esperanza, Santa Fe, Argentina*

8 *Instituto de Zoonosis Luis Pasteur, Buenos Aires, Argentina*

* *These authors contributed equally to this study.*

https://zoobank.org/5DA98512-20DF-4C06-B2E0-3F61D81B48DC

Corresponding authors: Diego A. Caraballo (dcaraballo@ege.fcen.uba.ar), Daniel M. Cisterna (dcisterna@anlis.gob.ar)

Academic editor Clara Stefen | **Received** 11 March 2023 | **Accepted** 14 May 2024 | **Published** 10 June 2024

Citation: Chambi Velasquez MA, Pavé R, Argoitia MA, Schierloh P, Piccirilli MG, Colombo VC, Beltrán FJ, Cisterna DM, Caraballo DA (2024) Revisiting *Molossus* (Mammalia, Chiroptera: Molossidae) diversity: Exploring southern limits and revealing a novel species in Argentina. Vertebrate Zoology 74 397–416. https://doi.org/10.3897/vz.74.e122822

Abstract

Understanding species diversity and delineating their boundaries are crucial for effective management and conservation efforts. In the case of bats, species identification holds particular importance from an epidemiological standpoint. The genus *Molossus* (Chiroptera: Molossidae) encompasses 15 species distributed across the Neotropics, ranging from the southeastern United States to Argentina. This genus exhibits two contrasting patterns of variation: some species are cryptic, while others are morphologically distinct yet genetically similar. This study explores the diversity of *Molossus* in Argentina through a molecular phylogenetic approach. We analyzed sequences from three molecular markers (cyt *b*, COI, and FGB) along with morphology data obtained from a sample of 64 individuals. Uni- and multivariate analyses of external and cranial measurements were conducted, alongside comparisons of external and cranial characteristics among species. Based on molecular and morphological differences, we describe a new species within the *Molossus* genus. This newly discovered species exhibits a broad distribution spanning the Paraná River basin across three distinct ecoregions. It is noteworthy that this species is pseudo-cryptic with respect to similar-sized species such as *M. molossus* and *M. melini*. Additionally, it is important to mention that all species in Argentina have overlapping distribution ranges. In summary, this study provides valuable insights into the diversity and distribution of *Molossus* bats in Argentina, employing molecular and morphological analyses. The discovery of a new species underscores the ongoing importance of comprehensive research efforts in understanding and conserving bat populations in the Neotropics.

Keywords

Mastiff bats, molecular phylogeny, morphology, morphometry, pseudo-cryptic species, South America

Introduction

Accurate species identification is of paramount importance for various reasons. Firstly, it serves as the foundation for understanding and documenting biodiversity, allowing the recognition and appreciation of the variety of life on Earth (Mora et al. 2011). Precise species identification is crucial for effective conservation efforts, as it enables the assessment of population sizes, monitoring distribution patterns, and evaluation of threats and vulnerabilities specific species face (Gaston and Spicer 2004; Pimm et al. 2014). Additionally, accurate species identification is essential for studying ecological interactions, unraveling evolutionary processes, as well as for the control, prevention, and elimination of pathogens (Heywood et al. 1995; De Benedictis et al. 2022). The advent of molecular techniques and modern taxonomic tools revealed differentiation within previously recognized species uncovering the existence of cryptic species in most types of organisms and habitats (Pfenninger and Schwenk 2007).

With over 1400 species comprising 20% of mammal diversity, bats (Order Chiroptera) hold a prominent position in the Tree of Life (Wilson and Mittermeier 2019; Simmons and Cirranello 2024). Genetic studies and molecular techniques have uncovered hidden diversity of cryptic species in a wide variety of bat families (see Tsang et al. 2016 and references therein), usually discovered within the boundaries of what has been previously considered a single species (Jones et al. 2009). In this respect, one particularly challenging group is the genus *Molossus* É. Geoffroy, 1805.

Bats of genus *Molossus* are distributed throughout the continental and insular Neotropics, from southeastern United States to central Argentina (Eger 2008), making them an ideal group to study speciation processes, diversity, and biogeographic patterns (Loureiro et al. 2019). The alpha taxonomy of this group has been historically unstable due to the occurrence of two opposing patterns that tend to generate uncertainty in the systematics of the genus. On one hand, there are morphologically similar species that exhibit higher levels of genetic differentiation than expected (e.g., *M. molossus*, *M. fentoni*, *M. milleri*, and *M. verrilli*) (Loureiro et al. 2018a). Many of these cryptic species have geographically restricted ranges in areas of high anthropogenic pressure, requiring high priority for conservation. The second pattern corresponds to morphologically divergent species with very low levels of genetic differentiation (e.g., *M. aztecus*, *M. pretiosus*, *M. currentium*, and *M. sinaloae*) (Lim and Arcila Hernandez 2016; Lindsey and Ammerman 2016; Lim 2017; Loureiro et al. 2019). The occurrence of these contrasting patterns can be explained by some typical attributes of the group such as low levels of morphological differentiation at the species level, significant differences in sexual characteristics, and a tendency towards genetic conservatism (Eger 2008; González-Ruiz et al. 2011; Loureiro et al. 2018a, 2019).

In a recent study, Loureiro et al. (2020) applied the genotyping-by-sequencing methodology (GBS) analyzing tens of thousands of single nucleotide polymorphisms (SNPs) covering the entire distribution of the genus providing the first high-resolution phylogeny for *Molossus*. This study offered new insights into species delimitation and taxon relationships, increasing the number of species from 11 to 14 (Fig. S1).

Recently, a fifteenth species, *M. melini*, was described in Argentina (Montani et al. 2021). Besides the new species, three other *Molossus* species are known to occur in Argentina (Barquez and Díaz 2020; Loureiro et al. 2020). *Molossus currentium* has a restricted distribution and few records in the country, while *M. molossus* and *M. fluminensis* have a broader distribution, principally in central and northern provinces of the country (Fig. S1). It should be noted that except for two specimens of *M. melini* (Montani et al. 2021), other populations of *Molossus* in Argentina have not been studied from a multi-locus perspective. This is a necessary approach since the use of a single molecular marker (cyt *b*) has proved to be insufficient for species identification in Argentinian mastiff bats (Caraballo et al. 2020).

Due to the peculiarity of the natural history of this bat genus, the identification of *Molossus* at the species level poses a common problem in epidemiological surveillance laboratories, fieldwork, and biological collections. Most specimens are cataloged as *Molossus* sp. or, what is worse, assigned an incorrect species name (typically *M. molossus*). Therefore, it is necessary to evaluate genes that can provide sufficient information for the identification of *Molossus* species without employing highly complex and costly methodologies such as GBS.

In this study, we conducted an extensive examination of the *Molossus* species diversity in Argentina, assessing the taxonomic differentiation at the species level using one nuclear and two mitochondrial markers. To facilitate further research, we compiled a meticulously curated dataset of genetic sequences, enabling the comprehensive assessment of the phylogenetic classification of any *Molossus* specimen across its entire geographic range. The discovery of a previously unrecognized lineage was confirmed as a distinct species through multivariate analysis of cranial morphology as well as comparisons of cranial and external characteristics with other known species

present in Argentina. In summary, our study significantly contributes to the understanding of the diversity and geographical distribution of mastiff bats in the southernmost extents of their range, shedding new light on the biology of this group.

Methods

Sampling

Genomic DNA was extracted from a set of 56 specimens belonging to the genus *Molossus*, sourced from various locations within Argentina (Table S1), and obtained through three distinct methods. A group of specimens was captured by fieldwork specialists during ecological studies of rabies virus, who followed a protocol involving the capture and subsequent release of the specimens. Wing membrane tissue samples were obtained using a biopsy punch with a diameter of 3 mm. Captures were carried out under the approval of the Ethics and Safety Committee of the Universidad Nacional del Litoral (Expte. FCV-0869428-17) and the Ministerio de Medio Ambiente de la Provincia de Santa Fe (Expte No. 02,101-00, 181, 129-9, Resolution No. 093/2018). Guidelines of the American Society of Mammalogists (Sikes and the Animal Care and Use Committee of the American Society of Mammalogists 2016) were followed. The second group consisted of ethanol-preserved tissue samples, including muscle and patagium, obtained from museum vouchers deposited in the Colección de Vertebrados of the Instituto Nacional de Limnología (**INALI-A**), Colección de Mamíferos of the Museo Provincial de Ciencias Naturales "Florentino Ameghino" (**MFA-ZV-M**), Colección de Mamíferos of the Museo Provincial de Ciencias Naturales "Dr. Ángel Gallardo" (**MG-ZV-M**), and Colección de Mamíferos of the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" (**MACN-Ma**). The remaining samples including intestine, patagium, and muscle tissue were collected from bat specimens from the "Instituto de Zoonosis Luis Pasteur" (Buenos Aires city, Argentina) and personal collections.

Sequencing

One nuclear gene, the intron 7 and short partial sequences of exons 7 and 8 of the β-fibrinogen (FGB) and two mitochondrial genes, cytochrome *c* oxidase I (COI) and cytochrome *b* (cyt *b*), were sequenced totaling 2567 aligned base pairs (COI — 657 bp; cyt *b* — 1140 bp; FGB — 770 bp). Primers and PCR conditions for each gene are given in Table S2. Sequences were obtained with both primers for each locus. Samples that failed to produce sequences were discarded, remaining 47 successfully sequenced specimens (Table S1). Coding sequences were checked for the presence of premature stop codons to discard the amplification of pseudogenes.

Data acquisition and curation

To test the resolution of each marker we downloaded all available sequences annotated as *Molossus* in GenBank. Although it is immensely valuable, this public database is susceptible to having lots of errors in the taxonomy of the sequences available in it, especially in taxa with cryptic species and taxonomic instability such as *Molossus*. Morphological species misidentification and/or lack of taxonomic update are the two sources of error that abound in *Molossus* sequences in GenBank.

To overcome this issue we designed a strategy consisting in updating all previous names according to the current taxonomy, taking into account species distributions (Loureiro et al. 2020; Montani et al. 2021, 2023; IUCN 2022; Pavé et al. 2023; Olímpio et al. 2024). For example, *M. rufus* was recently subdivided into *M. nigricans*, *M. rufus*, and *M. fluminensis*, with non-overlapping distributions in Central America, northern South America, and southern South America, respectively (Fig. S1). Another example is that of *M. milleri*, which is distributed in the Cayman Islands and Cuba, and sequences uploaded previously to its description were annotated as *M. molossus*. A second part of this strategy was the recognition of misidentified specimens. This was done by scrutinizing majority clusters (composed of more than 50% sequences) of a given species in each single-locus phylogeny and revising those sequences that behaved as outliers in those clusters. Again, this revision was done by contemplating the updated taxonomy, identifying also which species have low levels of genetic differentiation, and also checking if the putative wrong assignment was done to a specimen captured within the distribution range of the majority cluster. Even after the correction of erroneously annotated sequences, we noted that several species failed to produce monophyletic clades, so we included sequences representing all these lineages in the multi-locus analysis.

In addition to the analysis conducted for each marker separately, phylogenies were inferred from the concatenated mitochondrial loci on one hand, and from the three combined loci on the other. The combined analysis of cyt *b* and COI genes allows the reconstruction of the same evolutionary history (Hurst and Jiggins 2005). Different phylogenetic patterns have been reported between mitochondrial and nuclear DNA (Spinks and Shaffer 2009), which highlights the need to compare both datasets independently (Loureiro et al. 2019). For the full dataset, we concatenated the three genes including, whenever possible, specimens with sequences available for the three loci. In cases when this was not achievable, we constructed chimeras ensuring these were formed by individuals of the same species, country, and lineage in the single-locus trees (Table S3). At least two individuals/chimeras per species and lineage were included to test their monophyly. Since the FGB analysis resulted in a less informative topology, we gave priority to specimens with sequences from both mitochondrial loci. If a specimen lacked FGB data, we completed the chimera with another specimen, following the previously mentioned criteria. The sequences of specimens/chimeras that lacked a specific locus were coded as missing data.

Phylogenetic analyses

A Bayesian phylogenetic analysis was conducted for each locus and the concatenated dataset including representatives of all known species of *Molossus*. We used sequences of *Promops centralis* and *Eumops auripendulus* as outgroups. Nucleotide substitution models were estimated using MrModeltest2 (Nylander 2004) under the Akaike Information Criterion (correcting by the number of taxa), being HKY+G for FGB and COI, and HKY+I+G for cyt *b*. In the multi-locus analyses, we inferred a substitution model separating each codon position being K80+I, JC and HKY+G for 1st, 2nd, and 3rd positions of COI, and SYM+G, F81, and GTR+I+G for cyt *b*.

The phylogenetic analysis was performed in MrBayes version 3.2.7 (Ronquist et al. 2012), using computational resources from CCAD – Universidad Nacional de Córdoba (https://ccad.unc.edu.ar), which are part of SNCAD – MinCyT, República Argentina. For each single-locus analysis and for the concatenated mitochondrial (mtDNA) dataset, two independent runs for 1×10^7 MCMC (Markov chain Monte Carlo) generations, sampling every 1,000 generations, were carried out. The multi-locus analysis including nuclear and mitochondrial genes (FGB, COI, cyt *b*) was run for 2×10^7 MCMC generations, sampling every 1,000 generations. Convergence was assessed by analyzing the potential scale reduction factor (PSRF), and the average standard deviation of split frequencies (ASDSF). The burnin phase was set up in the generation that fulfilled PSRF values of 1.00–1.02 for all estimated parameters and standard deviations lower than 0.01. We also checked that the estimated sample sizes were >200. Trees were visualized with Figtree (http://tree.bio.ed.ac.uk/software/figtree). For the comparison of phylogenetic resolution, we considered a posterior probability (bpp) of 0.95, 0.75 and <0.95, and <0.75 as high, moderate, and low node support.

Network analysis

A median-joining network was constructed with PopART v1.7 (Leigh and Bryant 2015). The software necessitates that sequences have few or no undefined states. Since the nuclear marker (FGB) was absent in several individuals/chimeras, we opted to conduct the analysis solely considering the mtDNA loci. Additionally, individuals lacking sequences for either of the two mtDNA loci were excluded. Following this procedure, all lineages were represented, except for *Molossus* sp. 2. For this lineage, we created a chimera using a cyt *b* sequence and a COI sequence from two different individuals (*Molossus* sp. 2 Ecuador-A1 and *Molossus* sp. 2 Bolivia-A7, respectively).

Genetic distances

To compare intra- and interspecific distances we generated a p distances table with the concatenated matrix including the three loci. Pairwise genetic distances were calculated with MEGA X (Tamura et al. 2021). Of the two sequences of *M. currentium*, one was considered as *M. fluminensis* (MH185138), while the other was treated as *M. currentium* (MH185139), based on the results of the phylogenies obtained in this study (see below).

Morphological and specimens data

We recorded the following data of each specimen analyzed: locality, age class (i.e., subadult or adult), sex, external and cranial measurements, and body mass, following Barquez et al. (1999). External and cranial measurements were obtained for 64 adult specimens of *Molossus* species from Argentina, including six *M. currentium*, 12 *M. fluminensis*, 13 *M. melini*, 20 *M. molossus*, and 13 specimens of the novel *Molossus* species (*Molossus* sp. nov.). Measurements were taken using a digital caliper, supplemented in some cases by data obtained from the existing literature (Thomas 1901; Cláudio et al. 2020; Montani et al. 2021; 2023; Pavé et al. 2023; Olímpio et al. 2024). We included five external [total length (ToL), tail length (TL), hindfoot length (HFL), ear length (EL), forearm length (FA)] and 14 cranial measurements [greatest length of skull with incisors (GLS w.i), greatest length of skull excluding incisors (GLS wo.i), condylobasal length (CBL), postorbital constriction (PC), braincase breadth (BB), zygomatic breadth (ZB), mastoideal breadth (MB), maxillary toothrow length (LMxT), palatal length (PL), width across canines (C-C), width across second molars (M2-M2), mandible length (LM), mandibular toothrow length (LMdT), height of the sagittal crest (SAR)] for each species. In some cases, information was recorded directly from labels of museum specimens (File S1).

Comparative morphometry analyses

Univariate (using one-way ANOVA or Kruskal–Wallis test with corresponding pairwise post hoc tests) and multivariate statistical analyses (employing principal component analysis—PCA—coupled with PERMANOVA and its corresponding pairwise post hoc tests) of cranial and external linear measurements were conducted in R Studio (RStudio Team 2020) utilizing R version 4.2.1 (R Core Team 2021). The following R packages were utilized: FactoMineR version 2.8 (Lê et al. 2008), ggplot2 version 3.4.3 (Wickham 2016), Vegan version 2.6–4 (Oksanen et al. 2008) and pairwiseAdonis (downloaded from the author's github site: pmartinezarbizu/pairwiseAdonis/pairwiseAdonis). For shape based comparisons, an exploratory two dimensional geometric morphometric (2D GMM) study of nasal cavity perimeter of the three species that showed the lesser morphological differentiation (*M. molossus*, *M. melini*, and *Molossus* sp. nov.) was performed. The tpsDig2 version 2.32 (https://sbmorphometrics.org) was employed for manual definition of 13 landmarks and one semi-landmark on picture files (*.jpg) of rostral views. The R based geomorph package version 4.0.6 (https://cran.r-project.org/web/packages/geomorph/index.html) was used for Generalized Procrustes Analy-

sis (GPA) and PCA. Detailed listings of the datasets employed for univariate, multivariate, and 2D GMM morphometry, as well as the statistical methods applied and the resulting test outcomes, are provided in File S1.

Results

Single-locus and mtDNA phylogenies

The trees obtained for all nuclear and mitochondrial loci confirmed the monophyly of the genus *Molossus*. However, significant discrepancies were observed in internal nodes, particularly between FGB and both mitochondrial genes (Figs S2–S4; File S2). While the nuclear gene fails to recover most species as monophyletic, it can enhance the resolution of another dataset, such as the mtDNA genes (Fisher-Reid and Wiens 2011). The mtDNA dataset produced a similar but better-resolved phylogeny compared to cyt *b*, which is the better-resolved single-locus phylogeny (Fig. S5; Files S2, S3).

Multi-locus phylogeny

The phylogeny inferred from the concatenated dataset depicts higher levels of phylogenetic resolution, with the most robustly supported internal nodes (609 parsimony informative sites of a total of 2567) (Fig. 1). While there are minor discrepancies between the mtDNA tree and the multilocus tree, these are slight differences. The only significant disparity involves the positioning of sequences of *M. aztecus*, indicating potential introgression events (discussed in File S2). Consistent with the single-locus and mtDNA phylogenies, *M. fentoni*, *M. verrilli*, *M. milleri*, and *M. alvarezi* form highly-supported basally-diverging groups. In addition, *M. rufus* (with high support) and *M. bondae* (with low support) are also monophyletic groups.

The clade of *M. fluminensis* has moderate support (bpp = 0.84) but is still recovered as monophyletic, and splits into two well-supported subclades. Within this group, there are two previously annotated *M. rufus* that were updated according to their distribution to *M. fluminensis* (Fig. S1) and a sequence annotated as *M. currentium* from Paraguay, a region where the distribution of both *M. currentium* and *M. fluminensis* overlap. Notably, the other sequence of *M. currentium* (also from Paraguay) clusters with *M. nigricans*, *M. sinaloae*, and *M. rufus*. These three species do not overlap with *M. currentium*, so the most reasonable explanation is that this second haplotype represents the valid *M. currentium* and the former is, in fact, *M. fluminensis*.

The species *M. pretiosus* and *M. aztecus* from Mexico form a highly supported clade with *M. bondae*, but fail to be reciprocally monophyletic, indicating that these species are genetically close. Interestingly, the three representatives of *M. aztecus* from Brazil form a monophyletic clade, sister to the former, although there are two distinct lineages (A and B), as evidenced by the long branch within this group. In contrast, in the mtDNA phylogeny, *M. aztecus* from Brazil is divided in two non-related clades, one related to *M. fluminensis*, and the other related to *M. melini* (Fig. S5; File S2).

The clade of *M. melini* includes several specimens previously identified as *M. molossus*, not only from the originally described localities but extending also to the Buenos Aires province. Interestingly, several specimens that fall in the clade of *M. melini*, do not have the typical ochraceous to orange fur coloration, but instead are chocolate to grayish brown with the venter paler, and that could be the reason for their initial identification as *M. molossus* (Fig. S7). Sister to this clade is the *M. molossus* clade which includes the subspecies *M. molossus daulensis*. This clade groups two of the three *M. molossus* lineages found with COI: the canonical lineage, and a lineage composed of sequences from Panama and Brazil (Fig. S3; File S2). We conclude that these two lineages are indeed *M. molossus*. However, as occurs with the cyt *b*- and COI-based phylogenies, there is another clade of specimens identified as *M. molossus* with moderate support (bpp = 0.81), that splits into well/moderately supported clades, one (bpp = 0.81) including specimens from Ecuador, Peru, Guyana, Suriname, and Bolivia, while the other is exclusive of Argentinian bats (bpp = 0.97). This last group should be treated as *Molossus* spp., consisting of *Molossus* sp. nov. (Argentinian clade) and *Molossus* sp. 2 (sister clade), since it is reciprocally monophyletic to the canonical lineage of *M. molossus* (which has a wider distribution and includes the subspecies *M. molossus daulensis*). It is noteworthy that, except for one sample, *Molossus* sp. 2 formed part of the *M. molossus* clade in the mtDNA analysis (Fig. S5) and in the full dataset it groups with *Molossus* sp. nov.

Genetic distances

The genetic distance analysis illustrates the general panorama of *Molossus* (Fig. 1; Tables S4, S5). Intraspecific distances depict low values, ranging from 0 to 1.7% (mean 0.85%), the highest values belonging to *M. molossus*, *M. pretiosus*, *M. coibensis*, and *M. fluminensis*. The only exception is *M. aztecus*, depicting 2.5%, a remarkably high value for intraspecific variation (but see an extended discussion in File S2). As expected for this genus, there is a wide variation of interspecific distances, with a mean of 4.0%, but including extremely low values such as those found between *M. pretiosus*, *M. nigricans*, *M. bondae*, *M. rufus*, *M. sinaloae*, and *M. currentium*, all of which are <1.0%. The distance between *M. molossus* and *Molossus* sp. nov. is 2.8% which is indicative of interspecific variation. In contrast, the distances between *M. molossus* and *Molossus* sp. 2, and between *Molossus* sp. nov. and *Molossus* sp. 2 are 1.1 and 1.4, respectively, and will be discussed below. As a whole, *Molossus* appears to be a genetically stable group. The average genetic distance among morphologically recognized species using mtDNA markers was estimated to be approximately 3.3%

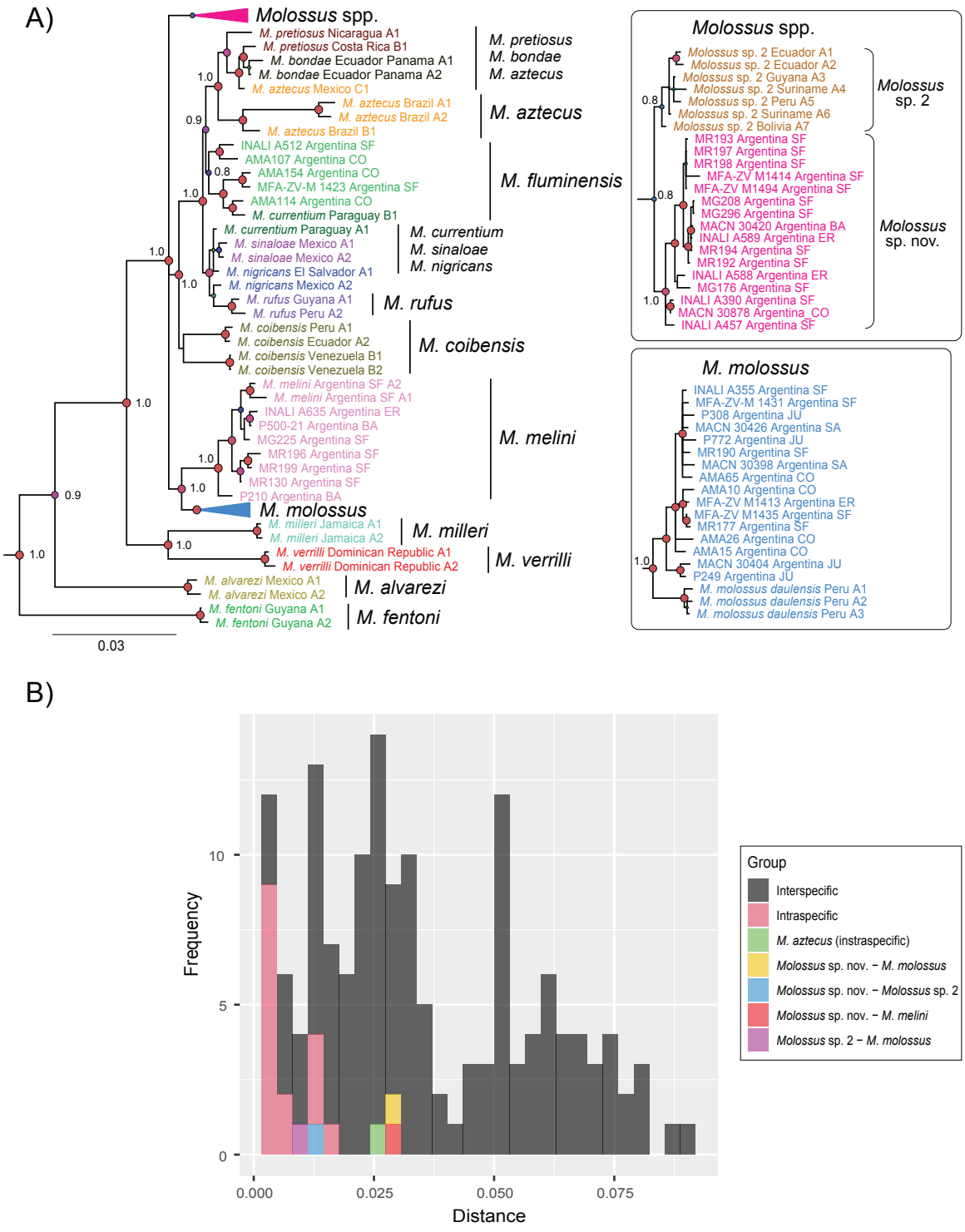

Figure 1. A Bayesian phylogeny obtained from the concatenated dataset (FGB, COI, and cyt *b*). Branch colors indicate lineage/species membership. The letters accompanying each terminal indicate the lineage based on the analysis of individual genes, while the numbers refer to the number of representatives of each lineage. Node sizes are proportional to their posterior probability (red: 1–0.95; purple-cyan: 0.94–0.75; green-yellow: <0.75). Node support above 0.8 is shown for main lineages. The scale is expressed in substitutions per site. **B** Uncorrected p distances. Histogram showing intra- and interspecific distances based on the concatenated dataset (FGB, COI, and cyt *b*). Different colors indicate intraspecific distances (pink), interspecific distances (dark gray), intraspecific distance of *M. aztecus* (green), distance between *Molossus* sp. nov. and *M. molossus* (yellow), distance within *Molossus* sp. nov. and *Molossus* sp. 2 (light blue), distance between *Molossus* sp. nov. and *M. melini* (red), and distance between *M. molossus* and *Molossus* sp. 2 (purple).

(Loureiro et al., 2019), a finding consistent with the results of this study. These low levels of genetic variation are comparable to certain molossid bats from the Old World but are notably lower compared to others such as *Cynomops*, *Mops*, and *Eumops*, where cyt *b* average distances range from 8.6% to 13.4% (Ratrimomanarivo et al. 2007;

Baker et al. 2009a; Moras et al. 2018). While interspecific distances in our study are notably lower compared to the >5% threshold suggested by Baker and Bradley (2006) for species delimitation, it is crucial to recognize that such thresholds serve as guidelines and can vary across different organisms (Baker et al. 2009b). These thresholds are not universally applicable and should be determined empirically for each taxonomic group, taking into account specific characteristics such as generation time, dispersal rates, and geographic range (Hebert et al. 2003). Finally, we believe that genetic distances should prompt taxonomic reassessments but should not be considered the sole criterion for species delimitation.

Network analysis

The mtDNA median-joining network illustrates significant differences among *M. melini*, *M. molossus*, and *Molossus* sp. nov. (Fig. S6). *Molossus molossus* exhibits more than 30 substitutions distinguishing it from the other two species, while *M. melini* and *Molossus* sp. nov. display twice this distance, indicative of their classification as distinct species. It is worth noting that certain taxa, such as *M. fluminensis*, *M. coibensis*, or the subspecies *M. molossus daulensis*, exhibit high levels of intraspecific divergence at the mitochondrial level. The chimera constructed using two sequences representing *Molossus* sp. 2

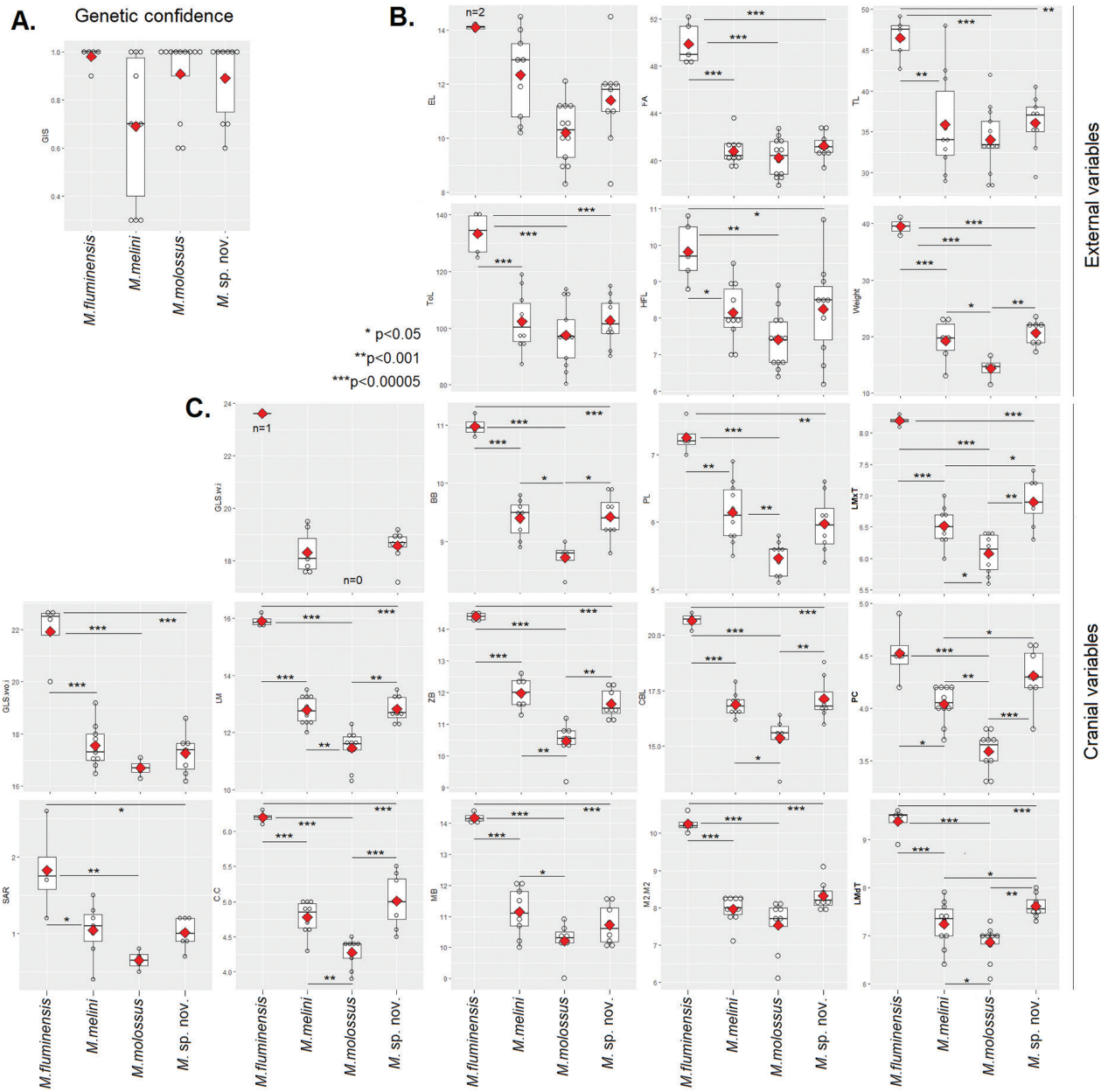

Figure 2. A Genetic confidence score (GIS) was defined for each species group by weighted consideration of known sequences (see File S1 for equation details). Univariate pairwise comparisons of external (**B**) and cranial measurements (**C**) of *Molossus* species present in Argentina (and Brazil) based on genetically confirmed specimens (*M. fluminensis*, *M. melini*, *M. molossus*, and *Molossus* sp. nov.). Box-and-whisker plots show data distributed across quartiles, the vertical lines indicate the ranges, and outliers are shown as separate data points. *, **, and *** represent significant pairwise differences among species.

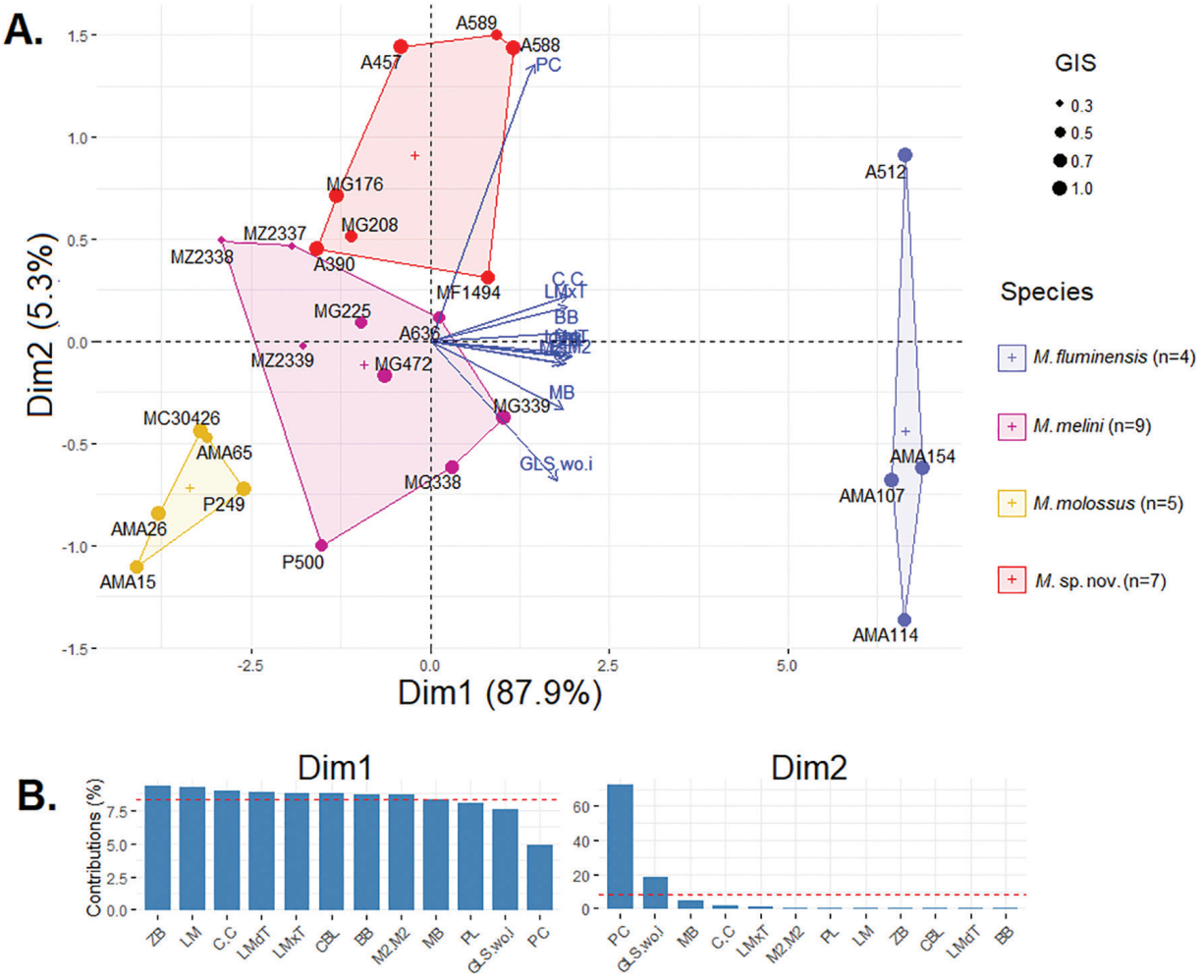

Figure 3. Principal component analysis (PCA) of selected cranial metric variables (GLS wo.i, CBL, PC, BB, ZB, MB, LMxT, PL, C-C, M2-M2, LM and LMdT) of genetically confirmed specimens of *Molossus* distributed in Argentina. **A** PCA results are presented in a biplot chart with individuals (dots) and variables (blue arrows) plotted together in the PC1 vs PC2 space. Individuals of the same species are enclosed by a convex hull. The mass centroid of each group is indicated by an unlabeled plus sign. Data labels are referenced in File S1 (Table M3). Dot sizes of each specimen is proportional to its genetic information availability score (GIS, see File S1 for score definition details). **B** Percentages of each cranial variable's contribution to the first two principal components (Dim1 and Dim2).

is closely associated with *Molossus* sp. nov. However, as will be discussed further, the fragmentary availability of sequences representing *Molossus* sp. 2 dictates a cautious approach in its treatment.

Comparative morphometric analysis of genetically (re)assigned specimens

In order to contrast the above inferred phylogeny with morphological data, we undertook morphometric comparisons using a subset of genetically identified specimens from which we have enough confident linear metric external and cranial measurements. *Molossus* sp. nov. clearly differentiates from *M. fluminensis* by consistently exhibiting lower linear measurement values across all external and cranial variables evaluated (one way ANOVA, Fig. 2). However, *Molossus* sp. nov. tends to overlap with the other small species, being *M. molossus* the more distinguishable specially when considering the skull measurements (Fig. 2; File S1 [Table SM2] for the analysis with genetically defined specimens plus misidentified genetically specimens). Furthermore, between *Molossus* sp. nov. and the recently described *M. melini* only three out of fourteen linear metric cranial variables evaluated were statistically different ($p<0.05$): LMxT, PC, and LMdT (Fig. 2).

Global skull size variation between species, analyzed by PCA using a subset of cranial measurements of genetically confirmed specimens of *M. fluminensis*, *M. melini*, *M. molossus*, and *Molossus* sp. nov. (see File S1 for specimen details), showed that the convex hull enclosing the specimens of *Molossus* sp. nov. maps separated from *M. molossus* ($p<0.001$) and *M. fluminensis* ($p<0.003$) clusters and in a lesser extent with *M. melini* ($p<0.05$) (Fig. 3; File S1 [Table SM4]). In this analysis, the first two components summarize 93% of the variation. PC2 explained only 5.3% of the variation, being clearly dominated by PC and GLS wo.i variables (Fig. 3B). The PC1 explained the largest amount of variation because most cranial vari-

Table 1. External and cranial measurements of the type series of *Molossus paranaensis* **sp. nov.** For descriptions of the abbreviations of measurements see "Methods".

	Holotype MFA-ZV-M 1494		Paratypes								
		INALI-A 389	INALI-A 390	INALI-A 457	INALI-A 588	INALI-A 589	MFA-ZV-M 1414	MG-ZV-M 176	MG-ZV-M 208	MACN-Ma 30420	MACN-Ma 30878
Sex	male	female	female	female	male	male	male	female	female	male	male
ToL	107.5	—	99	98	115	100	92	109.14	103	90.3	112.2
TL	40.5	—	39	—	38	33	35	35.4	37	29.5	37.4
HFL	8	—	8.5	8.5	8.5	9.2	7.2	10.73	9	6.7	6.2
EL	14.5	—	12	—	11	11.8	8.3	12	12	10	11
FA	42.84	39.4	41	42.7	40.7	40.6	41.8	40.68	41.3	39.4	41.4
W	22	—	—	17.3	23.5	22	—	22	19	—	18.8
CBL	18.8	16.3	16.5	16.7	18.2	17.2	—	16	16.7	—	16.9
ZB	12	11.4	11.1	11.5	12.2	12.3	—	11.4	11.5	—	11.2
GLS w.i	19	18.2	18.6	18.7	19.2	18.9	—	18.7	18.3	—	17.3
GLS wo.i	18.6	17.7	17.4	16.2	16.8	16.5	—	17.7	17.6	—	16.6
PC	4.3	4.3	4.2	4.5	4.6	4.6	—	4.3	4.2	—	3.8
BB	9.2	10.1	9.6	9.6	9.9	9.9	—	9.2	9.2	—	8.8
LMxT	7.4	6.7	6.5	6.9	7.2	7.2	—	6.8	6.9	—	6.3
PL	6.6	5.5	5.6	5.8	6.1	6.5	—	5.7	5.4	—	6.1
MB	10.4	11	10.1	11.2	11.5	11.6	—	10	10.2	—	10.8
LM	13.5	12.5	12.3	12.7	13.3	13.2	—	12.7	12.6	—	12.3
LMdT	7.9	7.8	7.3	7.4	7.6	8	—	7.5	7.7	—	7.5
C-C	5	4.9	4.6	5.3	5.5	5.4	—	4.8	5	—	4.5
M2-M2	8.6	8.6	8.2	8.4	9.1	8.2	—	8	8.1	—	7.9
SAR	1.2	1	1	0.9	1	1.2	—	1.2	0.9	—	—

ables contribute to it with almost equal strength (Fig. 3B). Accordingly, the centroids of species groups are distributed along PC1 from the smallest (*M. molossus*, *M. melini*, and *Molossus* sp. nov.) on the left to the largest (*M. fluminensis*) on the right side (Fig. 3A).

Based on the genetic and morphometric evidence shown above, we describe the members of the lineage *Molossus* sp. nov. as a new species, as follows:

Family Molossidae Gervais, 1856

Genus *Molossus* É. Geoffroy, 1805

Molossus paranaensis Caraballo, Pavé, Argoitia, Schierloh & Chambi Velasquez sp. nov.

https://zoobank.org/8608EDEB-5643-4482-9E73-C7699832742E

Chresonymy.

Molossus molossus – Pavé et al. (2017: 158)
Molossus molossus – Caraballo et al. (2020: 4)
Molossus molossus – Montani et al. (2021: 17)
Molossus molossus – Pavé et al. (2021: 17)
Molossus molossus – Pavé et al. (2023: 423)

Holotype. MFA-ZV-M 1494, adult male, preserved as skin, skull, and postcranial skeleton (Figs 4, 5), collected on 20 January 2018 by M. E. Montani (MEM) and V. Colombo (field number MEM 237). External and craniodental measurements for the type series are presented in Table 1. — *Type locality*. Sociedad Rural "Las Colonias", Esperanza, Santa Fe province, Argentina (lat. –31.4257, long. –60.9912, 44 m). This is a rural area in the Espinal ecoregion (sensu Olson et al. 2001) where the natural vegetation has been highly transformed in farmlands and there is very little tree vegetation. At the site, there are native tree species such as *Tipuana tipu* (Fabaceae) as well as exotic species like *Eucalyptus* sp. (Myrtales) and *Fraxinus* sp. (Oleaceae). Nine kilometers northeast of this site lies the Martín de la Peña Natural Reserve, characterized by its native vegetation.

Paratypes. INALI-A 389 and 390 adult females, with INALI-A 389 preserved as a skull specimen only, while both specimens are preserved in 70% alcohol, collected on October 2017 at "Desvío Arijón", San Jerónimo, Santa Fe province, Argentina (lat. –31.88, long. –60.89, 17 m); MG-ZV-M 176 adult female preserved as skin, skull and postcranial skeleton, collected on 16 May 2015 at "Reserva Hídrica Río Carcarañá", Area Natural Protegida, Pueblo Andino, Iriondo, Santa Fe province, Argentina (lat. –32.67, long. –60.87, 25 m); MG-ZV-M 208 adult female preserved as skin, skull, and postcranial skeleton, collected on 7 May 2016 at "Parque Villarino", Zavalla, Rosario, Santa Fe province, Argentina (lat. –33.03, long.

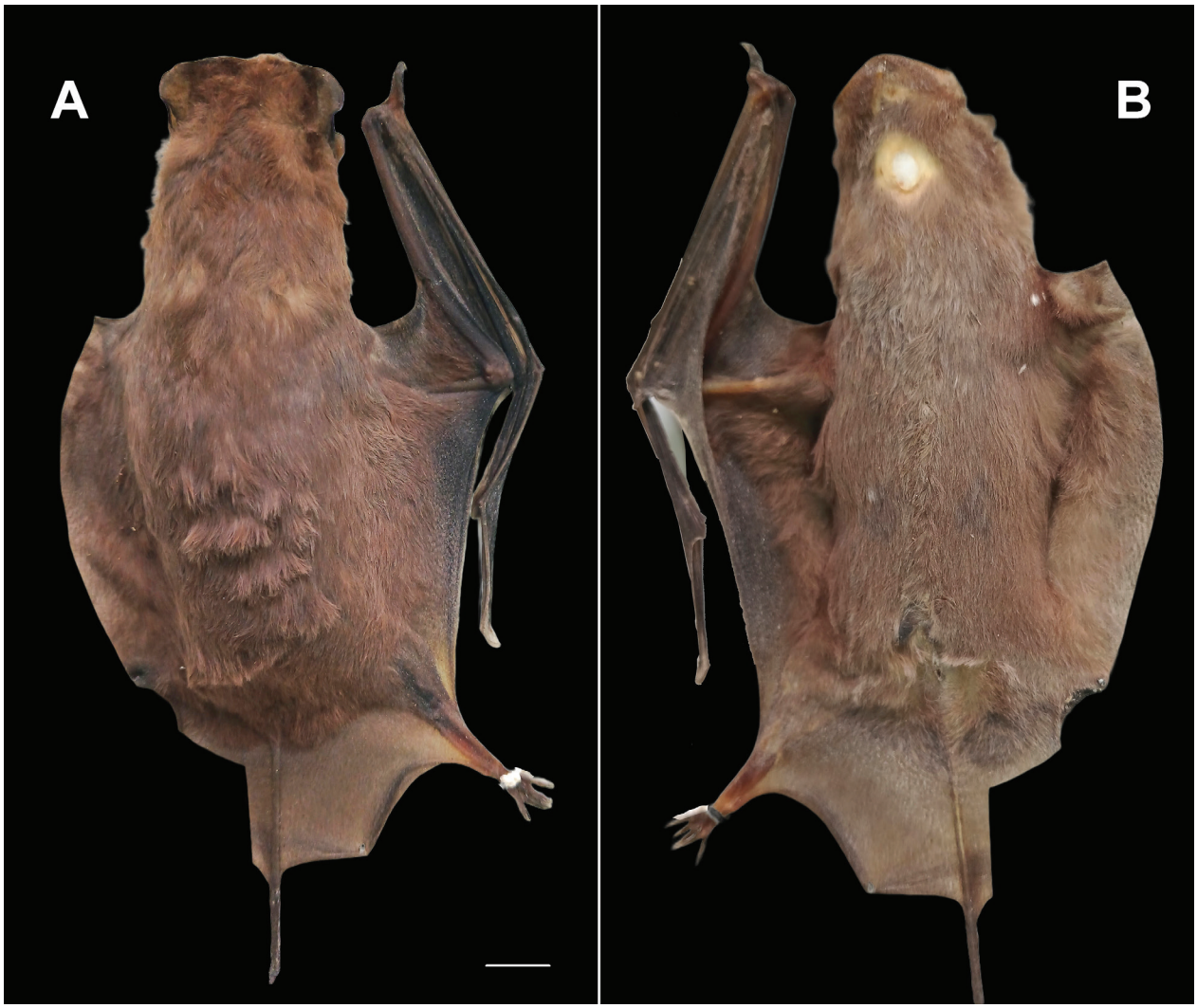

Figure 4. Dorsal (A) and ventral (B) views of the skin of the holotype of *Molossus paranaensis* (MFA-ZV-M 1494), adult male. Scale bar = 10 mm.

–60.89, 57 m); MG-ZV-M 296 and MFA-ZV-M 1414 subadult males preserved in 70% alcohol from Rosario, Santa Fe province, Argentina (lat. –32.69, long. –60.72, 30 m); INALI-A 457 adult female preserved as skull and in 70% alcohol, collected on 7 March 2018 at "Estancia Las Gamas", Vera, Santa Fe province, Argentina (lat. –29.42, long. –60.38, 62 m); INALI-A 588 and 589 adult males with scrotal testes, preserved as skin, skull and postcranial skeleton the first and in 70% alcohol the second, collected on 8 December 2018 at "Establecimiento Inchala", La Picada, Paraná, Entre Ríos province, Argentina (lat. –31.74, long. –60.26, 29 m); MACN-Ma 30878 an adult male preserved as skull and in 70% alcohol, collected on 7 April 2017 at "Campus Universitario FaCENA-UNNE", Corrientes city, Corrientes province, Argentina (lat. –27.47, long. –58.78, 61 m); MACN-Ma 30420 adult male preserved in 70% alcohol from Tigre, Buenos Aires province, Argentina (lat. –34.42, long. –58.57, 4 m).

Other specimens. Five individuals, three males and two females, were captured using mist nets at "Sociedad Rural", Santa Fe city, Santa Fe province, Argentina (lat. –31.63, long. –60.71, 20 m), obtained tissue samples, marked with a haircut and then released (collector Valeria Colombo number: MR 192, 193, 194, 197, 198).

Distribution and habitat. This species is known from ten localities in four provinces of eastern Argentina (Buenos Aires, Corrientes, Entre Ríos, and Santa Fe) in the Espinal, Humid Chaco, Humid Pampas, and Paraná Flooded Savanna ecoregions (sensu Olson et al. 2001). *Molossus paranaensis* inhabits natural environments in pampas grasslands, dry shrublands and wetlands, and anthropic environments such as human constructions in cities and agricultural fields.

It is conceivable that this newly discovered species may have a wider distribution, particularly within the region influenced by the Paraná River.

Etymology. The name *paranaensis* is bestowed in reference to the extended distribution of the new species along the Paraná River basin, one of the largest rivers in South America. Paraná is a word from the Mbyá people who speak Tupí (one of the native languages in Argentina), pará = "sea" and nã = "similar to" or "like", which means

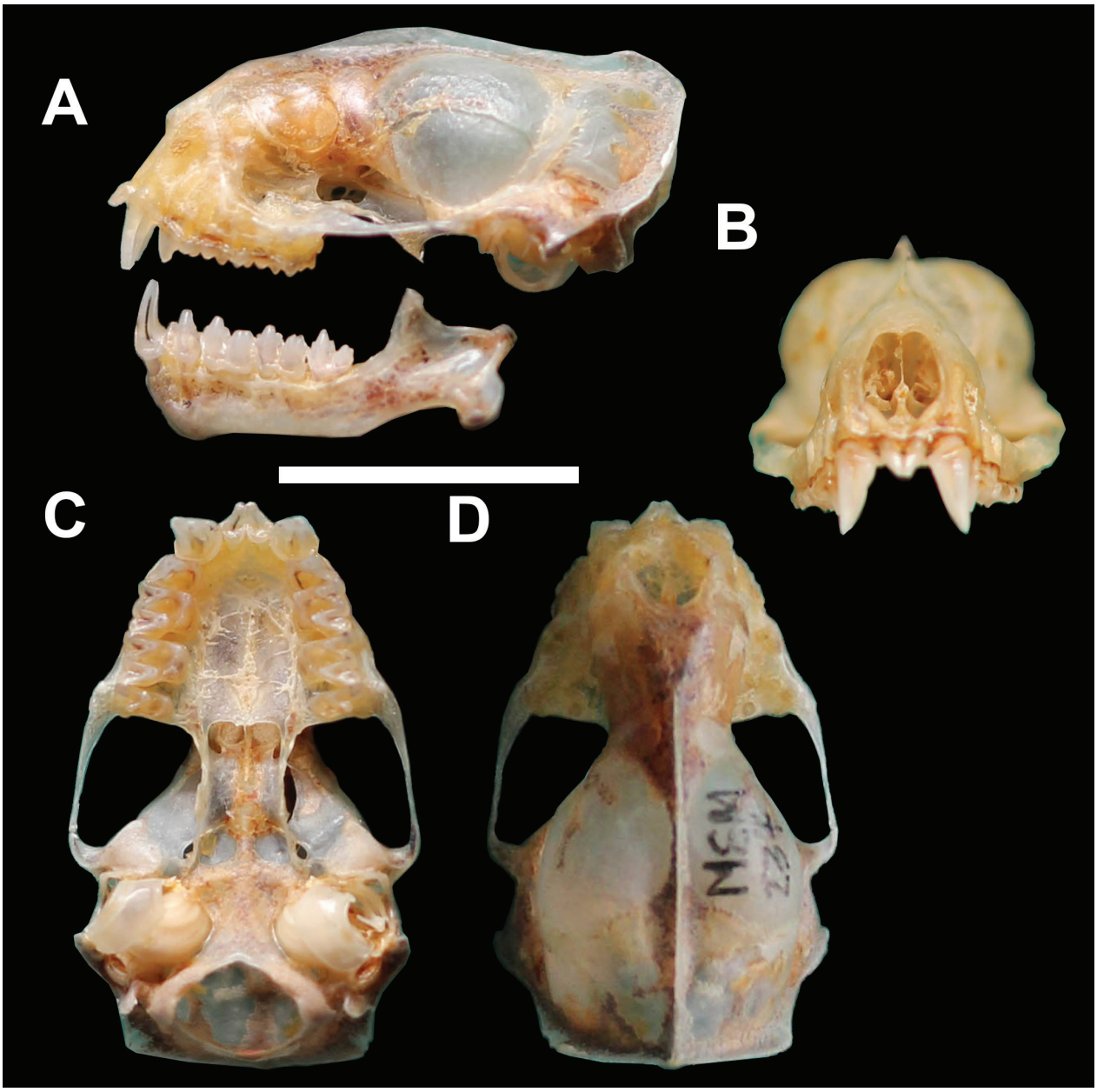

Figure 5. Lateral view of skull and mandible (A), and frontal (B), ventral (C) and dorsal (E) views of the skull of the holotype of *Molossus paranaensis* (MF-ZV-M 1494). Scale bar = 10 mm.

"that looks like the sea" or "similar to the sea". This river shelters a great biodiversity and natural beauty.

Diagnosis. *Molossus paranaensis* is distinguished from all other *Molossus* species by the following combination of characters: medium size (FA 39.4–42.8 mm; GLS w.i 17.3–19.2 mm; PC 3.8–4.6 mm; LMxT 6.3–7.4 mm); dorsal coloration medium brown (cinnamon to grayish brown sensu Ridgway 1912) with individual hairs bicolored, with a large and pale basal band reaching 1/4 to 1/2 to of total length of the hair, the venter is paler than dorsum (Fig. 4); in frontal view the rostrum is triangular (Fig. 5B), and the lambdoidal crests are moderately developed (Fig. 5).

Description. The new species is a medium-sized *Molossus* (ToL: 90.31–115 mm; FA: 39.4–42.84 mm, n = 13 specimens, 6 males and 7 females). Sexual dimorphism is observed in certain variables (body mass, ToL, CBL, MB, PL, C-C; see Table 1). Rostrum with a well-developed and straight keel, and the upper lip with a fringe of medium brown hard hairs on the border forming an upwardly projected mustache. Triangular and medium-sized ears (10–14.5 mm total length) with an oval-shaped antitragus that has an anterobasal constriction. Dorsal hairs measuring 5 mm long, markedly bicolored with a large pale band on the base from white to cream and the tips are variable from cinnamon to grayish brown (the holotype is cinnamon). Ventral hairs measuring 5 mm long, paler than the dorsal hairs and bicolored with grayish brown tips (buffy olive or citrine drab sensu Ridgway 1912; Fig. 4) and white or cream bases; ventrally, the pelage extends onto the plagiopatagium along the side of the body from the elbow to the knee. Wing membranes and uropatagium are

Table 2. Diagnostic external and cranial characters for each *Molossus* species following Loureiro et al. (2018b, 2019, 2020) and Montani et al. (2021). References: NA, not available; *: data from this study.

Species	Forearm (mm)	Dorsal hair color	Dorsal hair basal band	GLS with incisors (mm)	Upper incisors	Occipital region	Infra-orbital foramen direction	Format of rostrum
M. alvarezi	> 42	Chocolate (cocoa) brown	Bicolor. Large band (1/2)	19.3 (19.0–20.1)	Pincer-like	Triangular	Lateral	NA
M. aztecus	< 42	Dark brown	Unicolor	17.2 (16.5–18.3)	Spatulated	Quadrangular	Lateral	Triangular
M. bondae	< 44	Dark to reddish brown (coffee brown to blackish)	Unicolor	NA (17.3–19.4)	Spatulated	Quadrangular	Lateral	NA
M. coibensis	< 38	Medium to dark brown (cocoa brown to blackish)	Unicolor	16.0 (14.9–16.9)	Spatulated	Quadrangular	Frontal	Square
M. currentium	< 45	Dark brown (coffee brown to blackish)	Bicolor	18.3 (17.9–19.4)	Spatulated	Quadrangular	Lateral	Square
M. fentoni	< 36	Medium to dark brown	Bicolor. Short band (1/4)	15.3 (15.2–16.8)	Pincer-like	Triangular	Lateral	NA
M. fluminensis	> 46	Dark brown to blackish	Uni or bicolor. Short band (1/4)	NA (19.0–23.2)	Pincer-like	Quadrangular	Lateral	Triangular
M. melini	> 41	Orange to dark brown (yellow ocher, clay, cinnamon and grayish brown)	Bicolor. Short band (1/4)	18.7 (17.8–19.5)	Pincer-like	Triangular	Lateral	Triangular*
M. milleri	< 40	Medium to dark brown	Bicolor. Variable band (1/4–1/2)	16.1 (15.8–16.4)	Pincer-like	Triangular	Frontal	NA
M. molossus	< 40	Light to dark brown (cinnamon to cocoa and grayish brown)	Bicolor. Variable band (1/4–1/2)	17.4* (17.0–17.9)*	Pincer-like	Triangular	Frontal	Square
M. nigricans	> 47	Dark brown to blackish	Uni or bicolor. Short band (1/4)	20.1–24.1	Pincer-like	Quadrangular	Lateral	Triangular
M. paranaensis	> 39*	Medium brown (cinnamon to grayish brown)*	Bicolor. Variable band (1/4–1/2)*	18.5 (17.3–19.2)*	Pincer-like*	Triangular*	Frontal*	Triangular*
M. pretiosus	> 44	Dark brown to blackish or reddish (burnt umber to tawny)	Uni or Bbicolor. Short band (1/3)	20.5 (18.9–22.4)	Spatulated	Quadrangular	Lateral	Square
M. rufus	> 46	Dark brown to blackish	Uni or bicolor. Short band (1/4)	22.1 (19.9–23.8)	Pincer-like	Quadrangular	Lateral	Triangular
M. sinaloae	> 46	Dull, dark brown	Bicolor. Large band (2/3)	21.0 (19.4–22.4)	Pincer-like	Triangular	Lateral	Triangular
M. verrilli	< 41	Medium to dark brown	Bicolor. Large band (1/2)	17.2 (17.0–17.4)	Pincer-like	Triangular	Frontal	NA

pale brown. Feet have thickened first and fifth toes with short and curved hairs and all the toes are bordered with long, curved, and light hairs. The calcar is well-developed, longer than the hindfoot, and occupying ⅔ of the uropatagium edge.

The skull is elongated with a short rostrum (compared to the braincase); the infra-orbital foramen is frontally directed; the nasal cavity is taller than wide (in dorsal view the posterior edge of the nasal cavity is triangular); basisphenoid and basioccipitals pits moderately deep although the first more than the second; the occipital is triangular in posterior view; the sagittal crest has low development and the lambdoidal crest is moderately developed and has a quadrangular shape in posterior view as in *M. fluminensis*, both crests are more pronounced in males. The post-tympanic process of the squamosal is well developed and this is more visible dorsally (Fig. 5D). The dental formula is I 1/1, C 1/1, P 1/2, M 3/3, total = 26. The upper incisors are elongated, pincer-like, they are projected beyond the canines, and with parallel tips (Fig. 5A, B).

Comparisons. *Molossus paranaensis* is a medium-sized *Molossus* similar to *M. bondae*, *M. currentium*, *M. molossus*, *M. melini*, and *M. verrilli* (Loureiro et al. 2018a). The new species can be easily distinguished from *M. bondae* and *M. verrilli* by dorsal fur coloration and secondarily by geographical distribution. From the species occurring in Argentina, *M. paranaensis* differs from *M. currentium* in dorsal coloration and in some cranial characters such as the shape of upper incisors (see Table 2); from *M. molossus* in the length of forearm (in general < 40 mm in *M. molossus* and 39.4–42.8 mm in *M. paranaensis*) and LMTx (5.7–6.4 mm in *M. molossus* and 6.3–7.4 mm in *M. paranaensis*), the format of the rostrum in frontal view

(triangular in *M. paranaensis* and square in *M. molossus*; Loureiro et al. 2018a), the development of lambdoidal crests (moderately developed in *M. paranaensis* and underdeveloped in *M. molossus*), and in several cranial measurements (see Figs 2, 3; Table 2); from *M. melini*, in addition to the ochraceous or orange individuals of this last species, in the color of membranes (pale brown in *M. paranaensis* and dark brown in *M. melini*), in the direction of the infra-orbital foramen (frontal in *M. paranaensis* and lateral in *M. melini*), in the development of sagittal crest (higher in *M. melini*), and in the length of LMxT (6.0–7.0 mm in *M. melini* and 6.3–7.4 mm in *M. paranaensis*), PC (3.7–4.2 mm in *M. melini* and 3.8–4.6 mm in *M. paranaensis*), and LMdT (6.4–7.9 mm in *M. melini* and 7.3–8.0 mm in *M. paranaensis*) (Figs 2, 3; Table 2).

Molossus paranaensis can be readily differentiated from larger-sized *Molossus*, such as *M. alvarezi*, *M. fluminensis*, *M. nigricans*, *M. pretiosus*, *M. rufus*, and *M. sinaloae*, all of which have forearm longer than 42.8 mm and dorsal color in general dark brown. Furthermore, among large-sized species, *M. paranaensis* occurs in sympatry only with *M. fluminensis*. Finally, *M. paranaensis* differs from *M. aztecus*, *M. coibensis*, *M. fentoni*, and *M. milleri* due to its larger forearm (see Table 2). Additionally, *M. aztecus* and *M. coibensis* exhibit unicolored dorsal hairs, spatulated upper incisors, and a quadrangular occipital region, while *M. fentoni* and *M. milleri* are characterized by their dark fur. The external and cranial differences distinguishing *M. paranaensis* from the other *Molossus* species are presented in Table 2.

Discussion

As species are essential units of analysis in biology, their delimitation is the most fundamental aspect of systematics. However, there are many distinct, and partially incompatible, epistemological views of the species concept that emerge from considering different biological features (reviewed by Mayden 1997; de Queiroz 2005). In this study, we adopt two of the most commonly used species concepts to analyze the diversity of *Molossus* in Argentina. The evolutionary species concept, as a general principle, posits that a species constitutes a lineage resulting from populations or metapopulations evolving independently from any other lineage (Wiley and Mayden 2000; Zachos 2016). In a similar vein, the phylogenetic species concept proposes that a species represents a lineage that is evolutionarily distinct from any other, with its constituent populations forming a monophyletic group discernible through the pattern of character distribution, be it morphological or molecular (Wheeler 1999; Zachos 2016). In this study, we integrated molecular phylogenetic and morphological approaches to revise the diversity of *Molossus* at the southernmost limits of their distribution. The complexity of this genus, where both cases of morphological and genetic conservatism have been reported, makes the delimitation of species a difficult, at least non-straightforward, task. We consider that a species can be defined as a monophyletic group in the species-level phylogeny, that has enough genetic divergence from other species, and that can (or not) be morphologically distinguishable from other species of the genus. In this sense, we allow for the recognition of cryptic and non-cryptic species.

In this study, we created a multi-locus reference dataset for precise *Molossus* species-level identification. The first obstacle that we found in this way was to distinguish erroneously annotated sequences from possible biologically admissible conflicts (see a detailed discussion of this aspect in File S2). As mentioned above, the presence of cryptic taxa may contribute to this problem. In addition to morphological species misidentification, the lack of taxonomic updating in public databases also contributes to conflict in sequence annotation. To address these concerns, we implemented a strategy involving a thorough review of the current taxonomy and distribution of all species within the genus (see File S2).

Three groups of cryptic taxa are currently recognized in *Molossus*: 1) *M. molossus*, *M. fentoni*, *M. milleri*, and *M. verrilli* (Loureiro et al. 2018b); 2) *M. rufus*, *M. nigricans*, and *M. fluminensis* (Loureiro et al. 2020); 3) *M. bondae* and *M. currentium* (Loureiro et al. 2020). Regarding this aspect, it is important to distinguish between true cryptic species and pseudo-cryptic species. The latter can be discerned through fine-scale morphological analyses, although they may appear cryptic when using traditional morphological techniques (Lajus et al. 2015). One example of pseudo-cryptic species among bats is that of *Chiroderma gorgasi* and *C. trinitatum*, which are genetically distinguishable but only differ in the presence/absence of an accessory cusp on the second lower premolar (Lim et al. 2020).

In addition to recognized species, we could successfully identify two cryptic/pseudo-cryptic lineages that were previously classified as *M. molossus*. *Molossus* spp. comprises two lineages, one distributed in Argentina (*M. paranaensis*), and the other in northern countries of South America (*Molossus* sp. 2). The separation between *M. paranaensis* and *M. molossus*, is supported by both the phylogeny and genetic distances which positions this value within the range of interspecific distances (Fig. 1). Interestingly, *Molossus* sp. 2 groups with *M. molossus* in the mtDNA phylogeny, but is allied to *M. paranaensis* in the full dataset, and, depicts similar levels of genetic differentiation with both, within the range of intraspecific variation (Fig. 1). A possible scenario is that *M. paranaensis* and *M. molossus* are different species, and that *Molossus* sp. 2 is the product of hybridization between either species or other unsampled taxa, given the geographical separation between *M. paranaensis* and *Molossus* sp. 2. However, the fragmentary availability of sequences representing *Molossus* sp. 2 (there is no single individual with both mtDNA loci sequenced) requires further analyses to corroborate this hypothesis.

The morphological analyses mirror the results of the molecular analysis. The PCA of 12 cranial morphomet-

ric measurements allowed to distinguish *M. paranaensis* from all other genetically defined species occurring in Argentina. However, taking into account that the distinction from *M. melini* is only slightly statistically significant, and that all *M. paranaensis* specimens analyzed were previously misclassified as *M. molossus*, according to their external morphology, we can consider these species as pseudo-cryptic (based on differences in morphology and morphometry showed above), as occurs with many other species groups in the genus. It is noteworthy that all genetically corroborated specimens of *M. molossus* are more differentiated from *M. paranaensis*, compared to those that were not sequenced (File S1 [Fig. SM1]). Although there could be some degree of misidentification among those samples, we decided to keep them in the analysis shown in File S1 [Figure SM1] because PCA aims to find principal components that capture the maximum variance, and to accurately estimate the variance and covariance of a dataset, a representative sample of data points is needed. It is notable that *M. currentium*, the only species that lacks genetically confirmed specimens in the PCA dataset (File S1 [Fig. SM1, Table SM3]), is sufficiently distant from *M. paranaensis*, corroborating that these are two different entities. Furthermore, in the exploratory 2D geometric morphometric analysis conducted for nasal cavity shape comparisons (File S1 [Fig. SM3]), it was observed that the species *M. melini*, which exhibits significant size overlap with *M. paranaensis* and *M. molossus*, distinctly separates from them. This observation suggests that subtle shape variations may not be captured when only size-based comparisons (classic morphometry) are considered.

The integration of morphological and molecular findings leads to the conclusion that all *Molossus* lineages present in Argentina represent non-cryptic species. Among them, *M. fluminensis* stands out as the most distinct, notably larger than other species. Despite similar sizes and external morphologies, *M. molossus*, *M. melini*, *M. currentium*, and *M. paranaensis* can be reliably distinguished by cranial characteristics, categorizing them as pseudo-cryptic species. While field observers may struggle to differentiate these taxa, detailed examination of cranial characters or molecular diagnostic techniques can definitively identify each species. Fur color has been suggested as a marker for species identification in these bats; however, fur coloration can vary across habitats,

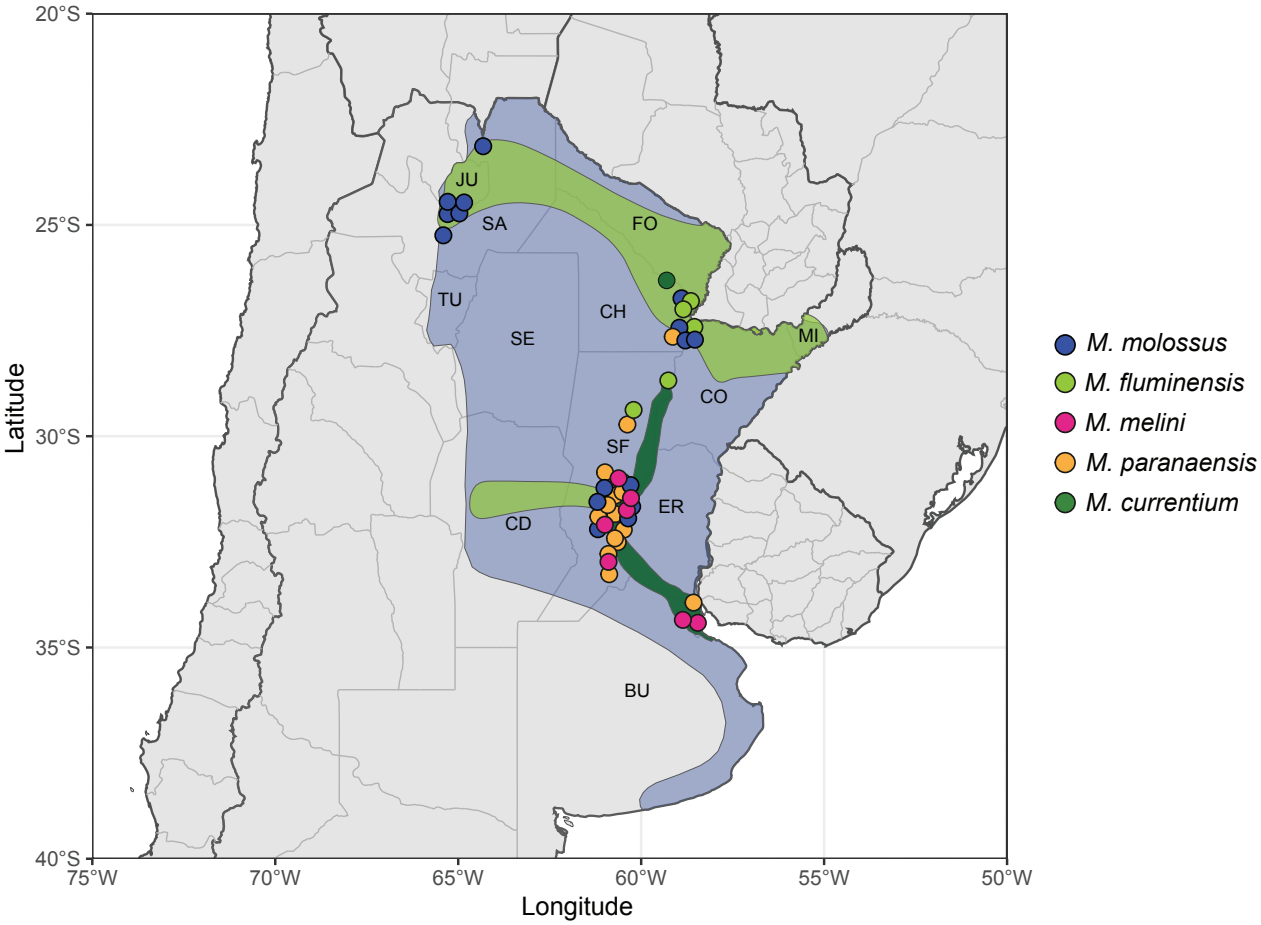

Figure 6. Map showing *Molossus* species distributions in Argentina according to Barquez and Díaz (2020) (*M. fluminensis* in light green, *M. currentium* in dark green and *M. molossus* in light blue) and the georeference records of the samples and their respective lineages identified in this study. Each species/lineage is shown with different colors: *M. molossus* (blue), *M. melini* (pink), *M. fluminensis* (bright green), *M. currentium* (dark green), and *M. paranaensis* (orange). Argentinian provinces where *Molossus* species are distributed are labelled with the following acronyms: JU (Jujuy), SA (Salta), FO (Formosa), CH (Chaco), MI (Misiones), CO (Corrientes), SF (Santa Fe), ER (Entre Ríos), CD (Córdoba), BU (Buenos Aires), TU (Tucumán), SE (Santiago del Estero).

leading to uncertain identifications. For instance, *M. melini* was previously distinguished from *M. molossus* by its characteristic ochraceous to orange fur coloration, but instances of chocolate to grayish brown specimens have been reported recently in Brazil and Argentina, indicating exceptions to this previously stated rule (Olímpio et al. 2024; this study) (Fig. S7).

The approach implemented in this study facilitated a revision of the diversity and geographic range of *Molossus* in Argentina, marking the southernmost limits of the genus' distribution. We confirmed the presence of three of the four species previously reported for the country, as well as reported the presence of the new species *M. paranaensis* (Fig. 6). *Molossus molossus* is the species with the broadest distribution, being the only species sampled in the northwestern region (Salta and Jujuy provinces) but also overlapping with the distribution of all remaining lineages, in agreement with previous assessments (Barquez and Díaz 2020). *Molossus fluminensis* was found in the northeastern region (Corrientes and center/north of Santa Fe), hence, according to our results, the distribution of the species (Barquez and Díaz 2020; Pavé et al. 2021) should be extended, being more likely that the two disjunct distributions are indeed connected (Fig. 6). *Molossus melini* was found in the south and center of Santa Fe and Entre Ríos, but also in Buenos Aires, expanding the known distributional boundaries of this species, which is characterized by an extremely limited distribution, with only four previous localities documented so far (Montani et al. 2021, 2023; Pavé et al. 2023; Olímpio et al. 2024). *Molossus paranaensis* is also distributed along the Paraná River, overlapping with *M. molossus*, *M. fluminensis*, and *M. melini*. Despite sampling mostly within the distribution range of *M. currentium*, no living samples compatible with this species were identified in our study. This result is particularly intriguing as the majority of our samples were collected from areas expected to harbor *M. currentium*, specifically the area of influence of the Paraná River (Barquez and Díaz 2020). One possibility that we considered is that *M. paranaensis* could indeed be *M. currentium*, but this was discarded by both molecular-phylogenetic and morphometric evidence. *Molossus currentium* appears to possess an elusive nature, as evidenced by the significant scarcity of available sequences and limited understanding regarding its distributional range and conservation status (Montani et al. 2019). Therefore, more research is needed to clarify its situation.

Conclusion

In this study, we conducted a review of *Molossus* bat diversity in Argentina and provided a curated dataset of genetic sequences. This dataset can serve as a valuable resource for testing the phylogenetic assignment of any *Molossus* specimen across its entire range. Additionally, we compiled a dataset of linear metric variables for five species within the genus, which inhabit central-east Argentina and south-east Brazil. This dataset can facilitate future morphometric comparisons. In the future, other lines of evidence such as echolocation data, or genomic approaches may complement the information provided in this study to have a deeper insight into the differences between the species of *Molossus*, especially those found at the southern limits of its distribution.

Based on molecular and morphometric evidence, we propose the existence of a novel species within the genus. We believe that this study can aid various stakeholders, including the research community, conservationists, policymakers, and zoonotic surveillance laboratories, to name some, in the challenging task of identifying unknown specimens. The systematics of this genus still has something to say.

Acknowledgments

We are grateful to the reviewers whose comments and suggestions have improved this manuscript. To all the people who contribute specimens to expand the biological collections. We would like to thank María Eugenia Montani of the vertebrate collection from the Museo Gallardo for her generosity in sample sharing and for a critical discussion of the main results of this study. We would also like to acknowledge Pablo Teta and Alejandro Giraudo, for their expert recommendations in the description of the species. We thank Alejandra Gavazza for some illustrations. To Leonardo Leiva, curator of the vertebrate collection from the Museo Florentino Ameghino and Germán Saigo, of the vertebrate collection from the Museo Gallardo, for providing access to and lending us specimens. Finally, we would like to thank Priscila Medeiros Olímpio for kindly sharing measurements of Brazilian samples. This study was funded by the grants PIP-0587 awarded by the National Scientific and Technical Research Council of Argentina to Diego Caraballo, PICT-2019-03347 awarded by the National Agency for the Promotion of Science and Technology (Ministry of Science) from Argentina to Valeria Colombo, the Instituto Nacional de Enfermedades Infecciosas (INEI-ANLIS) Dr. Carlos G. Malbrán to Daniel Cisterna, CAI+D Orientado 2016 (Res. C.S. N° 632/17), PIBAA (28720210100082CO) awarded by the National Scientific and Technical Research Council of Argentina (CONICET) to Romina Pavé, Universidad Nacional del Litoral and the Agencia Santafesina de Ciencia, Tecnología e Innovación (ASACTEI), Gobierno de la Provincia de Santa Fe (Código IO-2017-00068).

References

Baker RJ, Bradley RD (2006) Speciation in mammals and the genetic species concept. Journal of Mammalogy 87: 643–662. https://doi.org/10.1644/06-mamm-f-038r2.1

Baker RJ, McDonough MM, Swier VJ, Larsen PA, Carrera JP, Ammerman LK (2009a) New species of bonneted bat, genus *Eumops* (Chiroptera: Molossidae) from the lowlands of western Ecuador and Peru. Acta Chiropterologica 11: 1–13. https://doi.org/10.3161/150811009X465659

Baker AJ, Tavares ES, Elbourne RF (2009b) Countering criticisms of single mitochondrial DNA gene barcoding in birds. Molecular Ecology Resources 9: 257. https://doi.org/10.1111/j.1755-0998.2009.02650.x

Barquez RM, Díaz MM (2020) Nueva Guía de los Murciélagos de Argentina. Programa de Conservación de los Murciélagos de Argentina, Buenos Aires, 183 pp. http://hdl.handle.net/11336/156755

Barquez RM, Mares MA, Braun JK, Giannini N, Koopman KF (1999) The Bats of Argentina. Museum of Texas Tech University, Lubbock, TX, 275 pp.

Caraballo DA, Montani ME, Martínez LM, Antoniazzi LR, Sambrana TC, Fernández C, Cisterna DM, Beltrán FJ, Colombo VC (2020) Heterogeneous taxonomic resolution of cytochrome *b* gene identification of bats from Argentina: Implications for field studies. Yue B-S (Ed.). PLoS ONE 15: e0244750. https://doi.org/10.1371/journal.pone.0244750

Cláudio VC, Barbosa GP, Rassy FB, Rocha VJ, Moratelli R (2020) The bat fauna (Mammalia: Chiroptera) of Carlos Botelho State Park, Atlantic Forest of Southeastern Brazil, including new distribution records for the state of São Paulo. Zoologia 37: 1–32. https://doi.org/10.3897/zoologia.37.e36514.

De Benedictis P, Leopardi S, Markotter W, Velasco-Villa A (2022) The importance of accurate host species identification in the framework of rabies surveillance, control and elimination. Viruses 14: 492. https://doi.org/10.3390/v14030492

de Queiroz K (2005) Different species problems and their resolution. BioEssays 27: 1263–1269. https://doi.org/10.1002/bies.20325

Eger JL (2008) Bats. In: Gardner AL (Ed.) Mammals of South America. University of Chicago Press. Chicago, IL, 399–440.

Fisher-Reid MC, Wiens JJ (2011) What are the consequences of combining nuclear and mitochondrial data for phylogenetic analysis? Lessons from *Plethodon* salamanders and 13 other vertebrate clades. BMC Evolutionary Biology 11: 300. https://doi.org/10.1186/1471-2148-11-300

Gaston KJ, Spicer JI (2004) Biodiversity: An Introduction. 2nd Ed. Blackwell Publications, Malden, MA, 191 pp.

González-Ruiz N, Ramírez-Pulido J, Arroyo-Cabrales J (2011) A new species of mastiff bat (Chiroptera: Molossidae: *Molossus*) from Mexico. Mammalian Biology 76: 461–469. https://doi.org/10.1016/j.mambio.2010.06.004

Hebert PDN, Cywinska A, Ball SL (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society B 270: 313–321. https://doi.org/10.1098/rspb.2002.2218

Heywood VH, Watson RT, United Nations Environment Programme (Eds) (1995) Global Biodiversity Assessment: Summary for Policy-makers. Cambridge University Press, Cambridge, New York, NY, 46 pp.

Hurst GDD, Jiggins FM (2005) Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: The effects of inherited symbionts. Proceedings of the Royal Society B 272: 1525–1534. https://doi.org/10.1098/rspb.2005.3056

IUCN (2022) The IUCN Red List of Threatened Species. https://www.iucnredlist.org [accessed 2 June 2023].

Jones G, Jacobs D, Kunz T, Willig M, Racey P (2009) Carpe noctem: The importance of bats as bioindicators. Endangered Species Research 8: 93–115. https://doi.org/10.3354/esr00182

Lajus D, Sukhikh N, Alekseev V (2015) Cryptic or pseudocryptic: can morphological methods inform copepod taxonomy? An analysis of publications and a case study of the Eurytemora affinis species complex. Ecology and Evolution 5: 2374–2385. https://doi.org/10.1002/ece3.1521

Lê S, Josse J, Husson F (2008) FactoMineR: A package for multivariate analysis. Journal of Statistical Software 25: 1–18. https://doi.org/10.18637/jss.v025.i01

Leigh JW, Bryant D (2015) popart: Full-feature software for haplotype network construction. Methods in Ecology and Evolution 6: 1110–1116. https://doi.org/10.1111/2041-210X.12410

Lim BK (2017) Review of genetic diversification of bats in the Caribbean and biogeographic relationships to Neotropical species based on DNA barcodes. Genome 60: 65–73. https://doi.org/10.1139/gen-2015-0204

Lim BK, Arcila Hernandez LM (2016) DNA barcoding of Jamaican bats: Implications to Neotropical biodiversity. Mitochondrial DNA 27: 3013–3019. https://doi.org/10.3109/19401736.2015.1063047

Lim BK, Loureiro LO, Garbino, GST (2020) Cryptic diversity and range extension in the big-eyed bat genus *Chiroderma* (Chiroptera, Phyllostomidae). ZooKeys 918: 41–63. https://doi.org/10.3897/zookeys.918.48786

Lindsey LL, Ammerman LK (2016) Patterns of genetic diversification in a widely distributed species of bat, *Molossus molossus*. Museum of Texas Tech University 339: 1–15.

Loureiro LO, Lim BK, Engstrom MD (2018b) A new species of mastiff bat (Chiroptera, Molossidae, *Molossus*) from Guyana and Ecuador. Mammalian Biology 90: 10–21. https://doi.org/10.1016/j.mambio.2018.01.008

Loureiro LO, Gregorin R, Perini FA (2018a) Diversity, morphological phylogeny, and distribution of bats of the genus *Molossus* E. Geoffroy, 1805 (Chiroptera, Molossidae) in Brazil. Zoosystema 40: 425–452. https://doi.org/10.5252/zoosystema2018v40a18

Loureiro LO, Engstrom M, Lim B, González CL, Juste J (2019) Not all *Molossus* are created equal: Genetic variation in the mastiff bat reveals diversity masked by conservative morphology. Acta Chiropterologica 21: 51–64. https://doi.org/10.3161/15081109ACC2019.21.1.004

Loureiro LO, Engstrom MD, Lim BK (2020) Single nucleotide polymorphisms (SNPs) provide unprecedented resolution of species boundaries, phylogenetic relationships, and genetic diversity in the mastiff bats (*Molossus*). Molecular Phylogenetics and Evolution 143: 106690. https://doi.org/10.1016/j.ympev.2019.106690

Mayden RL (1997) A Hierarchy of Species Concepts: The Denouement in the Saga of the Species Problem. In: Claridge MF, Dawah HA, Wilson MR (Eds), Species: The units of diversity. Chapman & Hall, 381–423.

Montani ME, Díaz MM, Bárquez RM (2019) *Molossus currentium.* Categorización 2019 de los mamíferos de Argentina según su riesgo de extinción. Lista Roja de los mamíferos de Argentina, Buenos Aires. https://doi.org/10.31687/SaremLR.19.107 [accessed 10 March 2024].

Montani ME, Tomasco IH, Barberis IM, Romano MC, Barquez RM, Díaz MM (2021) A new species of *Molossus* (Chiroptera: Molossidae) from Argentina. Journal of Mammalogy 102: 1426–1442. https://doi.org/10.1093/jmammal/gyab078

Montani ME, Barberis IM, Días MM (2023) Nuevo registro de *Molossus melini* (Chiroptera, Molossidae) para Santa Fe, República Argentina. Notas sobre Mamíferos Sudamericanos 5. https://doi.org/10.31687/SaremNMS23.10.1 [accessed 10 March 2024].

Mora C, Tittensor DP, Adl S, Simpson AGB, Worm B (2011) How many species are there on earth and in the ocean? PLoS Biology 9: e1001127. https://doi.org/10.1371/journal.pbio.1001127

Moras LM, Gregorin R, Sattler T, Tavares VC (2018) Uncovering the diversity of dog-faced bats of the genus *Cynomops* (Chiroptera: Molossidae), with the re-description of *C. milleri* and the description of two new species. Mammalian Biology 89: 37–51. https://doi.org/10.1016/j.mambio.2017.12.005

Nylander J (2004) Mr Modeltest 2.2. Program distributed by author. Uppsala Evolutionary Biology Centre, Uppsala University.

Oksanen J, Kindt R, Legendre P, O'Hara B, Simpson GL, Solymos P, Stevens MHH, Wagner H (2008) vegan: Community Ecology Package. http://cran.r-project.org/, http://vegan.r-forge.r-project.org [accessed 10 March 2024].

Olímpio APM, Lima ACS, Mendes SB, Natividade BD, Fraga EC, Barros MC, Sampaio I (2024) *Molossus melini* Montani et al. 2021 (Chiroptera, Molossidae) in Brazil: New insights for distribution, morphology and genetics. Biodiversity Data Journal 12: e114261. https://doi.org/10.3897/BDJ.12.e114261

Olson DM, Dinerstein E, Wikramanayake ED, Burgess ND, Powell GVN, Underwood EC, D'amico JA, Itoua I, Strand HE, Morrison JC, Loucks CJ, Allnutt TF, Ricketts TH, Kura Y, Lamoreux JF, Wettengel WW, Hedao P, Kassem KR (2001) Terrestrial ecoregions of the World: A new map of life on Earth. BioScience 51: 933–938. https://doi.org/10.1641/0006-3568(2001)051[0933:TEOTWA]2.0.CO;2

Pavé R, Gavazza A, de Souza J, Giraudo AR (2021) Nuevos registros de murciélagos (Mammalia: Chiroptera) para las provincias de Entre Ríos y Santa Fe, Argentina. Revista del Museo Argentino de Ciencias Naturales N.S. 23: 15–25.

Pavé R, Cristaldi MA, Rodríguez ME, Barquez RM, Gavazza AI, Giraudo AR (2017) Nuevos registros de murciélagos para la provincia de Santa Fe, Argentina. Mastozoología Neotropical 24: 153–162.

Pavé R, Schierloh P, Chambi M, Piccirilli MG, Ulman S, Saavedra S, Cisterna DM, Caraballo DA (2023) Morphological and molecular analyses confirm the presence of the newly described bat species *Molossus melini* in the Espinal ecoregion from Argentina. Mammal Research 68: 417–425. https://doi.org/10.1007/s13364-023-00679-1

Pfenninger M, Schwenk K (2007) Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. BMC Evolutionary Biology 7: 121. https://doi.org/10.1186/1471-2148-7-121

Pimm SL, Jenkins CN, Abell R, Brooks TM, Gittleman JL, Joppa LN, Raven PH, Roberts CM, Sexton JO (2014) The biodiversity of species and their rates of extinction, distribution, and protection. Science 344: 1246752. https://doi.org/10.1126/science.1246752

R Core Team (2021) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org [accessed 10 March 2024].

Ratrimomanarivo FH, Vivian J, Goodman SM & Lamb J (2007) Morphological and molecular assessment of the specific status of *Mops midas* (Chiroptera: Molossidae) from Madagascar and Africa. African Zoology 42: 237–253. https://doi.org/10.3377/1562-7020(2007)42[237:MAMAOT]2.0.CO;2

Ridgway R (1912) Color standards and color nomenclature. Published by the author, Washington, DC. https://doi.org/10.5962/bhl.title.144788

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: Efficient Bayesian Phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539–542. https://doi.org/10.1093/sysbio/sys029

RStudio Team (2020) RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA. http://www.rstudio.com [accessed 10 March 2024].

Sikes RS, the Animal Care and Use Committee of the American Society of Mammalogists (2016) 2016 Guidelines of the American Society of Mammalogists for the use of wild mammals in research and education. Journal of Mammalogy 97: 663–688. https://doi.org/10.1093/jmammal/gyw078

Simmons NB, Cirranello AL (2024) Bat Species of the World: A Taxonomic and Geographic Database. Version 1.5. https://batnames.org [accessed 29 April 2024].

Spinks PQ, Shaffer HB (2009) Conflicting mitochondrial and nuclear phylogenies for the widely disjunct *Emys* (Testudines: Emydidae) species complex, and what they tell us about biogeography and hybridization. Systematic Biology 58: 1–20. https://doi.org/10.1093/sysbio/syp005

Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis version 11. Molecular Biology and Evolution 38: 3022–3027. https://doi.org/10.1093/molbev/msab120

Thomas O (1901) On a collection of bats from Paraguay. Annals and Magazine of Natural History 8: 435–443.

Tsang SM, Cirranello AL, Bates PJJ, Simmons NB (2016) The roles of taxonomy and systematics in bat conservation. In: Voigt CC, Kingston T (Eds) Bats in the Anthropocene: Conservation of Bats in a Changing World. Springer International Publishing, Cham, 503–538. https://doi.org/10.1007/978-3-319-25220-9_16

Wheeler QD (1999) Why the phylogenetic species concept?-Elementary. Journal of Nematology 31: 134–141.

Wickham H (2016) ggplot2: Elegant Graphics for Data Analysis. Springer, New York, NY https://ggplot2.tidyverse.org [accessed 29 April 2024].

Wiley EO, Mayden RL (2000) The evolutionary species concept. In: Wheeler QD, Meier R (Eds), Species Concepts and Phylogenetic Theory: a Debate. Columbia University Press, New York, NY, USA, 70–89.

Wilson DE, Mittermeier RA (2019) Volume 9: Bats. In: Wilson DE, Mittermeier RA (Eds) Handbook of the Mammals of the World. Lynx Edicions, Conservation International, IUCN, Barcelona, 1008.

Zachos FE (2016) Species concepts in biology: Historical development, theoretical foundations and practical relevance. Imprint: Springer, Cham.

Appendix

Specimens of *Molossus* included in this study.

For each specimen, the localities are listed alphabetically by province/state, department, and specific site, and between parentheses: Geographical coordinates, collection acronym and number. The biological collections and their specimens acronyms are: Natural History Museum (formerly British Museum of Natural History, London, United Kingdom (**BMNH**); Universidade Federal de São Carlos, Campus Sorocaba, Sorocaba, Brazil (**ZSP**); Colección de Mamíferos Lillo, San Miguel de Tucumán, Tucumán, Argentina (**CML**); Colección de Vertebrados of the Instituto Nacional de Limnología, La Capital, Santa Fe, Argentina (**INALI-A**); Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Ciudad Autónoma de Buenos Aires, Argentina (**MACN-Ma**); Colección de Mamíferos of the Museo Provincial de Ciencias Naturales "Florentino Ameghino", Santa Fe, Santa Fe, Argentina (**MFA-ZV-M**); Colección de Mamíferos of the Museo Provincial de Ciencias Naturales "Dr. Ángel Gallardo", Rosario, Santa Fe, Argentina (**MG-ZV-M**). Reference: * taken from bibliography.

With morphology and sequences:

Molossus fluminensis (n = 12). Argentina: Corrientes, Capital, Campus Universitário Deodoro Roca UNNE (lat. –27,47, long. –58,78; AMA 107, AMA 114, AMA 154); Santa Fe, General Obligado, Villa Ocampo, Portal del Humedal (lat. –28.501433, long. –59.264248; INALI A512); Santa Fe, Vera (lat. –29.46, long. –60.21; MFA-ZV-M 1423).

Molossus melini (n = 9). Argentina: Buenos Aires, Ciudad Autónoma de Buenos Aires (lat. –34.57, long. –58.45; P210); Buenos Aires, San Martín (lat. –34.57, long. –58.54; P500); Entre Ríos, Paraná, Paraná city (lat. –31.746864, long. –60.522371; INALI-A 635); Santa Fe, Rosario, Zavalla, "Parque Villarino" (lat. –33.03, long. –60.89; MG-ZV-M 225).

Molossus molossus (n = 21). Argentina: Corrientes, Capital, Campus Universitário Deodoro Roca UNNE (lat. –27.47, long. –58.78; AMA 10, AMA 15, AMA 26, AMA 65); Entre Ríos, Paraná (lat. –31.730, long. –60.527; MFA-ZV-M 1413); Santa Fe, La Capital, Santa Fe city (lat. –31.66, long. –60,71; MFA-ZV-M 1431, MFA-ZV-M 1435); Santa Fe, La Capital, Santa Fe city (lat. –31.633, long. –60.714; INALI-A 355); Salta, Orán (lat. –23.13, long. –64.32; P961); Jujuy, San Salvador de Jujuy (lat. –24.29, long. –65.29; MACN 30404, P249, P772); Salta, Salta (lat. –24.79, long. –65.41; MACN 30426).

Molossus paranaensis **sp. nov.** (n = 12). Argentina: Corrientes, Capital, Campus Universitário Deodoro Roca UNNE (lat. –27.47, long. –58.78; MACN 30878); Buenos Aires, Tigre (lat. –34,42, long. –58.57; MACN 30420); Entre Ríos, Paraná, La Picada, "Establecimiento Inchala" (lat. –31.736902, long. –60.260521; INALI-A 588, INALI-A 589); Santa Fe, Esperanza, Sociedad Rural "Las Colonias" (lat. –31.4257, long. –60.9912; MFA-ZV-M 1494); Santa Fe, San Jerónimo, Desvío Arijón (lat. –31.881212, long. –60.888899; INALI-A 389, INALI-A 390); Santa Fe, Rosario (lat. –32.94, long. –60.64; MFA-ZV-M 1414, MG-ZV-M 296), Santa Fe, Vera, Estancia "Las Gamas" (lat. –29.42319, long. –60.381607; INALI-A 457); Santa Fe, Iriondo, Pueblo Andino, Area Natural Protegida "Reserva Hídrica Río Carcarañá" (lat. –32.67, long. –60.87; MG-ZV-M 176); Santa Fe, Rosario, Zavalla, "Parque Villarino" (lat. –33.03, long. –60.89; MG-ZV-M 208).

Only with morphology:

Molossus currentium (n = 6). Argentina: Corrientes, Goya, Goya, 600 m (lat. –29.144, long. –59.264; BMNH 98.3.4.27*, BMNH 98.3.4.28 holotype*, BMNH 98.3.4.29*); Formosa, Pirané, El Colorado (lat. –26.309, long. –59.371; CML 1816*, CML 1817*). Brazil: São Paulo, Carlos Botelho State Park (lat. –24.183, long. –47.916; ZSP 050*).

Molossus fluminensis (n = 7). Argentina: Corrientes, Capital, Campus Universitário Deodoro Roca UNNE (lat. –27,47, long. –58,78; AMA 229, AMA 230); Corrientes, Concepción, Estancia "El Tránsito" (lat. –28.4221, long. –57.6939; INALI-A 114*, INALI-A 115*); Santa Fe, La Capital, Santa Fe city (lat. –31.63847, long. –60.68858; INALI-A 315*); Santa Fe, General Obligado, Villa Ocampo, Portal del Humedal (lat. –28.501433, long. –59.264248; INALI-A 513*); Villa Ocampo, Puerto Ocampo (lat. –28.520952, long. –59.123816; INALI-A 694*).

Molossus melini (n = 3). Argentina: Entre Ríos, Paraná, Paraná city (lat. –31.746864, long. –60.522371; INALI-A 651*, INALI-A 652*, INALI-A 653*); Santa Fe, Caseros, San José de la Esquina, Camping Comunal "El Río"(lat. –33.0933806, long. –61.7054111; MG-ZV-M 472*).

Molossus molossus (n = 8). Argentina: Entre Ríos, Paraná, Paraná city (lat. –31.730, long. –60.527; INALI-A 572*, INALI-A 712*); Santa Fe, La Capital, Ciudad Universitaria UNL (lat. –31.639771, long. –60.671765; INALI-A 150*, INALI-A 170*); Santa Fe, La Capital, Santa Fe city (lat. –31.633, long. –60.714; INALI-A 286, INALI-A 402*); Santa Fe, La Capital, Paraje Chaco Chico (lat. –31.56505, long. –60.639862; INALI-A 619*).

Molossus paranaensis **sp. nov.** (n = 3). Argentina: Santa Fe, La Capital, Santo Tomé (lat. –31.673, long. –60.774; INALI-A 147*, INALI-A 148*); Santa Fe, San Jerónimo, Desvío Arijón (lat. –31.881212, long. –60.888899; INALI-A 389*).

Only with sequences:

Molossus molossus (n = 3). Argentina: Jujuy, San Salvador de Jujuy (lat. –24.29, long. –65.29; P308); Santa Fe, La Capital, Santa Fe city (lat. –31.66, long. –60.71; 177, 190, released).

Molossus paranaensis **sp. nov.** (n = 5). Argentina: Santa Fe, La Capital, Santa Fe city, "La Rural" (lat. –31.63, long. –60.71; 192, 193, 194, 197, 198, released).

Molossus melini (n = 3). Argentina: Santa Fe, Esperanza, Sociedad Rural "Las Colonias" (lat. –31.4257, long. –60.9912, 130 released); Santa Fe, La Capital, Santa Fe city, "La Rural" (lat. –31.63, long. –60.71; 196, 199, released).

Supplementary Material 1

Figures S1–S7

Authors: Chambi Velasquez MA, Pavé R, Argoitia MA, Schierloh P, Piccirilli MG, Colombo VC, Beltrán FJ, Cisterna DM, Caraballo, DA (2024)

Data type: .pdf

Explanation notes: Figure S1. *Molossus* species distributions updated according to current taxonomy. The distribution shapes were downloaded from the IUCN Red List of Threatened species, and updated following Loureiro et al. (2020). *Molossus rufus* was subdivided in *M. rufus*, *M. fluminensis*, and *M. nigricans*. The distribution shapes of *M. fentoni*, *M. milleri*, *M. verrilli*, and of the subspecies *M. molossus daulensis* were created following Loureiro et al. (2020). The shape of *M. alvarezi* was updated following Loureiro et al. (2020), while the shape of *M. melini* was restricted to the points where the species was reported, following Montani et al. (2021, 2023), Pavé et al. (2023), and Olímpio et al. (2024). The shape of *M. pretiosus* was updated following Turcios-Casco et al. (2024) and Santos-Calvacante et al. (2024). — **Figure S2.** Bayesian tree obtained from partial sequences of the 7th intron of the gen B-fibrinogen (FGB). The colors of the branches indicate the species to which individuals were assigned based on external morphological characters. On the right, the names of the inferred groups based on phylogeny are shown. The size of the nodes is proportional to their posterior probability (red: 1–0.95; purple-cyan: 0.94–0.75; green-yellow: <0.75). The scale is expressed in substitutions per site. — **Figure S3.** Bayesian tree obtained from partial sequences of the mitochondrial gene cytochrome oxidase I (COI). The colors of the branches indicate the species to which individuals were assigned based on external morphological characters. On the right, the names of the inferred groups based on phylogeny are shown. The size of the nodes is proportional to their posterior probability (red: 1–0.95; purple-cyan: 0.94–0.75; green-yellow: <0.75). The scale is expressed in substitutions per site. — **Figure S4.** Bayesian tree obtained from partial sequences of the mitochondrial gene cytochrome *b* (cyt *b*). The colors of the branches indicate the species to which individuals were assigned based on external morphological characters. On the right, the names of the inferred groups based on phylogeny are shown. The size of the nodes is proportional to their posterior probability (red: 1–0.95; purple-cyan: 0.94–0.75; green-yellow: <0.75). The scale is expressed in substitutions per site. — **Figure S5.** Bayesian tree obtained from concatenated partial sequences of the mitochondrial genes cytochrome oxidase I (COI), and cytochrome *b* (cyt *b*). On the right, the names of the inferred groups based on phylogeny are shown. The size of the nodes is proportional to their posterior probability (red: 1–0.95; purple-cyan: 0.94–0.75; green-yellow: <0.75). The scale is expressed in substitutions per site. — **Figure S6.** Median-joining network for *Molossus*, constructed using cyt *b* and COI data. Mutational steps are represented by hatch marks and summarized within parentheses when exceeding 10. Observed haplotypes are indicated by colored circles, with the size reflecting the frequency of each haplotype, while black circles represent inferred hypothetical haplotypes. — **Figure S7.** Dorsal (A) and ventral (B) views of the skin of an adult female *Molossus melini* (MG-ZV-M 225) from Santa Fe province, Argentina. Scale bar = 10 mm.

Link: https://doi.org/vz.74.e122822.suppl1

Supplementary Material 2

Tables S1–S5

Authors: Chambi Velasquez MA, Pavé R, Argoitia MA, Schierloh P, Piccirilli MG, Colombo VC, Beltrán FJ, Cisterna DM, Caraballo, DA (2024)

Data type: .pdf

Explanation notes: Table S1. Sample ID, external morphology species identification, post-phylogeny species identification, state, locality, geographic coordinates, and GenBank Accession Numbers of the 47 specimens successfully sequenced in this study. — **Table S2.** Primers and PCR conditions for each locus analyzed in this study. — **Table S3.** Specimens/chimeras included in the multilocus dataset. We concatenated the three genes including, whenever possible, specimens with sequences available for the three loci. In cases when this was not achievable, we constructed chimeras ensuring these were formed by individuals of the same species, country and lineage in the single-locus trees. Letters denote different lineages of a given species while numbers enumerate the representatives of a same lineage. — **Table S4.** Intraspecific uncorrected pairwise distances of specimens/chimeras included in the concatenated

dataset (FGB, COI, cyt *b*). — **Table S5.** Interspecific uncorrected pairwise distances between all lineages included in the concatenated dataset (FGB, COI, cyt *b*).

Link: https://doi.org/vz.74.e122822.suppl2

Supplementary Material 3

File S1

Authors: Chambi Velasquez MA, Pavé R, Argoitia MA, Schierloh P, Piccirilli MG, Colombo VC, Beltrán FJ, Cisterna DM, Caraballo, DA (2024)

Data type: .pdf

Explanation notes: Extended methods, input data and results of the morphometric analyses [Tables SM1–SM4, Figures SM1–SM3[.

Link: https://doi.org/vz.74.e122822.suppl3

Supplementary Material 4

File S2

Authors: Chambi Velasquez MA, Pavé R, Argoitia MA, Schierloh P, Piccirilli MG, Colombo VC, Beltrán FJ, Cisterna DM, Caraballo, DA (2024)

Data type: .pdf

Explanation notes: Extended results and discussion of molecular phylogenies.

Link: https://doi.org/vz.74.e122822.suppl4

Supplementary Material 5

File S3

Authors: Chambi Velasquez MA, Pavé R, Argoitia MA, Schierloh P, Piccirilli MG, Colombo VC, Beltrán FJ, Cisterna DM, Caraballo, DA (2024)

Data type: .zip

Explanation notes: ZIP file containing the four annotated phylogenetic trees (FGB, COI, cyt *b*, mtDNA, concatenated multilocus data).

Link: https://doi.org/vz.74.e122822.suppl5