UFRGS
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PPGBAN
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA ANIMAL

INSTITUTO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA ANIMAL

DIEGO ALEJANDRO ESQUIVEL MELO

SISTEMÁTICA E EVOLUÇÃO DO GÊNERO DE MORCEGOS NEOTROPICAL *LOPHOSTOMA* d'Orbigny, 1836 (CHIROPTERA: PHYLLOSTOMIDAE)

PORTO ALEGRE
2021

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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Animal, Instituto de Biociências da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do título de Mestre em Biologia Animal.

Área de concentração: Biologia Comparada

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Dedico este trabalho à minha mãe Flor Alba Melo, pelo carinho, auxílio, energia, força e motivação despendidos durante minha trajetória acadêmica.

AGRADECIMENTOS

Eu gostaria de expressar a minha mais profunda gratidão ao Dr. Filipe Michels Bianchi pelo seu constante apoio, sua inesgotável paciência, suas valiosas ideias, sugestões e contribuições, assim como por compartilhar seu conhecimento e historias ao longo desses dois anos. Agradeço-o por me aceitar como orientado e por aceitar o desafio de entrar num novo mundo dominado pelos animais mais fantásticos que nós temos: os morcegos. Acredito que, mais do que se tornar meu orientador, ele se tornou um grande amigo.

Agradeço também à Profª. Drª Maria João Ramos pela oportunidade e confiança em me aceitar como orientado, pelo acompanhamento, incentivo, e tempo na minha formação. Também quero agradecer por compartilhar o gosto pelos morcegos, pelo aprendizado que me propiciou e, sobretudo, pela grande amizade que resultou deste trabalho.

Um agradecimento muito especial à Angie Penagos, minha companheira em todos os momentos, bons ou ruins, pelo carinho, amizade, paciência, ajuda e por tanto amor. Por se aventurar em sair de casa comigo e lutar pelos os nossos sonhos. Para você toda minha admiração, respeito e amor!

À minha mãe e irmã, por serem as propulsoras que me ajudaram a chegar até aqui, longe de casa, por trás de um ideal, um sonho de me formar no Brasil conseguindo entregar este trabalho, aqui.

Aos meus colegas de laboratório, pela amizade e a parceria, assim como por sua paciência para me compreender quando eu ainda nem falava alguma coisa em português.

Agradeço a UFRGS e ao Departamento de Zoologia pela oportunidade de ensino e pelo aprendizado obtido, assim como à bolsa de pesquisa financiada pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e auxílios fornecidos pelo PPGBAN.

Gracias totales!

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RESUMO

Morcegos *Lophostoma* d'Orbigny, 1836 (Chiroptera: Phyllostomidae) estão distribuídos desde o sul do México até o sudoeste do Paraguai e sudeste do Brasil. Este gênero inclui sete espécies de morcegos insetívoros, conhecidos como morcegos-de-orelhas-redondas. A sistemática e taxonomia do grupo têm sido objeto de diversos estudos, que utilizando dados morfológicos e moleculares revelaram uma enorme diversidade fenotípica e genotípica, indicando a possível existência de espécies crípticas, principalmente em *Lophostoma brasiliense* Peters, 1866 e *Lophostoma silvicola* d'Orbigny, 1836. O principal objetivo do presente estudo foi analisar a diversidade críptica de *Lophostoma* ao longo de sua distribuição geográfica, integrando análises moleculares, morfológicas e morfométricas para esclarecer os limites taxonômicos, distribuição e relações evolutivas das suas espécies. No primeiro capítulo, apresento uma avaliação taxonômica de *L. brasiliense*. Foram utilizados métodos de delimitação fenotípica e molecular das espécies com base no marcador mitocondrial *Citocromo c oxidase subunidade 1* (COI), dados morfológicos, morfométricos lineares e geométricos. Os resultados reconheceram *L. brasiliense* como duas linhagens distintas com distribuições alopátricas. Uma corresponde à *L. brasiliense sensu stricto*, com distribuição cis-andina; outra corresponde à *L. nicaraguae* (Goodwin, 1942), atualmente sinônimo de *L. brasiliense*, com distribuição transandina. O status de espécie válida é proposto para *L. nicaraguae* considerando as evidências apresentadas. No segundo capítulo, faço uma avaliação taxonômica de *L. silvicola* baseada em dois genes mitocondriais (COI, Cyt-*b*) e múltiplas evidências fenotípicas (morfologia qualitativa e quantitativa). As análises suportam a validação de duas entidades taxonômicas adicionais à *L. silvicola*: *L. laephotis* (Thomas, 1910) e *L. amblyotis* Peters, 1867. Por fim, apresento informações sobre a distribuição de cada uma das espécies aqui reconhecidas, bem como hipóteses sobre a sua diversificação, e discuto as possíveis implicações das barreiras geográficas na origem destas espécies.

Palavras chave: espécies crípticas, delimitação de espécies, Phyllostomidae, revisão taxonômica, taxonomia integrativa.

ABSTRACT

Bats of genus *Lophostoma* d'Orbigny, 1836 (Chiroptera: Phyllostomidae) are distributed from southern Mexico to southwestern Paraguay and southwestern Brazil. This genus includes seven species of insectivorous bats, which are known as round-eared bats. Systematics and taxonomy of the group have been the subject of several studies, which using morphological and molecular data revealed an enormous phenotypic and genetic diversity, indicating the possible existence of cryptic species, mainly in *Lophostoma brasiliense* Peters, 1866 and *Lophostoma silvicola* d'Orbigny, 1836. The main objective of the present study was to analyze the cryptic diversity of *Lophostoma* throughout its geographic distribution, integrating molecular, morphological and morphometric analyzes to clarify the taxonomic limits, distribution and evolutionary relationships of its species. In the first chapter, I present a taxonomic assessment of *L. brasiliense*. Phenotypic and molecular species delimitation methods were used based on the mitochondrial marker Cytochrome c oxidase subunit I (COI), morphological, linear and geometric morphometric data. The results recognized *L. brasiliense* as two distinct lineages with allopatric distributions. One corresponds to *L. brasiliense sensu stricto*, with a cis-Andean distribution; another corresponds to *L. nicaraguae* (Goodwin, 1942), currently synonymous with *L. brasiliense*, with trans-Andean distribution. Valid species status is proposed for *L. nicaraguae* considering the evidence presented. In the second chapter, I perform a taxonomic assessment of *L. silvicola* based on two mitochondrial genes (COI, Cyt-b) and multiple phenotypic evidences (qualitative and quantitative morphology). The analyzes supported the validation of two additional taxonomic entities to *L. silvicola*: *L. laephotis* (Thomas, 1910) and *L. amblyotis* Peters, 1867. Finally, I present information on the distribution of each of the species recognized here, as well as hypotheses about their diversification, and discuss the possible implications of geographic barriers in the origin of these species.

Key words: cryptic species, integrative taxonomy, species delimitation, Phyllostomidae, taxonomic revision.

CAPÍTULO I – Introdução Geral

Sistemática e Taxonomia: desafios e novas tendências

A sistemática e a taxonomia desempenham um papel fundamental no estudo da biodiversidade. A sistemática classifica, organiza e estabelece hipóteses sobre as relações evolutivas entre os táxons, enquanto a taxonomia os caracteriza e os nomeia (Mayr 1999). Ambas disciplinas têm implicações diretas sobre a conservação das espécies e possibilitam a compreensão dos processos ecológicos e evolutivos a que os organismos estão sujeitos (Huber & Langor 2004).

Após mais de dois séculos de avanços no conhecimento da biodiversidade sob a nomenclatura Lineana, os cientistas descreveram aproximadamente 1.9 milhões de espécies (Roskov, 2019), mas estima-se que a quantidade de espécies presentes na Terra possa estar entre 5 e 9 milhões (Mora et al., 2011). Assim, embora um conhecimento profundo de nossa biodiversidade seja essencial para diversas áreas da biologia, sejam básicas ou aplicadas, na verdade só conhecemos uma pequena parte dela. Essa lacuna de conhecimento taxonômico é chamada de déficit Lineano (Lomolino et al. 2004), e afeta drasticamente outras subáreas relacionadas à biologia, pois tem a ver com a unidade fundamental de qualquer estudo em ecologia, evolução, biogeografia e conservação: as espécies (Funk et al. 2002). A taxonomia busca reduzir esse déficit, descobrindo, delimitando e descrevendo as espécies.

Apesar de ser tão importante para a biodiversidade, a ciência por trás da delimitação do mundo natural em "espécies" é frequentemente negligenciada e mal compreendida. A taxonomia enfrenta vários desafios: i) a descrição de novas espécies pode ser um processo longo e mais lento do que seu desaparecimento, o que leva os taxonomistas a correrem contra o tempo (Chenuil et al. 2019); ii) não há taxonomistas suficientes para a elevada quantidade de grupos taxonômicos, o que nos permite supor que a taxa de extinção em organismos pouco estudados é muito maior do que a descoberta de novas espécies (Chenuil et al. 2019); iii) a taxonomia não recebe o necessário reconhecimento social e, subsequentemente, financiamento (Lee 2000; Agnarsson & Kuntner 2007).

Um desafio adicional é o desvendar de espécies crípticas, definidas como a ocorrência de diferentes linhagens evolutivas de organismos fenotipicamente semelhantes reconhecidas como uma mesma espécie (Bickford et al. 2007). Essas

espécies são muito semelhantes fenotipicamente, mas eventualmente distinguíveis do ponto de vista ecológico, comportamental ou molecular. Com efeito, historicamente, as delimitações taxonômicas têm sido baseadas em características morfológicas diagnósticas para separar populações e reconhecer diferenças entre espécies e subespécies (Matos-Maraví et al., 2019). No entanto, a delimitação de espécies com base somente na morfologia falha quando se trata de espécies crípticas, porque muitas vezes não se consegue fazer delimitações explícitas e claras entre elas (Struck et al., 2018).

Com o avanço de novas metodologias moleculares e de ferramentas e arcabouços estatísticos, novos conceitos de espécies têm sido propostos buscando delinear as espécies de forma mais adequada (ver Zachos 2016). Contudo, a delimitação das espécies depende do conceito de espécie utilizado e dos critérios de delimitação apropriados para esse conceito (Sites & Marshall, 2004; de Queiroz, 2007); portanto, o conceito usado afetará o número de espécies finais. Com o objetivo de tentar resolver o problema levantado pelo uso de diferentes conceitos de espécies, foi proposto o conceito de espécie unificada, que identifica as espécies como linhagens metapopulacionais evoluindo separadamente (de Queiroz, 2007). Assim, os "limites" das espécies podem ser descobertos pela análise de diferentes tipos de dados de acordo com a biologia (em suas mais distintas facetas) das espécies (Jaiswara et al., 2012).

Para delimitar com precisão as espécies crípticas e, assim, reduzir o déficit Lineano, os taxonomistas usam diferentes linhas de evidência (por exemplo, dados genômicos, morfológicos, acústicos, ecológicos, climáticos, geográficos, entre outros), procurando reconhecer padrões entre os dados e, assim, identificar as unidades taxonômicas não reconhecidas (DeSalle et al., 2005; Bickford et al., 2007). O uso dessas diferentes linhas de evidência para elucidar questões taxonômicas em estudos sistemáticos tem sido designado por "taxonomia integrativa" (Padial et al. 2010; Yeates et al. 2010). Neste contexto, múltiplas linhas de evidência fornecem um suporte mais robusto para hipóteses que propõem a existência de diferentes linhagens evolutivas (de Queiroz 2007). O rigor na delimitação de espécies pode, portanto, ser aumentado quando várias abordagens escolhidas para complementaridade são usadas (Schlick-Steiner et al. 2010).

Chiroptera: Avanços na sistemática dos morcegos neotropicais

Os morcegos são a segunda ordem mais diversa de mamíferos, tendo menos espécies apenas que os roedores. Atualmente são reconhecidas 21 famílias, 233 gêneros e 1432 espécies de morcegos no mundo (Simmons & Cirranello 2020), sendo que mais de 380 espécies ocorrem no Neotrópico (Solari & Martínez-Arias, 2014). Estes organismos fornecem diferentes serviços ecossistêmicos como a dispersão de sementes, polinização, controle biológico de insetos e pequenos vertebrados, e transporte de nutrientes para ambientes cavernícolas, contribuindo para o equilíbrio e para a manutenção geral dos ecossistemas. Desta forma, devido às funções ecológicas que cumprem, os morcegos têm um potencial de importância econômica e geração de impactos positivos sobre o bem-estar humano (Kunz et al. 2011; Castillo-Figueroa 2020).

Na região neotropical a família mais diversificada morfologicamente e representativa é Phyllostomidae. Os estudos sistemáticos morfológicos (Wetterer et al., 2000; Dávalos et al., 2014) e moleculares (Baker et al., 2003; Rojas et al., 2011; Dumont et al., 2012; Dávalos et al., 2014) neste grupo são numerosos e resultaram na definição de 11 subfamílias (Macrotinae, Micronycterinae, Desmodontinae, Phyllostominae, Glossophaginae, Lonchorhininae, Lonchophyllinae, Glyphonycterinae, Carolliinae, Rhinophyllinae e Stenodermatinae), 12 tribos (Diphyllini, Desmodontini, Macrophyllini, Phyllostomini, Vampyrini, Glossophagnini, Brachyphyllini, Choeronycterini, Lonchophyllini, Hsunycterini, Sturnirini e Stenodermatini), e nove subtribos (Brachyphyllina, Phyllonycterina, Anourina, Choeronycterina, Vampyressina, Enchisthenina, Ectophyllina, Artibeina e Stenodermatina) (Baker et al. 2016). Porém, uma parte importante dos estudos filogenéticos na família têm se concentrado em resolver a filogenia profunda, ou seja, a relação entre categorias taxonômicas mais elevadas, pelo que ainda existem muitas lacunas de informação nas categorias inferiores como as relações entre gêneros e espécies, particularmente dentro de algumas subfamílias. Os avanços na sistemática e taxonomia de morcegos, e em particular em Phyllostomidae, têm ajudado a aumentar consideravelmente o conhecimento da sua diversidade de espécies e padrões de distribuição no neotrópico (Solari & Martínez-Arias, 2014; Solari et al., 2019). No entanto, existem ainda muitas lacunas sobre muitos táxons, especialmente aqueles que podem incluir diversidade críptica.

Estudos recentes sugerem elevada diversidade críptica em algumas espécies de morcegos (Clare, 2011), razão pela qual é fundamental a geração de novos dados, especialmente moleculares, que permitam não só hipotetizar as relações filogenéticas em certos grupos, mas também delimitar suas espécies, esclarecendo os mecanismos que favoreceram a sua diversificação. Desvendar a diversidade críptica presente num grupo taxonômico é um passo fundamental, não só para compreender profundamente a diversidade biológica em uma região, mas também para compreender a evolução e fatores na conservação que envolvem esta realidade biológica "oculta" (Funk et al. 2002). Os morcegos *Lophostoma* d'Orbigny, 1836, (Phyllostomidae: Phyllostominae: Phyllostomini), constituem um grupo onde parece existir elevada diversidade críptica por explorar, o que tem sido sugerido através de estudos morfológicos e moleculares (Velazco & Cadenillas 2011). Por esse motivo, constituem um excelente grupo-alvo para contribuir para a redução do défice Lineano em morcegos neotropicais.

Os morcegos de orelhas redondas, *Lophostoma* d'Orbigny, 1836

Morcegos do gênero *Lophostoma* d'Orbigny, 1836, (Phyllostomidae: Phyllostominae) são insetívoros comuns na região Neotropical, onde ocupam uma grande variedade de habitats ao longo da sua distribuição, desde regiões semiáridas a florestas tropicais secas e úmidas (Williams & Genoways 2008). O gênero é endêmico das Américas e encontra-se distribuído do sul do México ao sudoeste do Paraguai (Simmons 2005; Williams & Genoways 2008). Atualmente, compreende sete espécies de tamanho pequeno a médio (antebraço 33-56 mm; Williams & Genoways 2008): *Lophostoma brasiliense* Peters, 1867, *L. carrikeri* (Allen,1910), *L. evotis* (Davis & Carter, 1978), *L. kalkoae* Velazco & Gardner, 2012, *L. occidentale* (Davis & Carter, 1978), *L. schulzi* (Genoways & Williams, 1980) e *L. silvicola* d'Orbigny, 1836.

Relações filogenéticas a nível do gênero foram já estudadas (Lee et al. 2002, Porter et al. 2003) assim como revisões taxonômicas para algumas espécies (Velazco & Cadenillas 2011; Camacho et al. 2016). Contudo, tem sido sugerida a necessidade de uma revisão mais profunda, particularmente para o táxon *L. silvicola*, que inclui três subespécies reconhecidas, embora sem limites taxonômicos claramente definidos, e para *L. brasiliense* que pode representar um complexo de linhagens evolutivas distintas ainda não investigado adequadamente (chamado de *hidden diversity* por Velazco & Cadenillas 2011).

Em análises filogenéticas utilizando o gene mitocondrial Citocromo-*b*, Velazco & Cadenillas (2011) revelaram a parafilia entre linhagens de *L. silvicola* com três clados definidos (Figura 1). No entanto, utilizando apenas dados morfológicos, não foi possível diferenciar conjuntos agrupados a partir das análises filogenéticas. Isso demonstra a necessidade de uma revisão mais profunda desse complexo de potenciais espécies, por meio de uma amostragem mais completa não só de indivíduos, como também de outras fontes de informação.

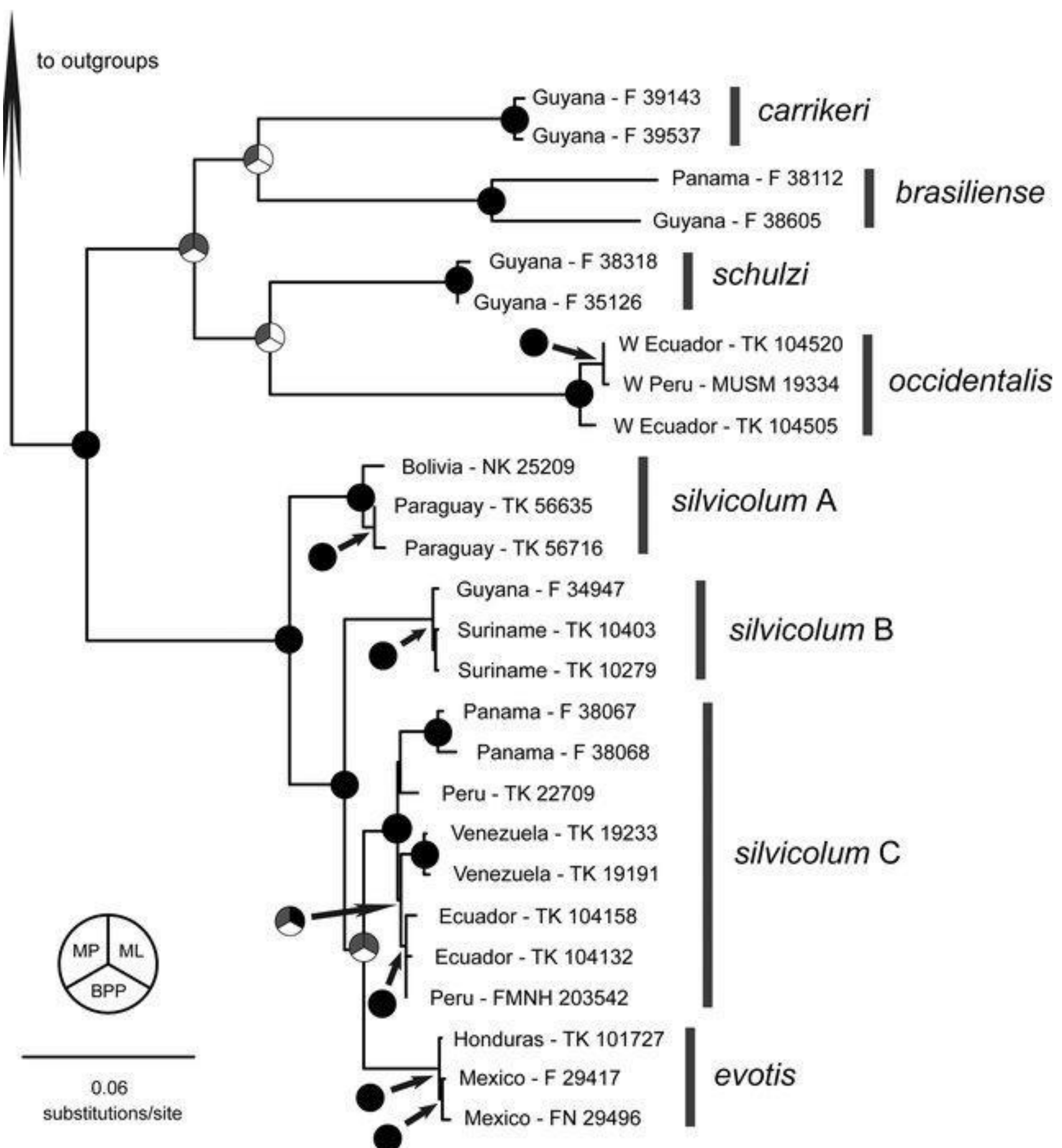

Figure 1. Filograma de máxima verossimilhança com base em sequências de Citocromo-*b* para seis espécies de *Lophostoma. Sensu* Velazco & Cadenillas (2011).

Histórico taxonômico

Lophostoma foi descrito por A. d'Orbigny em 1836 como um gênero monotípico, usando somente o holótipo de *Lophostoma silvicola* d'Orbigny, 1836, coletado na Bolívia. Posteriormente, outras espécies foram adicionadas ao gênero: *Lophostoma bidens* (Spix, 1823), transferida por W. Peters (1865) por combinação de nomes, *L. brasiliense* Peters, 1867, e *L. amblyotis* Peters, 1867. Dobson (1878) indicou *L. silvicola* como um sinônimo júnior de *L. amblyotis*; no entanto Palmer (1898) reconheceu erros neste tratamento e tratou *Lophostoma* como sinônimo júnior de *Tonatia* Gray, 1827. Esta decisão taxonômica perdurou por 80 anos, quando novas evidências citogenéticas, de aloenzimas e imunológicas (Patton & Baker, 1978; Baker & Bickham 1980; Arnold et al., 1983; Honeycutt & Sarich 1987) indicaram divergências entre as espécies agrupadas em *Tonatia*. Lee et al. (2002) examinaram as sequências de DNA e evidenciaram *Tonatia* como um grupo parafilético, sugerindo elevar *Lophostoma* novamente à categoria de gênero, incluindo as espécies *L. brasiliense*, *L. carrikeri*, *L. evotis*, *L. schulzi* e *L. silvicola*. A partir deste momento, *L. kalkoae*; Velazco & Gardner, 2012 foi descrita e *L. occidentale* (Davis and Carter, 1978) foi revalidada. Estudos recentes reconhecem sete espécies em *Lophostoma*, (Camacho et al. 2016), das quais pelo menos duas apresentam elevada diversidade críptica: *L. silvicola* e *L. brasiliense.*

Neste contexto, esta dissertação foca na diversidade críptica presente em *Lophostoma* e no levantamento de hipóteses sobre os processos de diversificação do táxon. Os resultados obtidos permitem melhorar o conhecimento taxonômico deste grupo de morcegos no neotrópico, bem como parte da sua diversidade genética e a sua distribuição geográfica, informações necessárias ao subsequente reconhecimento de áreas prioritárias para sua conservação. Para atingir os objetivos, diferentes linhas de evidência foram utilizadas, incluindo dados genéticos mitocondriais, morfológicos e de morfometria geométrica e dados geográficos para esclarecer os limites e a diversidade das espécies dentro do gênero *Lophostoma*, com especial ênfases nos grupos *L. brasiliense* e *L. silvicola*.

Objetivos

Objetivo geral

O presente trabalho tem como objetivo geral analisar a diversidade críptica em *Lophostoma* a partir de uma abordagem taxonômica integrativa.

Objetivos específicos

– Delimitar e separar espécies crípticas nos complexos *L. brasiliense* e *L. silvicola* a partir de evidências genéticas, morfométricas e morfológicas.
– Descrever e redescrever espécies dentro de cada complexo visando melhorar a diagnose dentro do gênero.
– Determinar a distribuição geográfica das espécies de *Lophostoma.*

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CAPÍTULO II – Diversidade críptica no complexo *Lophostoma brasiliense*

Artigo submetido para o Zoological Journal of the Linnean Society

Multiple lines of evidence support cryptic diversity in the bat *Lophostoma brasiliense* (Chiroptera: Phyllostomidae)

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Short running title: Cryptic diversity in *Lophostoma brasiliense*

ACKNOWLEDGMENTS

We are especially thankful to museum curators for allowing access to the specimens under their care, as well as the loan of tissue samples. We particularly thank Adam Ferguson and Bruce Patterson (FMNH), Nancy Simmons and Neil Duncan (AMNH), Heath Garner (TTU), Jessica Light (TCWC), Ludmilla Aguiar (UnB), Marcelo Weksler and João Oliveira (UFRJ), Ciro Líbio (UFMA), Thiago Bernardi Vieira (UFPA), Leonora Pires Costa and Monique Nascimento (UFES), Fernando Cervantes, Mariana Figueroa, and Viridiana Marcos (CNMA), Sergio Guillermo Pérez (USAC), and Octavio Saldaña from Programa para la Conservación de los Murciélagos de Nicaragua (PCMN). We would like to thank Juan Díaz-Nieto and Juan Martinez-Ceron (Universidad EAFIT) for kindly sharing their sequences with us. This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES - Finance Codes 001 and 88882.439397/2019-01), given in the form of a MSc scholarship to DAE and a post-doctoral fellowship to FBM. MJRP was supported by a National Council for Scientific and Technological Development (CNPq) productivity.

ABSTRACT

Phenotypically similar species – often called cryptic species – represent a challenge for taxonomy and conservation biology because they are usually undetectable to science. To unravel these cryptic taxa, studies now employ data from different sources under an integrative approach. We present an assessment of the cryptic diversity of the *Lophostoma brasiliense* species complex (Phyllostomidae, Chiroptera) based on multiple lines of evidence (molecular, morphological, morphometric, geometric morphometric and geographic data) and using molecular (ABGD, ASAP, GMYC and bPTP) and phenotypic (distance-based approaches and Normal Mixture Model Analyses) species delimitation methods. Our analyses recognized two distinct lineages with clear allopatric distributions. One of these lineages corresponds to *L. brasiliense* with a cis-Andean distribution and the second lineage corresponds to the formerly species *Lophostoma nicaraguae* with a trans-Andean distribution. The distribution of both species is limited by the Andes, constituting an ideal taxonomic group to test the effect of the rise of this biogeographic barrier in the mammalian diversification. This study evaluates the intraspecific relationships within *Lophostoma brasiliense* complex, and underline the need for multiple lines of evidence to solve the remaining taxonomic problems among species of *Lophostoma*.

ADDITIONAL KEYWORDS: Cryptic species – species delimitation – systematics – taxonomy – neotropical bats – Phyllostominae – round-eared bat

INTRODUCTION

The discovery of cryptic species together with the advancement of molecular techniques and the use of multiple lines of evidence have revealed an increase in the number of new species of mammals in the last few years (Solari *et al*., 2019). Cryptic species are defined as different evolutionary lineages of phenotypically similar organisms classified within a single species (Bickford *et al*., 2007). Unveiling cryptic diversity is essential for understanding biogeographic, evolutionary, and ecological processes; it is also crucial in conservation biology, because undescribed cryptic diversity may already be seriously threatened, by occurring in restricted areas or being endemic to areas suffering severe anthropogenic pressures (Delić *et al*., 2017; Theodoridis *et al*., 2019; Ramesh *et al*., 2020).

Unveiling and describing cryptic species involves the use of different lines of evidence, including molecular, morphological, acoustic, ecological, climatic and geographic data, allowing the recognition of patterns across data for the identification of candidate species (DeSalle *et al*., 2005; Bickford *et al*., 2007). Since the beginning of the use of taxonomy as a practice to classify and name diversity on Earth, species delimitation was mostly based on phenotypic characteristics as a single line of evidence (the typological-morphological concepts: Cronquist, 1978; Mayr, 1996). Approaches grounded in new sources of evidence provided new perspectives, methodologies, and species concepts, such as the Henningian (Hennig, 1966) and the Ecological (Van Valen, 1976) species concepts. Different methods and species concepts challenge taxonomists to decide which data types are most likely to uncover new species, and how to handle them, simultaneously. The use of these different lines of evidence to elucidate taxonomic issues in systematic studies has been called “integrative taxonomy” (Padial *et al*., 2010, but see Yeates *et al*., 2010).

As cryptic species are unveiled, it becomes apparent that many of the sister taxa exhibit allopatric distributions, also pinpointing the geographical barriers that may have played a fundamental role in their formation (Chenuil *et al*., 2019). In South America, the Andes represent the most important biogeographic feature, affecting rivers, precipitation, biome limits, and species distributions (Patterson *et al*., 2012; Viale & Garreaud, 2015). The role of the Andes in limiting gene flow and promoting species diversification has been widely reported for many vertebrate taxa, such as birds (Brumfield & Capparella, 1996), amphibians (Hutter *et al*., 2017), and mammals (Patterson *et al*., 2012). The complex and relatively recent Andean uplift promoted high diversification in multiple taxa, but also the division of reasonably recent species that rapidly accumulated high genetic differentiation but poor morphological differentiation (i.e., formation of cryptic species).

Bats have experienced a considerable increase in the number of species as a result of surveys in underexplored regions or from taxonomic reviews using integrative taxonomy (Burgin *et al*., 2018; Solari *et al*., 2019). However, due to their nocturnal habits and the difficulty of assessing many taxa in the wild, the current number of bat species is probably still underestimated considering the potential species richness of this group (Solari *et al*., 2019). Indeed, recent studies not only suggest the existence of high cryptic diversity in many bat lineages, and the need to revise them in depth (e.g., Clare, 2007, 2011; Lim & Lee, 2018), but also the potential areas for the occurrence of new species, particularly in megadiverse regions (Aguiar *et al*., 2020).

Neotropical bats of the genus *Lophostoma* d'Orbigny, 1836, (Phyllostomidae: Phyllostominae) exhibit a wide distribution in the Neotropics, ranging from southern Mexico southward to southwestern Paraguay and into eastern Brazil (Simmons, 2005; Williams & Genoways, 2008). The seven insectivorous species of *Lophostoma* are

known as round-eared bats. Three of these species are medium-sized (forearm > 49 mm, greatest length of skull > 26 mm): *L. silvicola* d'Orbigny, 1836; *L. evotis* (Davis & Carter, 1978); and *L. occidentale* (Davis & Carter, 1978); and four are small-sized (forearm 36 – 49 mm, greatest length of skull 18 – 26 mm): *L. brasiliense* Peters, 1867; *L. carrikeri* (Allen, 1910); *L. schulzi* (Genoways & Williams, 1980); and *L. kalkoae* Velazco & Gardner, 2012 (Williams & Genoways, 2008; Velazco & Gardner, 2012). During the past decade, advances in the use of morphological and molecular data have improved the knowledge on the systematics and taxonomy of *Lophostoma* (e.g., Velazco & Cadenillas, 2011; Velazco & Gardner, 2012; Camacho *et al*., 2016). Some studies suggest high cryptic diversity, particularly in *L. brasiliense* and *L. silvicola* which are considered species' complexes (Velazco & Cadenillas, 2011; Lim & Lee, 2018).

Since its original description, the taxonomy of *Lophostoma brasiliense* has been the subject of contention. Peters (1867) described the species based on a single specimen from "Baia" (= Salvador), Bahia, Brazil. Later, Robinson & Lyon (1901) described *Lophostoma venezuelae*, a species phenotypically similar to *L. brasiliense*, based on specimens from Macuto, Venezuela. Subsequently, Trouessart (1904) transferred these species to the genus *Tonatia* Gray, 1827. Four decades later, *Tonatia nicaraguae* Goodwin, 1942 and *Tonatia minuta* Goodwin, 1942 were described from Nicaragua and Peru, respectively. For many years, the taxonomic status of these taxa was puzzling. Handley (1966) synonymized *T. nicaraguae* under *T. minuta*, nomenclature followed by some authors such as LaVal (1969) and Valdez & LaVal (1971). However, Jones *et al*. (1971) pointed out that *T. nicaraguae* had page priority over *T. minuta* and that the former should be the valid name. Koopman (1976) returned to the synonymy raised by Handley (1966), arguing that the holotype of *T. nicaraguae* was an immature specimen

with a broken and decalcified skull, invalidating that assignment. This taxonomic arrangement was followed by Gardner (1976) and Greenbaum & Jones (1978), until Koopman (1978), and later Genoways & Williams (1984), found no consistent character to consider them as different taxa, considering only one species: *Tonatia brasiliensis*. Baker (1979) and Baker *et al*. (1982) reported karyotype information in which they retained the names *T. brasiliensis*, *T. minuta*, and *T. venezuelae* suggesting the possibility of more than one taxon involved. In 1989, Eisenberg & Redford considered four subspecies: *Tonatia brasiliensis brasiliensis*, *T. b. minuta*, *T. b. nicaraguae*, and *T. b. venezuelae*. Finally, Lee *et al*. (2002) transferred *Tonatia brasiliensis*, as well as *evotis*, *schulzi* and *silvicola*, to the genus *Lophostoma* with no subspecies recognized (Simmons, 2005; Williams & Genoways, 2008). Nevertheless, no comprehensive analysis on this putative species complex has ever been made throughout its entire distribution, which includes populations on both sides of the Andes.

Here, using multiple lines of evidence, including molecular (gene tree, distance and tree-base methods), morphological (discrete characteristics), morphometric (linear and geometric morphometrics), and geographic data, we aim to assess the taxonomic limits of the *L. brasiliense* complex. We hypothesize that *L. brasiliense* is a complex of species with at least two separate evolutionary lineages given its wide distribution across the Neotropics, where a wide range of environmental conditions and geographical barriers may have promoted its genetic isolation and morphological diversification. Because the signature of vicariance caused by the uplift of the Andes is visible in numerous mammalian taxa and, particularly, many bat lineages (see Patterson *et al*., 2012), we predict that the trans-Andean and cis-Andean populations represent different evolutionary lineages.

MATERIAL AND METHODS

Molecular analyses

To assess the phylogenetic relationships of the *Lophostoma brasiliense* complex, we obtained genetic data from specimens in different geographic regions housed in the following museums: Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; Coleção de Tecidos e DNA da Universidade Federal do Espírito Santo, Vitória, Brazil; Museu Nacional do Rio de Janeiro, Rio de Janeiro, Brazil; Universidade Federal do Maranhão, São Luís, Brazil; Universidade Federal do Pará, Altamira, Brazil and The Museum of Texas Tech University, Lubbock, USA.

DNA extraction, amplification and sequencing

We extracted total genomic DNA from tissue samples of wing membrane (c. 1 mm^2) and muscle preserved in ethanol under -20ºC. The extractions were made using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions, eluting to a final volume of 100 µL. The total genomic DNAs were stored at -20°C before amplification. The pair of primers used was designed by Folmer *et al.* (1994), targeting the mitochondrial gene Cytochrome Oxydase subunit I, LCOI 1490 (5'-GGTCAACAAATCATAAAGATATTGG -3') and HCOI 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). The PCR conditions consisted in an initial denaturation step at 92ºC for 10 min, followed by 35 cycles of 95ºC for 30 s (denaturation), 49–51ºC for 40 s (annealing), 72ºC for 90 s (polymerization), 72ºC at 10 min (final extension), and an infinite hold at 4ºC. The PCR products were purified using Exonuclease I and shrimp alkaline phosphatase (Affymetrix, Inc. USB Products, Cleveland, OH, U.S.A.). The two DNA strands for the PCR products were sequenced by Macrogen, Inc. (Seoul, South Korea). We visually inspected, verified and manually

edited the sequence chromatograms using the Staden package (Staden *et al*., 2000). We verified the sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), confirming the high similarity of our submitted sequences to *Lophostoma* species.

Phylogenetic analyses and molecular species delimitation

To evaluate the monophyly of *brasiliense*, we conducted a phylogenetic analysis including all COI sequences available on GenBank and additional sequences provided by us, totaling 31 sequences of *brasiliense*, plus five sequences for each *Lophostoma* species available on GenBank, and rooted the tree in *Vampyrum spectrum* (Phyllostomidae: Phyllostominae) (see Supporting Information, Table S1, for voucher numbers, locality data, and GenBank accession numbers). We used PhyloSuite (Zhang *et al*., 2020) for the workflow of the analyses. We aligned the sequences with MAFFT (Katoh & Standley, 2013) using '--auto' strategy and normal alignment mode. We removed ambiguously aligned fragments using Gblocks (Talavera & Castresana, 2007). We selected the best partitioning schemes and evolutionary models for pre-defined partitions using PartitionFinder2 (Lanfear *et al*., 2017), with all algorithms and AICc criterion. Bayesian Inference phylogenies were inferred using MrBayes 3.2.6 (Ronquist *et al*., 2012) under HKY+G model (2 parallel runs, 10 million generations), discarding the initial 20% of sampled data as burn-in. For the GMYC analysis (see below), we built an ultrametric tree using the BEAUti2.5/BEAST v2.5 (Bouckaert *et al*., 2019) with the same substitution models of the previous analyses. We assumed a strict clock model and a coalescent tree prior with constant population size. We did two independent runs for each dataset with 50 million generations, sampling the parameters every 5000 generations. We used Tracer v.1.7 (Rambaut *et al*., 2018) to inspect the convergence to the stationary distribution of the chains. The first 10% of the generations were discarded as ‘burn-in’ and then combined the chains: the combined ESS for each parameter was

higher than 200. We visualized and edited the trees using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). We are aware of the limitations of a gene tree to reproduce phylogenetic hypotheses, however our main aim using the COI analysis was species delimitation (DeSalle & Goldstein, 2019).

For delimiting species using the molecular data, we applied four DNA-based single-locus species delimitation approaches, two distance-based and two tree-based. The methods used were: (a) the automatic barcode gap discovery method (ABGD; Puillandre *et al*., 2012), (b) the assemble species by automatic partitioning (ASAP; Puillandre *et al*., 2021), (c) the Generalized Mixed Yule Coalescent approach (GMYC; Pons *et al*., 2006), and (d) a Bayesian version of the Poisson Tree Processes model approach (bPTP; Zhang *et al*., 2013). These analyses were performed using the ABGD web server (ABGD – https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) setting the parameters Simple Distance (*p*-distances) with relative gap width (X = 1.5); the ASAP web (https://bioinfo.mnhn.fr/abi/public/asap/) also setting Simple Distance (*p*-distances); and the Exelixis Lab's web server (bPTP – http://species.h-its.org/ptp/) setting unrooted, 500 000 MCMC generations, burn-in of 0.2; (GMYC – http://species.h-its.org/gmyc/) setting "single threshold" method. For distance methods the uncorrected *p*-distances yield more accurate (or at least similar) results when compared to other models of nucleotide evolution (e.g., K2P; see Srivathsan & Meier, 2012; Collins & Cruickshank, 2012).

Morphological analysis

We examined 165 adult specimens of *Lophostoma brasiliense* (87 males, 75 females and three specimens of undetermined sex) which represent the entire distribution of the species throughout the Neotropics (Fig. 1). The revised material included fluid-preserved specimens, dry skins, skeletons and skulls held in the following institutions: Coleção de Mamíferos da Universidade Federal do Rio Grande do Sul, Porto Alegre,

Brazil (DZUFRGS); Museu de Ciências Naturais da Fundação Zoobotânica, Porto Alegre, Brazil (MCN); Museu Nacional do Rio de Janeiro, Rio de Janeiro, Brazil (MNRJ); Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil (MZUSP); Colección Zoológica Universidad del Tolima, Ibagué, Colombia (CZUT); Instituto de Investigaciones Biológicas Alexander Von Humboldt, Villa de Leyva, Colombia (IAvH); Museo de Historia Natural Universidad de Caldas, Manizales, Colombia (MHN-UCa); Museo de Historia Natural Universidad Distrital Francisco José de Caldas, Bogotá, Colombia (MUD); Museo de Zoología de Nicaragua (MZN); Colección Nacional de Mamíferos-UNAM, Ciudad de México, México (CNMA); American Museum of Natural History, New York, USA (AMNH); Field Museum of Natural History, Chicago, USA (FMNH); National Museum of Natural History (U.S. National Museum), Smithsonian Institution, Washington D.C., USA (USNM); The Museum of Texas Tech University, Lubbock, USA (TTU); and Biodiversity Research and Teaching Collections, Texas A&M University, College Station, USA (TCWC). The examined material included the type specimens of *Lophostoma venezuelae* Robinson & Lyon, 1901 (USNM 102919), *Tonatia nicaraguae* Goodwin, 1942 (AMNH 41184), and *Tonatia minuta* Goodwin, 1942 (AMNH 71619). A list of specimens included in this study, with their respective localities is presented in the Supporting Information (Appendix S1).

In the examined specimens, we observed patterns of dorsal and ventral pelage coloration, ear and foot coloration, ear shape, and presence or absence of pinna folds. We also analyzed aspects of cranial morphology such as rostrum and skull shape, presence or absence of processes (e.g., clinoid, paraoccipital, postorbital, among others), and the morphology of the teeth (size and cusps). The nomenclature used to describe the skull and its structures follows Velazco (2005).

Morphometric analysis

For each specimen, we examined the quantitative variation in 20 morphological measurements that included three externals, 16 craniodental and one postcranial. These measurements were defined based on, but not restricted to, those defined by Velazco & Cadenillas (2011), and are presented in Table 1.

We took all measurements using a digital caliper with 0.01 mm resolution on each specimen, and all were $\log_{10}$ transformed for the subsequent statistical analyses. We calculated mean, standard deviation, and range for each character, and visually examined them to assess non-overlapping differences in individual trait measurements between groups (candidate species and sex) using box and density plots. We also tested for differences in individual traits between groups using Student's t-test, or the nonparametric Mann-Whitney U-test when normality and homoscedasticity assumptions were not met. We assessed normality and homoscedasticity using the Shapiro–Wilk and Levene tests. Outliers were detected using descriptive methods such as histogram, boxplot and percentiles.

To determine whether morphometric data would diagnose distinct phenotypic groups, we conducted two types of analyses. First, we separate and grouped geographically specimens in Operational Taxonomic Unit (OUT´s) following a consensus of molecular delimitation (see Results), and performed a Principal Component Analysis (PCA) and Discriminant Function Analysis (DFA) to investigate whether these previously determined groups could be distinguished based on external and craniodental morphology using the package ‘MASS’ (Ripley *et al.*, 2013) in R version 4.1.0 (R Core Team, 2021). Differences in the multivariate space were evaluated using a MANOVA. Second, while not assigning groups of specimens a priori, we applied normal mixture model analyses (NMMs) to estimate the number of distinct normal distributions that

best fitted the pooled morphological data following procedures described by Cadena *et al*. (2018). For these procedures we used the R packages 'clustvarsel' and 'mclust' in three main operations: (1) finding the variables that most effectively delimit morphological clusters; (2) using these variables to fit different normal mixture models; and (3) selecting the best model determining the optimal number of clusters best supported by our dataset. In a complementary analysis, we log-transformed our morphometric dataset and performed a PCA on the covariance matrix. Here, rather than exploring the species limits using the principal components accounting for most of the variation (standard procedure in most taxonomic studies), we reduced the dimensionality of the data and selected the set of principal components most useful for group discrimination in NMMs using the R package 'clustvarsel' (Scrucca & Raftery, 2018). After identifying those key traits, we examined support for models specifying the existence of one to four morphological groups. The group with only one morphological cluster represents the current taxonomic treatment of *Lophostoma brasiliense* as a unique species, the group with two clusters consider the results from our phylogenetic analyses and the group with four clusters represent previous hypotheses where up to 4 species were considered (*T. nicaraguae*, *T. venezuelae*, *T. minuta*, and *T. brasiliensis*). We fitted the models using the R package 'mclust' (Scrucca *et al*., 2016) and used the Bayesian Information Criterion (BIC) to measure the support for different NMMs.

Geometric morphometric analysis

We obtained two-dimensional images of the skull in ventral and dorsal views and the mandible in lateral and dorsal views of each specimen using a digital camera (Nikon Coolpix P900, Tokyo, Japan). All photos were taken following the same standardized protocol in which skulls and mandibles were in the same position and perpendicular to the axis of the camera.

Two-dimensional landmarks and semilandmarks configurations on the skull and mandible were digitized from these pictures using tpsDig version 2.3 (Rohlf, 2017). We defined the landmarks based on criteria of homology, consistency of relative position, coverage of the form, and repeatability (Zelditch *et al*., 2012). To examine how many landmarks and semilandmarks could effectively capture the information of shape and size, we employed a Landmark Evaluation Curve analysis (Watanabe, 2018) with the *lasec* function in the R package 'LaMDBA'. This function produces a sampling curve and a table with fit values that allows it to recognize the number of anatomical points necessary to characterize the shape variation and size. We determined the number of landmarks and semilandmarks for each region considering a required fit of 0.9, 0.95, and 0.99 (Supporting Information, Table S2). Then, the shape and size of the skull were obtained through 22 landmarks in ventral view and 9 landmarks with 16 semilandmarks in dorsal view of 88 adult specimens, while the shape and size of the mandible were obtained from 9 landmarks in lateral view and 12 landmarks in dorsal view of 85 adult specimens. Detailed descriptions of the landmarks and semilandmarks and a figure with details about these anatomical points on each view are presented in the Supporting Information (Appendix S2-A).

The landmarks dataset was subjected to superimposition using a Generalized Procrustes Analysis (GPA) that removes undesirable effects of scale, position, and orientation using the *gpagen* function in the R package 'geomorph' (Adams *et al.*, 2020). We obtained procrustes shape coordinates, and a size estimator called centroid size (CS) as the square root of the sum of squares of the distance of each landmark to the centroid (mean of all coordinates) of the configuration (Bookstein, 1997). Also, consensus shapes that summarizes the skull and mandible shape variation among species were

generated. Here, each individual was compared against the consensus shape, which allows to visualize differences between species.

Statistical analyses of size and shape

Differences in centroid size between females and males (sexual dimorphism) and also among candidate species were graphically summarized using series boxplots in each view. The effects of (1) size, (2) sex, and (3) species on skull and mandible shape and their interactions were tested by evaluating the fit of models using the randomized residual permutation procedure (RRPP) with the *lm.rrpp* function in the R package 'RRPP' (Collyer & Adams, 2018, 2020). Using the same function, we quantified the differences in size among groups, employing the (log) centroid size of the specimens as the response variable, and sex and species as independent predictors. Although results from these models showed significant differences between sexes for shape in all views (see Results), a separate analysis by sex did not alter the overall patterns; therefore, we present the results with both sexes included. All models were fit using the type-II (hierarchical) sum of squares, and its significance was based on 10,000 permutations of residual randomization. We used the *anova.lm.rrpp* function to compute analysis of variance (ANOVA) tables for each model, which are based on random statistical distributions and use the F distribution to calculate effect sizes. Pairwise comparisons were conducted on significant factors using the *pairwise* function in the R package 'RRPP' (Collyer & Adams, 2018, 2020).

Differences in skull and mandible shape among groups (candidate species and sex) were also explored using ordination methods. We carried out two main steps: first, we performed a PCA on the Procrustes-aligned data using the *gm.prcomp* function in the R package 'geomorph' (Adams *et al*., 2020). Of the PCs produced, we chose those containing significant cumulative variance of shape in each view (~90%). Then we

generated deformation grids with the extremes (maximum and minimum) of shape variation along the principal components 1 and 2 (PC1 and PC2). Second, we used a DFA to determine whether the groups could be reliably distinguished. Procrustes distances between groups were tested for significance with a 10,000 permutations procedure. The probability of a specimen belonging to any of the predefined groups was estimated via jackknife cross-validation of the scores.

Estimating missing landmarks and error measurement

When one side was damaged or incomplete in structures with bilateral symmetry (ventral/dorsal skull and dorsal mandible), we first used the *reflectMissingLandmarks* function in the R package 'StereoMorph' (Olsen & Westneat, 2015), where missing landmarks on either side are imputed from the mirrored specimen. For those missing landmarks that could not be estimated taking advantage of bilateral symmetry, we used the least-squares regression (REG), and the Bayesian PCA (BPCA) in the cranium and mandible respectively, to estimate missing values in our datasets using functions from the R package 'LOST' (Arbour & Brown, 2014). These methods proved to be the best missing data estimators for those structures in our dataset, among three standard approaches [Bayesian PCA (BPCA), least-squares regression (REG), mean substitution (MS)] and a geometric-morphometric-specific method [Thin-plate spline interpolation (TPS)] after a rigorous evaluation carried out by us that included simulations of missing data, test for the impact of missing data estimation and analyses on the performance of those methods. All evaluation and simulation processes with incomplete specimens and comparing the resulting methods are presented in detail in the Supporting Information (Appendix S2-B).

Finally, we tested the error of digitization of the landmarks through a Procrustes ANOVA, which measures and compares random errors (Klingenberg & McIntyre,

1998; Klingenberg *et al*., 2002). In this sense, we digitized all skulls and mandibles twice and performed a Procrustes ANOVA between these two replicates, which yielded very high replicability (> 90% all views).

Taxonomic decision

We defined the candidate species according to the results of the following lines of evidence: (1) monophyletic lineages using the gene tree; (2) genetic distance-based approaches; (3) tree-based methods; (4) delimitation methods using morphometric linear distances; (5) normal mixture models (NMMs); and (6) geometric morphometric analyses. We evaluated the accumulation of evidence from each method, recognizing candidate species to support a distinct species hypothesis. Under this approach, the support increases when more data validate clades obtained through previous analyses. Finally, we assumed the unified species concept, understanding species as a lineage evolving separately from other lineages (De Queiroz, 2007).

RESULTS

Phylogenetic analysis of *Lophostoma brasiliense* and molecular species delimitation

The gene tree based on 56 COI sequences of *Lophostoma* recovered *L. brasiliense* as a monophyletic group with high posterior probability (PP = 0.99). This clade presents two deep lineages that split the specimens from Mexico, Belize, Panama and northern Colombia (PP = 1) from the remaining *L. brasiliense* specimens from South America (PP = 0.99); also the monophyly of *L. carrikeri* (PP = 0.99) and *L. schulzi* (PP = 1) were strongly supported. *Lophostoma silvicola* was the only non-monophyletic species, recovered as paraphyletic (PP = 1) including all specimens of *L. evotis* (PP = 1).

The four DNA-based single-locus species delimitation approaches (ABGD, ASAP, GMYC, and bPTP) resulted in a similar number of taxonomic units for our samples and

were consistent in recognizing two lineages in *L. brasiliense*. ABGD analyses found from 11 to 13 groups within our samples (for prior maximal distance from 0.001000 to 0.007743), and found barcode gap distance around 0.033. The first (p = 0.0629) and second (p = 0.357) best results of ASAP recovered respectively 11 (threshold distance = 0.027523) and 10 (threshold distance = 0.041284) taxonomic units for our dataset. GMYC analysis recovered 13 entities with a confidence interval between 12–13. The bPTP identified 11 entities with good support as the best result. ABGD, ASAP, and bPTP analyses identified two lineages within the *L. brasiliense* samples. A cluster comprising the sequences from South America (*L. brasiliense sensu stricto*), and another including the sequences from Mexico, Belize, Panama and northern Colombia (hereafter, considered as the candidate species). GMYC suggests 4 clusters for *L. brasiliense*: Mexico, Belize, Panama and northern Colombia (candidate species); Sergipe and Espírito Santo states in Brazil (Atlantic Forest); Tocantins and Goiás states in Brazil (Cerrado) with Guyana-Demerara (Guiana Shield/Amazon); and the remaining samples from Guyana (Guiana Shield/Amazon). All algorithms identified *L. carrikeri*, *L. schulzi*, and *L. evotis* as one taxonomic unity each, while *L. occidentale* and *L. silvicola* split in two and three putative species respectively.

Morphological variation

Paired tests and density plots did not show evidence of sexual dimorphism, so subsequent analyses were carried out considering the pooled sexes (Supporting Information, Fig. S1). Although univariate tests recovered significant differences among candidate species and *L. brasiliense* (*sensu stricto*) for all traits, these exhibited partially or largely overlapping distributions indicating that none of the individual traits can reliably diagnose the candidate species (Supporting Information, Fig. S2). However, considering the whole dataset, MANOVA found significant differences ($p < 0.05$)

between two putative species. In general, considerable variation in the values of individual traits was observed between species (Table 2), where one species (the candidate species) is smaller than the other (*Lophostoma brasiliense sensu stricto*).

The PCA analysis based on 20 morphometric traits (four external and 16 craniodental) showed the first principal component (PC1) accounting for 67.93% of the variation, PC2 for 8.35% and PC3 for 5.50%, together accounting for more than 80% of the variation (Fig. 2A). It appears that most differences between species are related to size: PC1 describes variation associated with cranial and mandibular lengths (CIL, GLS, CCL, and DENL), while PC2 relates to the variation in ear and hindfoot lengths. Although there is overlap between species values, the DFA performed on different suites of morphological characters suggests that at least 84% of the individuals within the candidate species may be distinguished from those of *L. brasiliense* based on the analyzed measurements (Table 3; Fig. 2B). DFA results on morphological characters were similar considering both PCs or log-transformed data.

The normal mixtures analysis, with no *a priori* species definition, provided maximum support for models specifying two morphological groups (i.e., two distinct phenotypic distributions). Model support for the presence of one, two, three or four morphological groups according to different taxonomic proposals is larger for two groups (BIC = -840.1137), rejecting the current scenario of recognition of a single group (Fig. 3).

Overall, our results pointed to the existence of two phenotypic groups defined by morphological variation in our sample of *Lophostoma brasiliense*, supporting the results obtained with the genetic data.

Geometric morphometrics

Variation in cranium and mandible size

The centroid size (CS) in the cranium and mandible was significantly different between the two genetic and morphological identified groups, showing the candidate as smaller than *Lophostoma brasiliense sensu stricto* ($p < 0.05$ in all views; see graphical summaries in Supporting Information, Fig. S3-A). We did not find evidence of sexual dimorphism in size in any of the views examined, although the interaction term (species x sex) indicates significant sexual dimorphism inside the candidate species, where males are larger than females (Supporting Information, Fig. S3-B). Likewise, when comparing individuals of the same sex between the two putative species, we found significant differences between females in the dorsal skull and dorsal mandible, with females of *L. brasiliense sensu stricto* being larger. The variance of the factors tested, represented by mean squares value and the R^2, showed that most of the variance in skull and mandible size is found between species (Table 4).

Variation in cranium and mandible shape

Models showed significant sexual dimorphism in shape in all views (except dorsal mandible; Table 5). When we tested for cranial and mandibular shape variation in the entire Procrustes shape space, we found significant differences among the two tentative species in all views tested ($p < 0.05$ in all cases). When we evaluated differences between females and males separately, significant differences in shape between species remained (results not shown: $p < 0.05$ in all cases). Fitted linear models showed significant effect of size on shape variation in all the examined views except dorsal mandible; however, morphological variation explained by size was low (< 7% in all cases; Table 5) so the allometric effect was ruled out, and analyzes and graphical representations were carried out on the original shape coordinates.

The PCA showed a clear ordination for the dorsal skull and lateral mandible, but not for the ventral skull and dorsal mandible. The first three PC scores accounted for ~57 and

~64% of the skull and mandible total shape variation, respectively. Results are shown from the first two PCs, which accounted for ~55% of the variation (Fig. 4). Specimens with positive scores on PC1 presented thinner and elongated rostrum, smaller braincase, larger distance between the anteriormost point of premaxilla to nasal, and shorter distance between basion and opisthion. In the PC2 individuals with positive scores had shorter braincase. Concerning the mandible, the PC1 positive scores were associated with shorter, thicker, and more robust jaws, while negative scores with more elongated, thin, and slender ones. Most individuals of the putative new species presented positive scores on PC1 for the skull and negative for the mandible, showing characteristics that may help to differentiate the species (Fig. 4).

The DFA also showed that the two putative species are significantly different in the shape of both the skull and the mandible (Fig. 4). The percentage of correct classification using DFA shows high values for the putative new species in three views (dorsal skull – 78.38%, ventral skull – 97.29%, and lateral mandible – 90.91%). So, the morphological structures with the higher percentage of correct classification and probably the most useful to discriminate between the two taxonomic entities are the lateral mandible and the ventral skull. The above-mentioned evidence supports the recognition of the candidate species as a "confirmed candidate species".

ON THE APPLICABILITY OF NAMES IN THE *LOPHOSTOMA BRASILIENSE* SPECIES COMPLEX

Three epithets are under synonymy of *L. brasiliense*: *venezuelae* (Robinson & Lyon, 1901: 154), *nicaraguae* (Goodwin, 1942: 205) and *minuta* (Goodwin, 1942: 209). Based on analyses with linear and geometric morphometrics data, which included all type specimens, we conclude that *nicaraguae* should be applied to the trans-Andean populations of the *Lophostoma brasiliense* species complex (Supporting Information,

Appendix S3). We do not agree with Handley (1966) and Koopman (1976) in considering invalid the holotype of *nicaraguae*. The holotype represents an adult female and does not constitute an immature specimen as stated by these authors. Despite having a partially broken skull, the holotype allows an evaluation of the diagnostic characteristics of the species. In addition, the single specimen used by Goodwin (1942) for the description of this taxon is located in the morphometric space represented by the *L. nicaraguae* samples using both linear and shape measurements (Supporting Information, Appendix S3). The holotype of *L. venezuelae* shares the morphometric space with all individuals identified as *Lophostoma brasiliense* (including the nominal type of *L. brasiliense*) from Brazil, Guyana, Trinidad, Venezuela, eastern Colombia and Peru, and is well-separated from specimens of *L. nicaraguae* from Central America, which rules out the use of this name. Lastly, *T. minuta* clusters with *L. nicaraguae* using linear distances but splits when the mandible shape is analyzed, showing similarities in size but differences in shape (Supporting Information, Appendix S3). Moreover, our findings suggest no sympatric distribution between populations of *L. nicaraguae* and *T. minuta* (i.e., *L. brasiliense*).

Based on our results, we consider that the name *Lophostoma nicaraguae* is the appropriate name for the trans-Andean populations of the *L. brasiliense* species complex. To clarify the species limit and morphological characteristics of both lineages of the *L. brasiliense* species complex, we provide a emended diagnosis and comparisons of both taxa.

SYSTEMATICS

ORDER CHIROPTERA BLUMENBACH, 1779

FAMILY PHYLLOSTOMIDAE GRAY, 1825

SUBFAMILY PHYLLOSTOMINAE, GRAY, 1825

GENUS *LOPHOSTOMA* D'ORBIGNY, 1836

LOPHOSTOMA BRASILIENSE **PETERS, 1867**

PIGMY ROUND-EARED BAT

(FIG. 7)

Lophostoma brasiliense Peters, 1867:674; type locality "Baía" (= Salvador), Bahia, Brazil.

Lophostoma venezuelæ Robinson and Lyon, 1901:154; type locality "Macuto, [Distrito Federal,] Venezuela."

[*Tonatia*] *brasiliense*: Trouessart, 1904:111; name combination.

T[*onatia*]. *venezuelæ*: Miller, 1907:129; name combination.

Tonatia minuta Goodwin, 1942:209; type locality "Boca Curaray, Ecuador" (= Boca del Río Curaray, Loreto, Peru).

Tonatia brasiliensis: Handley, 1976:16; name combination and correct gender concordance.

[*Lophostoma*] *brasiliense*: Lee, Hoofer, and Van Den Bussche, 2002:55; first modern use of current name combination.

Type specimen: Holotype: Adult female, deposited at the British Museum of Natural History (BMNH 1849.11.7.14), prepared as dry skin and skull (broken) [purchased from] Brandt; data of capture not specified.

Type locality: Brazil [Bahia,] "Baıa" (= Salvador).

Distribution: Lophostoma brasiliense occurs from eastern versant of the Andes in Colombia, Ecuador, Peru and Bolivia to Orinoquia and eastern Amazonia in Venezuela,

Guyana, Suriname, French Guiana and Brazil. The southern limit of this species includes the states of Presidente Hayes in central Paraguay [lower (wet) Chaco], Mato Grosso do Sul (Cerrado), Sao Paulo, Rio de Janeiro and Espirito Santo (Atlantic Forest) in Brazil. The species is also present in the Island of Trinidad (Fig. 5).

Measurements: External and craniodental measurements are presented in Table 2.

Emended diagnosis: Lophostoma brasiliense can be distinguished from all Central and South American congeners (only exception of *L. nicaraguae*) by its smaller size (no overlapping in greatest length of skull, condyloincisive length, condylocanine length, braincase breadth, greatest breadth across the mastoid processes, and mandibular toothrow length).

Description and comparisons: *Lophostoma brasiliense* is a small-sized bat (FA = 32.8–42.0 mm; GLS = 18.7–21.7 mm; Table 2). Dorsal pelage presents geographical variations with a coloration ranging from dark brown, reddish brown to gray. Ventral pelage paler than dorsal. Ears are long and round. The skull is small. The sagittal crest is finely development. The dentary is robust. One pair of weakly bifid lower incisors. First lower premolar with posterior portion overlapping anterior part of second lower premolar, second lower premolar with cutting edge slightly above anterior border of cingulum of third lower premolar. For comparisons see *Lophostoma nicaraguae* account.

LOPHOSTOMA NICARAGUAE **(GOODWIN, 1942)**

MESOAMERICAN ROUND-EARED BAT

FIGS. 6–7

Tonatia nicaraguae Goodwin, 1942:205; type locality "Kanawa Creek, near Cukra, north of Bluefields, [Zelaya,] Nicaragua."

Type specimen: Holotype: Adult female, deposited at the American Museum of Natural History (AMNH 41184), prepared as a body in alcohol with extracted skull (broken), and collected on 7 August 1916 by Halter and Mannhardt.

Type locality: Nicaragua [Zelaya,] Kanawa Creek, near Cukra, north of Bluefields, collected at 30 m a.s.l.

Distribution: Lophostoma nicaraguae is a widely distributed species from Mexico, throughout Central America, to northern South America. The records located at the northernmost point of its distribution include the states of Oaxaca, Chiapas, Campeche and Quintana Roo in Mexico, with records in Belize, Guatemala, Honduras, Nicaragua, Costa Rica, Panama, Colombia and Ecuador. The southern limit of this species includes the northern and central part of Colombia, and the western slope of the Andes in Colombia and Ecuador (Pacific region, Fig. 5). *Lophostoma nicaraguae* is distributed in an elevational range from sea level to 1,300 m.

Measurements: External and craniodental measurements are presented in Table 2.

Emended diagnosis: Lophostoma nicaraguae can be easily distinguished of all other species in the genus (*L. evotis*, *L. occidentale*, *L. silvicola*, *L. carrikeri*, *L. schulzi*, and *L. kalkoe*) by its smaller size and shorter skull (AB < 37; GLS < 21). All linear measurements of *Lophostoma nicaraguae* overlap with those of *Lophostoma brasiliense* but are generally smaller.

Description and comparisons: Lophostoma nicaraguae is a small-sized bat (FA = 31.4–36.4 mm; GLS = 18.1–20.5 mm; Table 2). The dorsal fur is tricolored and mummy brown while the ventral fur is bicolored and clearer with a pale brown coloration. Dorsal individual hairs have a white base (approximately 25% of the length of each hair), with a long mummy brown subterminal band (approximately 70% of each hair),

and a very short, pale to whitish terminal band. The ears are mummy brown and are connected by a low band across the forehead with well-marked folds in the naked pinna.

The skull is small, narrow, delicate, and longer than wide (Fig. 6). In dorsal view, the rostrum is elongated, slender, and narrow at the level of the ventral ethmoidal crest, parallel-sided and not constricted in the orbital region. The braincase is low, rounded and without sagittal crest. The distance between the anteriormost point of premaxilla and the nasal is long. Well-developed process of the glenoid fossa. The dentary is thin and slender. Small teeth. Labial cingulum of the upper canine is not well developed. Posterior border of palate ending on a line across front of last molar. Middle lower premolar minute but completely separating anterior and posterior teeth.

Lophostoma nicaraguae does not present post-auricular patches (present in *L. occidentale*, and *L. evotis*) or small wartlike granulations on head, wings, legs and dorsal surfaces of forearms as in *L. schulzi*. Fur in the gular region is dark brown in *Lophostoma nicaraguae*, *L. evotis*, and *L. schulzi*, but is dark to pale brown in *L. silvicola* and *L. brasiliense*, and whitish in *L. occidentale* and *L. carrikeri*. Abdominal fur is pale brown (white in *L. carrikeri* and *L. kalkoe*; dark brown in *L. evotis* and *L. silvicola*; and dark to pale brown in *L. brasiliense*). Lower lip with a naked "V" shaped broken into wart-like protuberances. Noseleaf is broad in the middle and it is gradually attenuated to form a sharp point. The wing membranes are dark brown with the phalanges of a lighter color. Uropatagium sparsely haired on the proximal third and nearly naked posteriorly. Tail is short and does not perforate the uropatagium. The dorsal surface of the forearm is covered with short hair in *Lophostoma nicaraguae*, *L. brasiliense*, *L. carrikeri*, and *L. evotis* (naked in *L. occidentale*, *L. schulzi*, and *L. silvicola*) while ventrally has the proximal third of the forearm with long pale brown hair in *Lophostoma nicaraguae*, *L. occidentale*, *L. brasiliense*, *L. carrikeri*, and *L. evotis*

(short pale brown hair in *L. schulzi* and *L. silvicola*). Metacarpal III is shorter than metacarpal V. Tibia is naked. Dorsal surfaces of the feet are naked, and calcar longer than foot.

Some additional characteristics separate *L. nicaraguae* from its sister species *L. brasiliense*: ears and feet are mummy brown (black to dark brown in *L. brasiliense*) in the same way that the dorsal fur, which is reddish brown (in some geographic regions from Brazil) to dark brown (Colombia and Peru) in *L. brasiliense*. The skull is smaller, narrower, and more delicate than in *L. brasiliense*. Sagittal crest is absent, but weakly developed in *L. brasiliense*. The distance between the anteriormost point of premaxilla and the nasal is long (short in *L. brasiliense*; Fig. 7A). Rostrum is more elongated and narrower at the level of the ventral ethmoidal crest (thick in this area in *L. brasiliense*; Fig. 7B). Presence of well-developed process of the glenoid fossa (absent in *L. brasiliense*; Fig. 7C). The mandible is thinner and slender (robust in *L. brasiliense*). Teeth are generally smaller than in *L. brasiliense*. The cingula of the upper canines are not as well developed as that of *L. brasiliense* (Fig. 7D).

Ecology: Ecological information on *L. nicaraguae* is confusing because of its taxonomic history. Ecological aspects have been published under the names *Tonatia nicaraguae*, *Tonatia minuta*, *Tonatia brasiliensis*, and *Lophostoma brasiliense*. The species has been recorded occupying a wide variety of lowland ecosystems throughout its range, including: tropical rainforest, gallery forest, thorn forest, and areas with secondary vegetation and strong human intervention as coffee crop and banana groves in Honduras and Nicaragua (LaVal, 1969; Valdez & LaVal, 1971; Medina-Fitoria *et al.*, 2020). In Colombia, it inhabits dry tropical and moist tropical forests in the Caribbean and Pacific region (see Esquivel *et al.*, 2020a). This species specializes in the modification and use of termite nest as shelters (Esquivel *et al.*, 2020b), and has been

reported using this type of shelter in Belize (Reid, 1997), Costa Rica (York *et al*., 2008), Panama (Handley, 1966) and Colombia (Esquivel *et al*., 2020b). Dental anomalies have not been reported for this species (Esquivel et al. 2021).

DISCUSSION

Using multiple lines of evidence, we provide a comprehensive view on the taxonomic status of the *L. brasiliense* complex and we revalidate *L. nicaraguae* to the species level. Our results are consistent in recognizing *Lophostoma nicaraguae* as an independent evolutionary lineage apart from *L. brasiliense* and geographically isolated by the Andes, confirming our predictions. The morphology, cranial/mandibular shape and genetics of *Lophostoma nicaraguae* are distinct from *Lophostoma brasiliense* (Fig. 8), and all other species of *Lophostoma*. This species increases the number of valid taxonomic units in *Lophostoma* from seven to eight, with four of them occurring in Central America.

Species with wide distribution across diverse habitat types in Neotropical region often exhibit high genetic diversity or form cryptic species complexes, for instance in the Phyllostomidae, *Carollia castanea* (Solari & Baker, 2006), *Glossophaga soricina* (Calahorra-Oliart *et al*., 2021), *Platyrrhinus helleri* (Velazco *et al*., 2010), and *Trachops cirrhosus* (Clare *et al*., 2007). Accordingly, the wide distribution of *L. brasiliense* from Mexico to southern Brazil would indeed suggest the probable existence of unrecognized lineages. Here, our analyses recognized two distinct lineages distributed over different geographic regions, among what was traditionally known as *L. brasiliense*, supporting our initial hypothesis.

The diversification process of the genera of the Phyllostominae occurred during the Miocene (23–16.9 mya) (Hoffmann *et al*., 2008). Specifically, divergence of

Lophostoma from the remainder of the Phyllostomini must have occurred at least at 15.4 mya, in the Mid-Miocene. Hoffmann *et al*. (2008) estimated the divergence between *L. brasiliense* and *L. carrikeri* about ~ 6.0 mya, in the Late-Miocene. So, the separation of *Lophostoma nicaraguae* from *L. brasiliense* was more recent and probably accompanied by events favoring its geographical isolation, such as the latest rise of the Eastern Cordillera, Northern Andes. We hypothesize that an ancestral panmictic population split due to the surface uplift of the Andes, resulting in allopatric speciation of those two lineages. Between 6 and 3 mya ago, the Northern Andes experienced a topographic growth from elevations <1000 m to the present >2500 m, favoring vicariance processes (Mora *et al*., 2008; 2020). The known distribution of *L. nicaraguae* and *L. brasiliense* suggests elevational restrictions, with none of the species recorded above 1400 m. Thus, the Andes seem to have played a fundamental role as a barrier to gene flow, delimiting the northern distribution of *L. brasiliense* and the southern distribution of *L. nicaraguae*, separating these two lineages (Fig. 5).

A usual first step for species discovery and delimitation processes are the single-locus methods. Due to their limitations, convergent results from different algorithms should be considered a robust primary species hypothesis (Puillandre *et al*., 2021). The distance-based and tree-based approaches used here, which are grounded on distinct species criteria, converged to recognize *L. nicaraguae* as a taxonomic entity distinct from *L. brasiliense*. Moreover, GMYC split *L. brasiliense* into three other candidate species, corresponding to populations from Amazonia, Cerrado, and Atlantic Forest. This algorithm tends to be more sensitive than others used here (Pentinsaari *et al*., 2017) but, like the putative physical barrier that split *L. nicaraguae* from *L. brasiliense*, the wide range of environmental conditions in historical and contemporary South America may have promoted a restriction to gene flow among the populations of *L. brasiliense*.

Distance-based methods are grounded on threshold values to recognize species limits. The use of fixed threshold neglects the evolutionary heterogeneity and coalescence within diverse lineages (Fujita *et al*., 2012; Pentinsaari *et al*., 2017). For any taxon, empirical data should be used to look for barcoding gaps, and then setting a threshold value (Gonçalves *et al*., 2021). Our sampled data of COI suggested the threshold value for *Lophostoma* between 2.75 and 4.28%. For the two closest species of *L. nicaraguae*, *L. brasiliense* and *L. carrikeri* (Fig. 8), divergence is about 6.8 and 11.7%, respectively. The inclusion of new data, mainly sequences from unsampled populations, may refine these values. Thus, the evaluation of threshold values should be iterative (Bianchi & Gonçalves, 2021).

Further distributional and molecular evidence may reinforce *Lophostoma nicaraguae* as an independent lineage from *L. brasiliense*. For instance, Velazco & Cadenillas (2011) using the mtDNA cytochrome-*b* found a population of *L. brasiliense* from Suriname presenting 7.81% of divergence from Panamanian populations, here named *L. nicaraguae*. Other pairs of sister species within the Phyllostomidae show similar distribution (Western *vs.* Eastern Andean Cordilleras) and cytochrome-*b* divergence, for instance, *Carollia castanea* and *C. benkeithi* (8.1%) (Solari & Baker, 2006), *Gardnerycteris keenani* and *Gardnerycteris crenulata* (12.3%) (Hurtado & D'Élía, 2018), and *Tonatia bakeri* and *Tonatia maresi* (7.65%) (Basantes *et al*., 2020). The similar distribution and genetic divergence between these species suggest that all those species diversified under the same evolutionary scenario (i.e., allopatric speciation with the Andes as the vicariant barrier).

Despite the molecular and geographic differences mentioned above, our morphometric analyses indicate high phenotypic similarity between *L. nicaraguae* and *L. brasiliense*. Using quantitative data gathered from over 160 specimens we were not able to recover

any non-overlapping differences in univariate data that would permit a reliable diagnosis of the species. Although univariate tests recovered significant differences for all 20 traits among *L. nicaraguae* and *L. brasiliense*, differences in mean trait values do not allow for unambiguous species diagnoses. Therefore, considering only univariate quantitative data, the separation of the two species is a complicated task. We also found continuities in the morphometric space between these species. This scarce phenotypic separation between species is typical of cryptic complexes and may be explained by at least three different mechanisms: (1) recent divergence, (2) convergent evolution (parallelism or convergence), and (3) phylogenetic niche conservatism (Fišer *et al.*, 2018; Struck *et al.*, 2018). We consider that a relatively recent divergence in *L. nicaraguae* better explains its poor morphological differentiation in univariate traits from its sister species, *L. brasiliense*. It is worth noting that this morphological similarity, added to the limited availability of specimens in the museums, were the reasons for the confusing taxonomic history of this species complex.

Geometric morphometrics is being increasingly used in bat systematics to analyze variation in shape and discriminate among species and populations (Evin *et al*., 2008; Velazco *et al*., 2010; Taylor *et al*., 2018; Calahorra-Oliart *et al*., 2021). Here, the geometric morphometrics was doubtless in supporting the recognition of *L. nicaraguae* as a species. Contrary to the use of linear measurements, the shape of the cranium and mandible were useful in distinguishing the two species. Geometric morphometrics analyses clearly demonstrate that *L. nicaraguae* and *L. brasiliense* largely differ in centroid size, and exhibit large differences in mandibular and skull shape. The most informative components were the lateral mandible and ventral skull. These components reflect specializations for feeding and echolocation (Herrel *et al*., 2008; Santana *et al*.,

2010; Arbour *et al*., 2019) and could be prioritized in future studies seeking solving taxonomic problems between closely related species.

Considering the cranial and mandibular shape, the pattern of sexual dimorphism was similar for both *L. nicaraguae* and *L. brasiliense*. While the size is similar between males and females, the mandibles of females are significantly thinner than those of males. We see two explanations for this pattern: first, at the physiological level it has been found that the females of some insectivorous bats, such as *Eptesicus fuscus*, decrease their skeletal mass during pregnancy and lactation due to the increased calcium requirements associated with raising the offspring (Booher & Hood, 2010). This is also seen in other mammals, including humans (Grizzo *et al.,* 2020); second, from a functional ecology perspective, it is known that males of *Lophostoma* use their teeth to modify active termite nests and use them as roosts, suggesting adaptations to roost excavation (Esquivel *et al*., 2020b), and thus explaining a positive pressure for thicker mandibles.

Lophostoma constitutes an example of a successful phyllostominae radiation, with its species occupying many ecosystems of the Neotropics (Williams & Genoways, 2008). The use of multiple lines of evidence unveiled new lineages of these bats, placing them among the most diverse Phyllostominae genera (Velazco & Gardner, 2012). Despite these advances, our results still suggest a greater number of species within *Lophostoma*. We found considerable molecular divergence in *L. silvicola* dispersed in different lineages. *Lophostoma silvicola* has been recovered as paraphyletic, clustering the entire lineage of *L. evotis* (Baker *et al*., 2004; Velazco & Cadenillas, 2011). Further studies should use multiple lines of evidence to solve doubts about the taxonomic status of the currently recognized subspecies of *L. silvicola*, as well as the taxonomic identity of *L. evotis*.

Data Availability statement

Genetic sequences are available in Genbank. Supplementary data are available with this article in its online version. Data and R codes are available upon request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. List of specimens included in this study.

Appendix S2. A- Detailed descriptions of the landmarks and semilandmarks used in this study and a figure with details about these anatomical points on each view. **B-** Selection of the best missing data estimator to our dataset using simulations with incomplete specimens.

Appendix S3. Analysis of linear and shape measures including holotypes.

Table S1. Specimens used for phylogenetic analyses. Species, vouchers numbers, GenBank/BolSystems accession numbers and geographic information are given for the *Lophostoma* samples used in the phylogenetic analyses.

Table S2. Results from performing LaSEC with 1000 iterations.

Figure S1. Paired test and density plots showing differences between sex.

Figure S2. Density plots of individual traits between species. Dark = *Lophostoma brasiliense*, Blue = *Candidate species*.

Figure S3. A- Plot centroid size by species. **B** – Plot centroid size by species/sex.

Figures

Figure 1. Sampling localities of *Lophostoma* specimens analyzed in this study. Dark dots represent localities from specimens in museums, the red star indicates the type locality for *L. brasiliense*, while the green, blue, and orange triangles point out type localities for synonyms (*nicaraguae*, *venezuelae*, and *minuta*, respectively). A list of revised specimens with their respective localities is presented in the Supporting Information, Appendix S1.

Figure 2. Morphometric variation in *Lophostoma brasiliense*. **A**- Principal Components Analysis (PCA) on 20 externals, craniodental and mandibular measurements performed for all samples of the *Lophostoma brasiliense* complex. Each individual is represented by a dot, painted according to the group to which it belongs (*L. brasiliense*: black; candidate species: light blue). Outer solid-line ellipses delimit the area enclosing 95% of the individual points in each group, whereas inner broken-line ellipses encompass 50% of those points. **B**- Linear Discriminant Function (LD1) after cross-validation tests. Even though there is overlap between the two putative species showing morphological similarity, individuals tend to cluster together with their respective group.

Figure 3. Support for species delimitation scenarios without *a priori* information generated from normal mixture models. Plot shows results for normal mixture models specifying one to nine morphological clusters, as well as models fitting both previous and current hypotheses of subspecies.

Figure 4. Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) of *Lophostoma brasiliense* and the candidate species obtained from four different views. Each individual is represented by a dot, painted according to the group to which it belongs (*L. brasiliense*: black; candidate species: light blue).

Figure 5. Geographic distribution of *Lophostoma nicaraguae* (light blue) and *Lophostoma brasiliense* (light dark) based on the localities of the specimens analyzed in this study.

Figure 6. Dorsal, ventral, and lateral views of the skull and lower jaw of the holotype of *Lophostoma nicaraguae* (AMNH 41184♀).

Figure 7. Comparative morphology of *Lophostoma brasiliense* (left, FMNH 75140♂) and *Lophostoma nicaraguae* (right, TTU 28009♂).

Figure 8. Phylogram of 56 *Lophostoma* COI sequences showing results of species delimitation methods. Numbers indicate support values of the adjacent node retrieved in the Bayesian inference. Titles of columns indicate the current treatment of *L. brasiliense* as one group (CurT), and the results from species delimitation methods employed (see Taxonomic decision section). Boxes in different colours indicate species inferred with each method.

Tables

Table 1. External and craniodental variables used in this study.

Table 2. External and craniodental measurements (mm), including mean, standard deviation, range, and sample size of *Lophostoma brasiliense* and the candidate species. p-values from univariate tests are shown. Measurement acronyms follow Table 1.

Table 3. DFA classification results without (DFA) and with (DFA-CVs) leave-one-out cross-validation for all morphometrics analyses and datasets. MANOVA statistically significant differences between the candidate species and *Lophostoma brasiliense sensu stricto* are marked with an asterisk.

Table 4. ANOVA results regarding effects of sex, species and their interaction on centroid size (log CS).

Table 5. ANOVA results regarding effects of size (allometry), sex (sexual dimorphism), species and their interactions on shape.

Figure 1

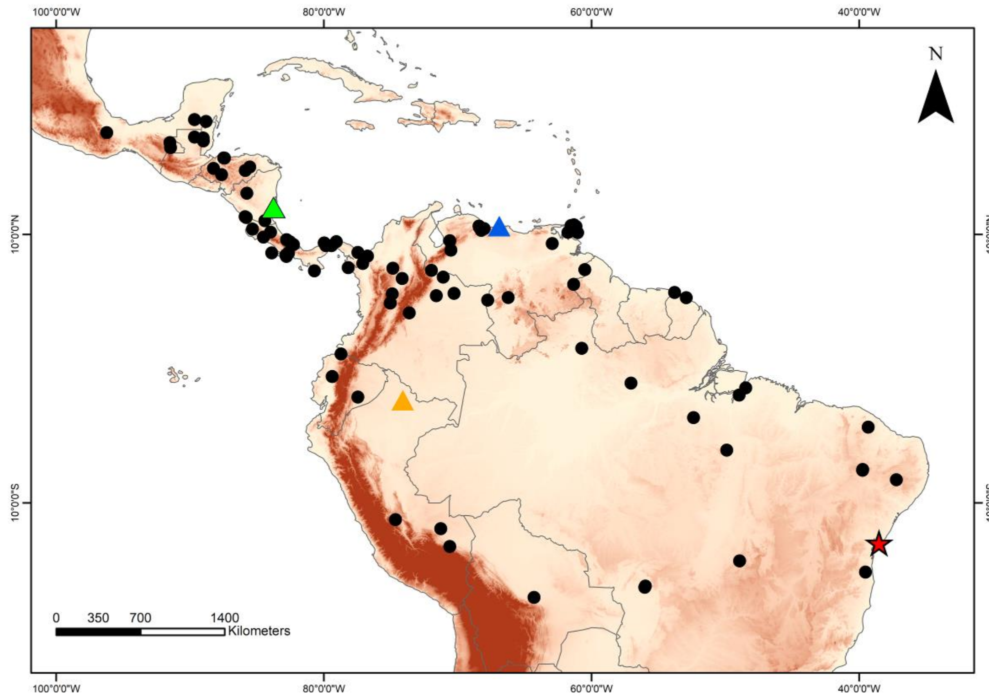

Figure 2

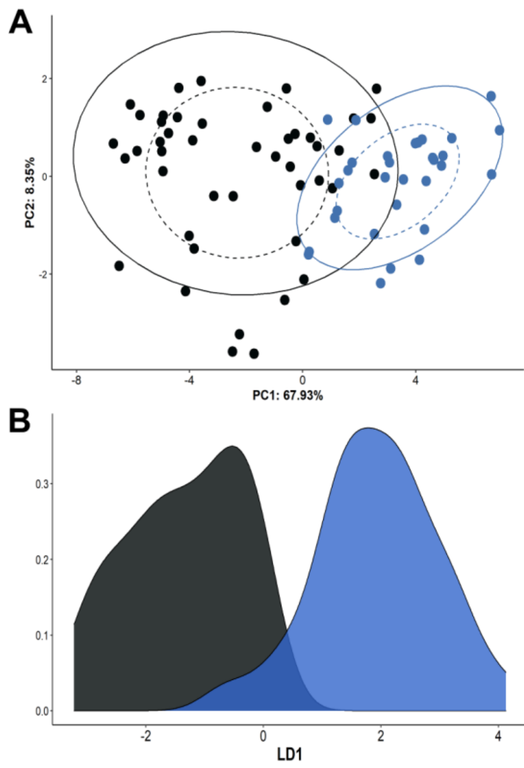

Figure 3

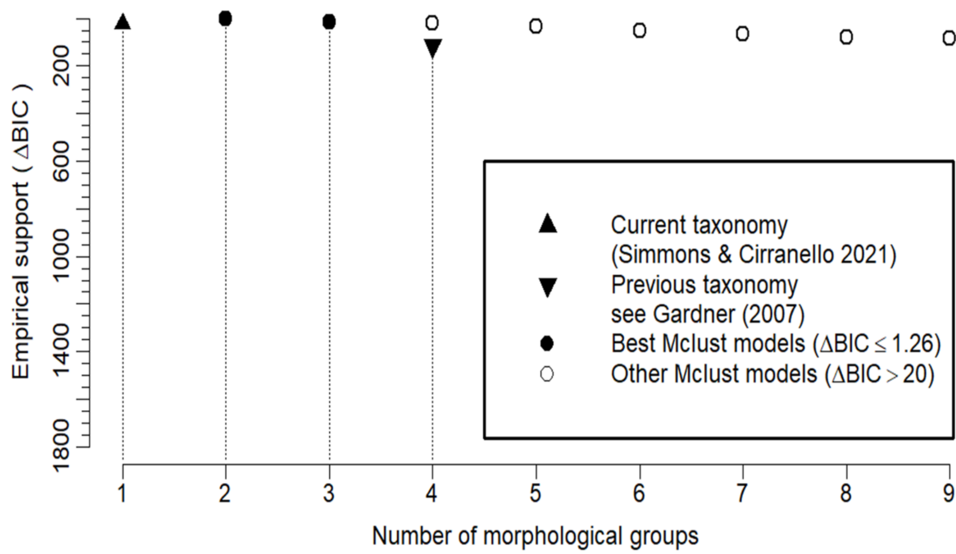

Figure 4

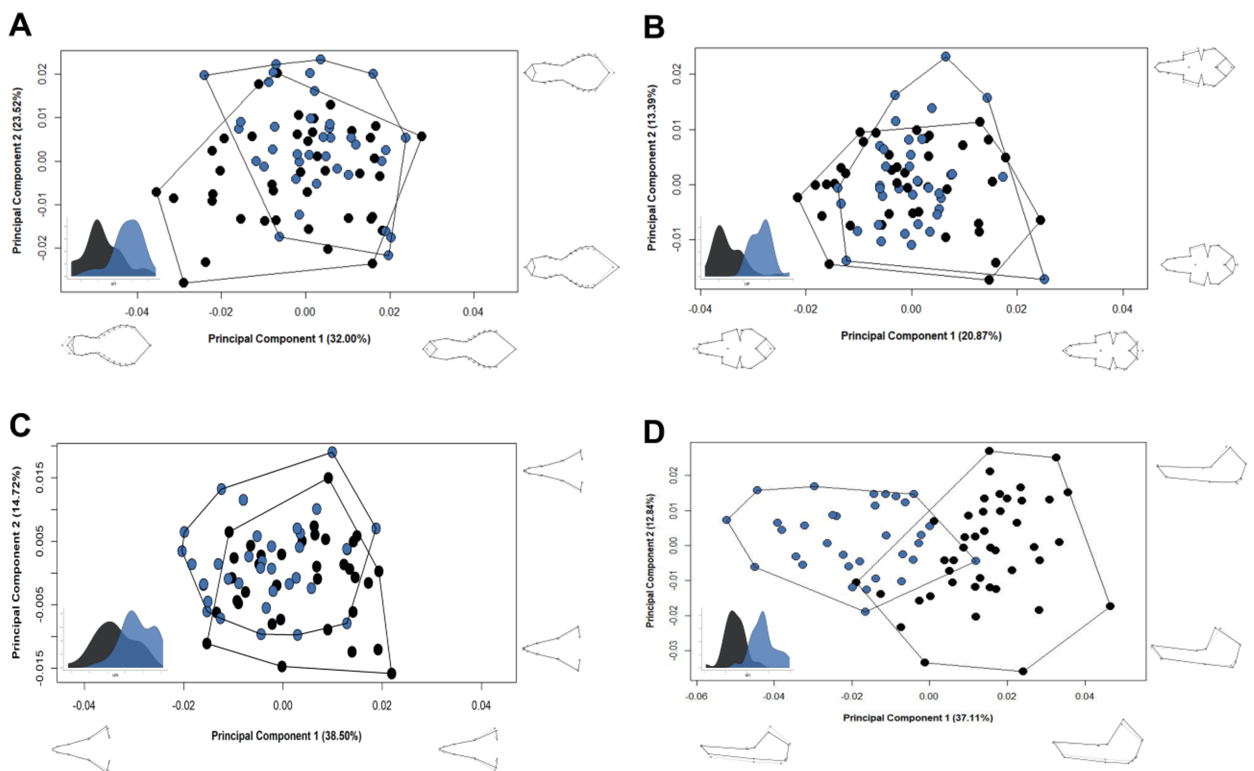

Figure 5

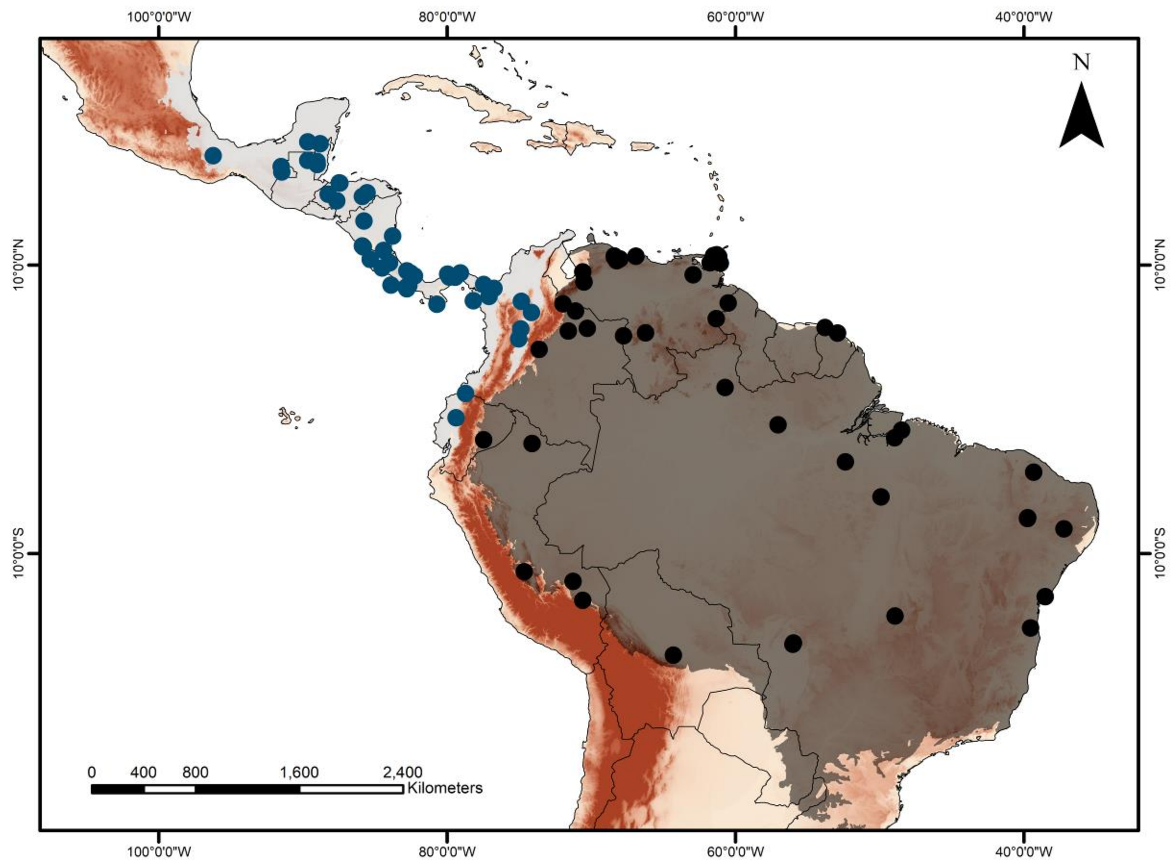

Figure 6

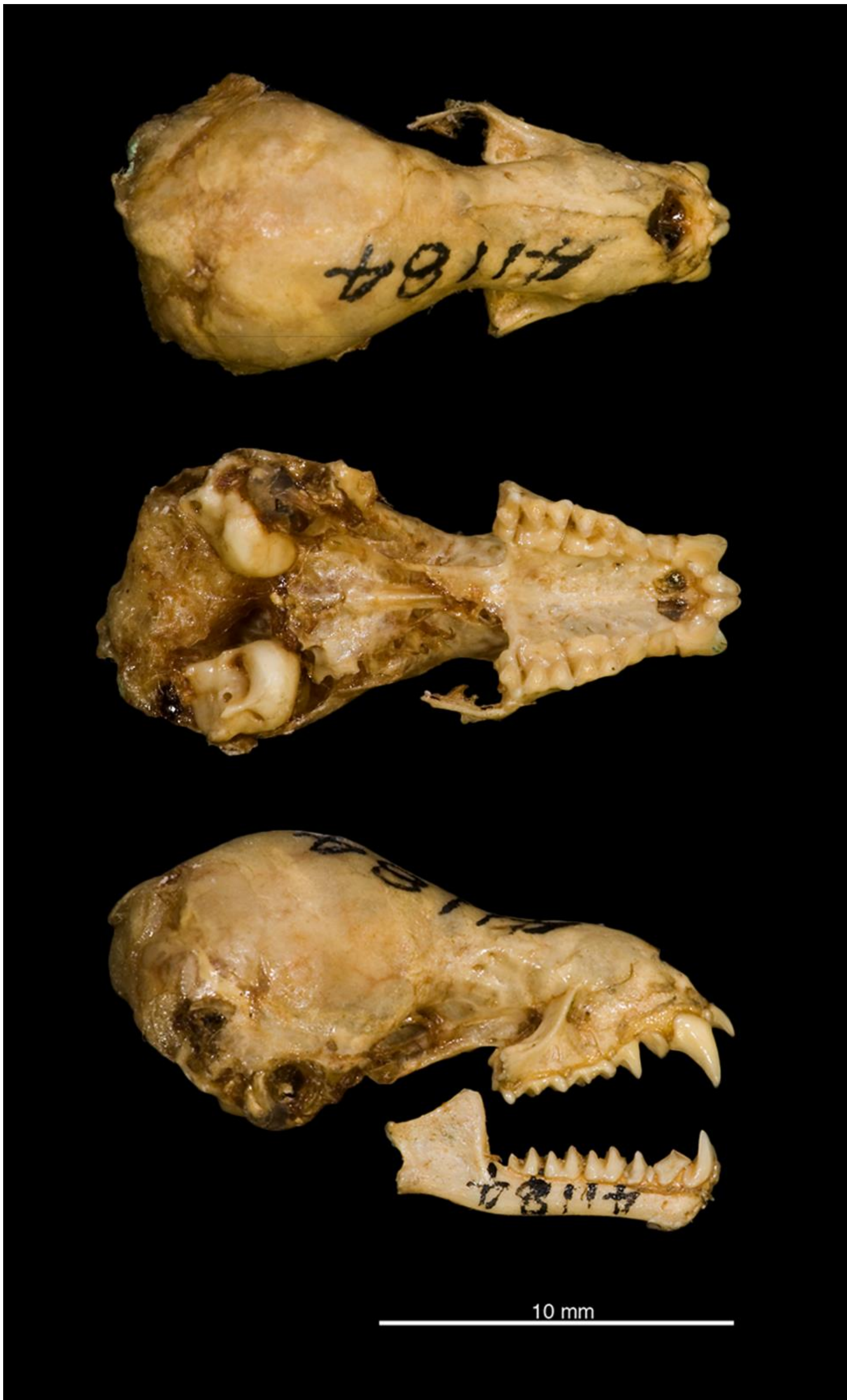

Figure 7

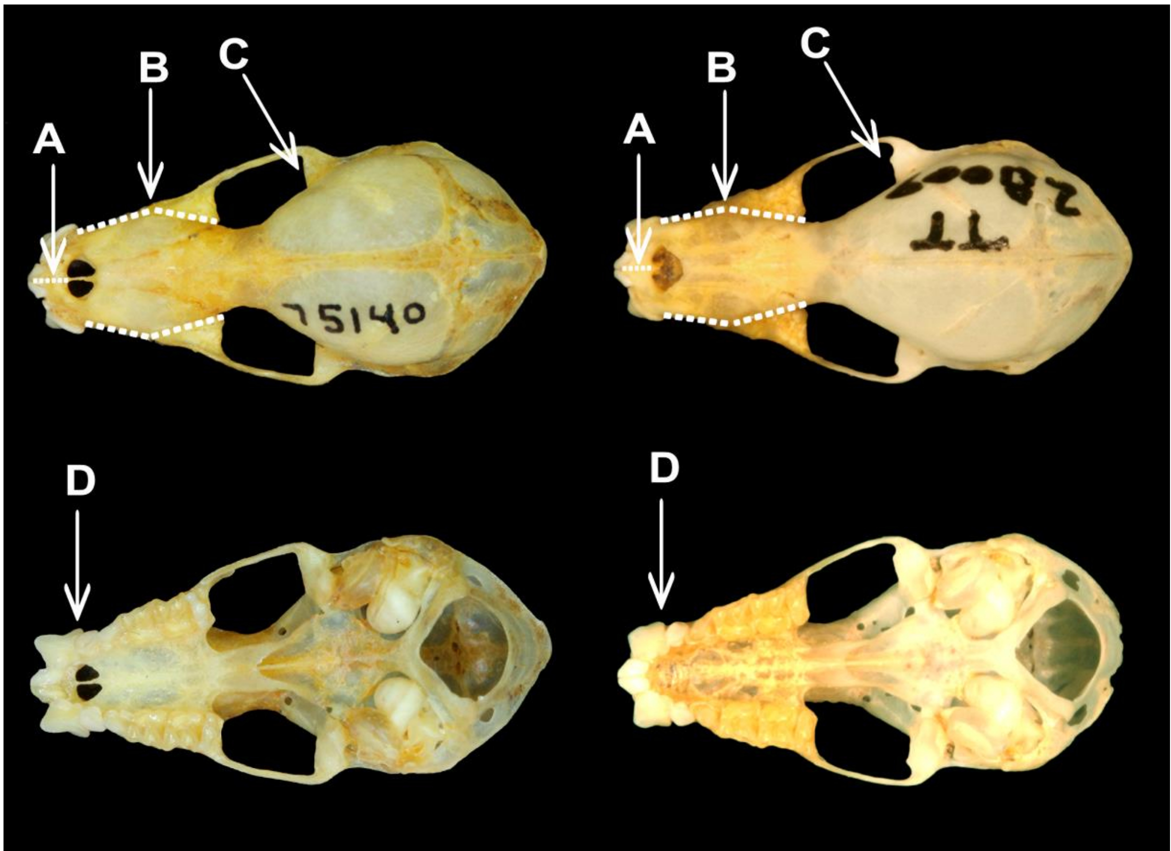

Figure 8

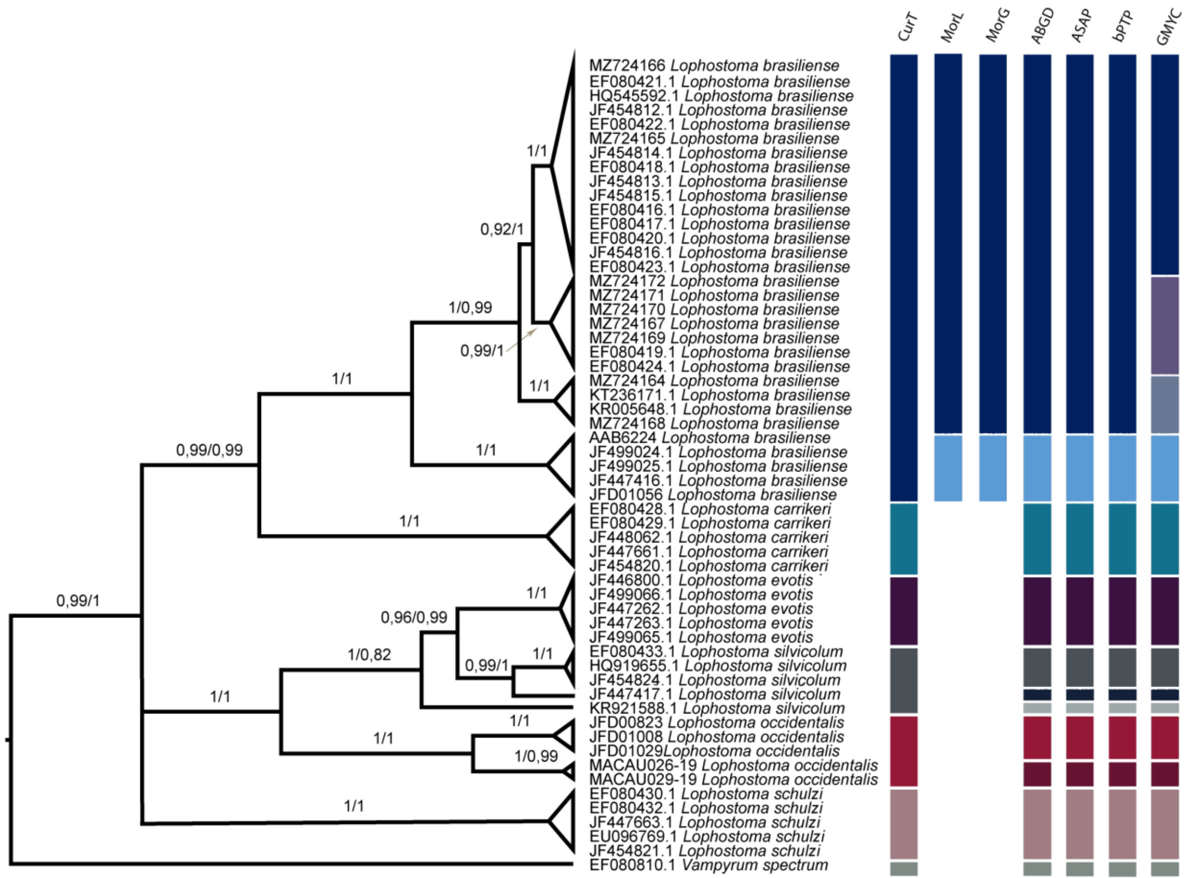

Table 1

	Measurement	Definition
External	Forearm length (FA)	Distance from the tip of the olecranon process to the wrist (including the carpals). This measurement is made with the wing at least partially folded.
	Ear length (EL)	Distance from basal notch to the tip of the pinna.
	Hind-foot length (HF)	From the proximal edge of the base of the calcar to the tip of the claw of the longest toe.
	Bands of contrast in the dorsal fur	Bicolored (0), tricolored (1), tetracolored (2).
	Bands of contrast in the dorsal ventral	Unicolored (0), bicolored (1), tricolored (2), tetracolored (3).
Cranial	Greatest Length of Skull (GLS)	Greatest distance from the occiput to the anteriormost point on the premaxilla (including the incisors).
	Condyloincisive Length (CIL)	Distance between a line connecting the posteriormost margins of the occipital condyles and the anteriormost point on the upper incisors.
	Condylocanine Length (CCL)	Distance between a line connecting the posteriormost margins of the occipital condyles and a line connecting the anteriormost surfaces of the upper canines.
	Braincase Breadth (BB)	Greatest breadth of the globular part of the braincase, excluding mastoid and paraoccipital processes.
	Zygomatic Breadth (ZB)	Greatest breadth across the zygomatic arches.
	Postorbital Breadth (PB)	Least breadth at the postorbital constriction.
	Palatal Length (PL)	Distance from the posterior palatal notch to the anteriormost border of the incisive alveoli.
	Palatal Width at Canines (PWC)	Least width across palate between lingual margins of the alveoli of upper canines.
	Mastoid Width (MSTW)	Least breadth across skull immediately behind jugal base of zygomatic arches.
	Mastoid Process Width (MPW)	Greatest breadth across the mastoid processes.
	Maxillary Toothrow Length (MTRL)	Distance from the anteriormost surface of the upper canine to the posteriormost surface of the crown of M3.
	Molariform Toothrow Length (MLTRL)	Distance from the anteriormost surface of P3 to the posteriormost surface of the crown of M3
	Palatal Width at M2 (PWM2)	Greatest width across palate between labial margins of the M2s
	Dentary Length (DENL)	Distance from midpoint of condyle to the anteriormost point of the dentary.
	Mandibular Toothrow Length (MANDL)	Distance from the anteriormost surface of the lower canine to the posteriormost surface of m3.
	Coronoid Height (COH)	Perpendicular height from the ventral margin of mandible to the tip of coronoid process.
	Posterior border of the hard palatal (MP)	‘‘U’’ shaped (0), ‘‘V’’ shaped (1).
	Clinoid process (CP)	Absent (0), Present (1).
	Base Foramen (BF)	‘‘U’’ shaped (0), ‘‘V’’ shaped (1), Other (2)
Postcranial	Metacarpal III Length (MET-III)	Distance from the joint of the wrist (carpal bones) with the 3rd metacarpal to the metacarpophalangeal joint of 3rd digit.

Table 2

	Lophostoma brasiliense	*Candidate species*	***p*-value**
FA	36.24 ± 1.93	34.70 ± 0.88	**< 0.01**
	(32.87–42.00) 69	(31.41–36.40) 41	
HF	11.45 ± 1.10	10.40 ± 0.89	**< 0.01**
	(8.80–14.00) 51	(9.00–13.00) 36	
E	23.86 ± 1.81	23.33 ± 1.12	0.057
	(18.60–28.00) 52	(20.00–25.00) 37	
MET-III	28.44 ± 1.53	27.46 ± 1.25	**< 0.05**
	(25.25–32.90) 74	(21.92–29.90) 41	
GLS	20.34 ± 0.79	19.41 ± 0.48	**< 0.01**
	(18.70–21.79) 81	(18.18–20.50) 60	
CIL	17.87 ± 0.69	17.15 ± 0.41	**< 0.01**
	(16.20–19.27) 81	(16.12–18.05) 60	
CCL	17.31 ± 0.64	16.59 ± 0.39	**< 0.01**
	(16.12–18.77) 81	(15.59–17.42) 60	
BB	8.23 ± 0.24	7.89 ± 0.20	**< 0.01**
	(7.59–8.97) 81	(7.40–8.33) 60	
ZB	9.71 ± 0.42	9.16 ± 0.27	**< 0.01**
	(8.79–10.59) 81	(8.18–9.60) 60	
PB	3.27 ± 0.13	3.17 ± 0.13	**< 0.05**
	(3.00–3.60) 82	(2.82–3.50) 60	
PL	8.90 ± 0.51	8.37 ± 0.38	**< 0.01**
	(7.85–9.97) 81	(7.60–9.13) 60	
PWC	3.97 ± 0.29	3.78 ± 0.25	**< 0.05**
	(3.40–4.60) 82	(3.33–4.41) 60	
MSTW	8.57 ± 0.35	8.13 ± 0.28	**< 0.01**
	(7.69–9.48) 81	(7.28–8.96) 60	
MPW	9.47 ± 0.36	9.02 ± 0.27	**< 0.01**
	(8.71–10.25) 81	(8.23–9.53) 60	
MTRL	7.08 ± 0.28	6.84 ± 0.18	**< 0.01**
	(6.58–7.73) 82	(6.28–7.15) 60	
MLTRL	5.83 ± 0.31	5.55 ± 0.18	**< 0.01**
	(5.17–6.46) 81	(5.00–5.80) 60	
PWM2	6.38 ± 0.25	6.07 ± 0.21	**< 0.01**
	(5.75–6.96) 81	(5.62–6.63) 60	
DENL	12.79 ± 0.58	12.15 ± 0.26	**< 0.01**
	(11.54–14.05) 82	(11.48–12.64) 60	
MANDL	7.88 ± 0.35	7.60 ± 0.22	**< 0.01**
	(6.97–8.60) 82	(7.12–8.19) 60	
COH	4.90 ± 0.32	4.42 ± 0.27	**< 0.01**
	(4.11–5.64) 81	(3.63–4.95) 60	

Table 3

Dataset	N	DFA % correct		DFA-CVs % Correct		MANOVA
		L. brasiliense	*Candidate species*	*L. brasiliense*	*Candidate species*	
Log-Transformed Traditional Data						
External only	77	86.36	90.91	79.54	84.85	< 0.001*
Cranial only	130	91.67	91.38	84.72	84.48	< 0.001*
Cranial and External	76	100	90.32	91.11	87.10	< 0.001*
Principal Components (~90%)						
External only	77	81.82	84.85	75.00	84.85	< 0.001*
Cranial only	130	90.28	93.10	86.11	91.38	< 0.001*
Cranial and External	76	100	90.32	100	90.32	< 0.001*

Table 4

	Df	SS	MS	R^2	F	Z	P
Centroid Size (CS)							
(A) DORSAL SKULL							
Sex	1	0.0004	0.0004	0.0030	0.2599	-0.2555	0.6027
Species	1	0.0091	0.0091	0.0673	5.7949	1.9221	**< 0.05**
Sex x species	1	0.0091	0.0091	0.0671	5.7809	1.9609	**< 0.05**
Residuals	74	0.1164	0.0016	0.8591			
Total	77	0.1355					
(B) VENTRAL SKULL							
Sex	1	0.0001	0.0001	0.0003	0.0240	-1.2212	0.8750
Species	1	0.0558	0.0558	0.1380	12.9191	2.8282	**< 0.001**
Sex x species	1	0.0327	0.0327	0.0807	7.5575	2.2228	**< 0.05**
Residuals	73	0.3155	0.0043	0.7797			
Total	76	0.4047					
(C) DORSAL MANDIBLE							
Sex	1	0.0008	0.0008	0.0064	0.5283	0.1025	0.4721
Species	1	0.0198	0.0198	0.1619	13.3558	2.8506	**< 0.001**
Sex x species	1	0.0088	0.0088	0.0719	5.9324	1.9604	**< 0.01**
Residuals	63	0.0933	0.0015	0.7637			
Total	66	0.1221					
(D) LATERAL MANDIBLE							
Sex	1	0.0008	0.0008	0.0031	0.2312	-0.3511	0.6392
Species	1	0.0157	0.0157	0.0577	4.3726	1.6908	**< 0.05**
Sex x species	1	0.0117	0.0117	0.0430	3.2551	1.4301	0.0701
Residuals	68	0.2443	0.0036	0.8979			
Total	71	0.2721					

Table 5

	Df	SS	MS	R^2	F	Z	P
Shape							
(A) DORSAL SKULL							
Size	1	0.0034	0.0034	0.0721	6.5178	3.8334	**< 0.001**
Sex	1	0.0024	0.0024	0.0512	4.6274	3.0086	**< 0.001**
Species	1	0.0017	0.0017	0.0370	3.3401	2.4674	**< 0.001**
Size x sex	1	0.0007	0.0007	0.0144	1.3040	0.7271	0.2364
Size x species	1	0.0009	0.0009	0.0188	1.6979	1.1702	0.1233
Sex x species	1	0.0004	0.0004	0.0089	0.8057	-0.1179	0.5430
Size x sex x species	1	0.0004	0.0004	0.0092	0.8314	-0.0572	0.5212
Residuals	70	0.0364	0.0005	0.7745			
Total	77	0.0471					
(B) VENTRAL SKULL							
Size	1	0.0023	0.0023	0.0628	5.5333	4.5838	**< 0.001**
Sex	1	0.0009	0.0009	0.0258	2.2708	2.2296	**< 0.01**
Species	1	0.0020	0.0020	0.0555	4.8839	4.4583	**< 0.001**
Size x sex	1	0.0004	0.0004	0.0111	0.9729	0.1371	0.4426
Size x species	1	0.0008	0.0008	0.0225	1.9830	1.8623	**0.0309**
Sex x species	1	0.0004	0.0004	0.0115	1.0153	0.2160	0.4127
Size x sex x species	1	0.0007	0.0007	0.0190	1.6715	1.4122	0.0808
Residuals	69	0.0281	0.0004	0.7835			
Total	76	0.0359					
(C) DORSAL MANDIBLE							
Size	1	0.0004	0.0004	0.0165	1.2111	0.5777	0.2798
Sex	1	0.0005	0.0005	0.0238	1.7469	1.2371	0.1073
Species	1	0.0009	0.0009	0.0435	3.1931	2.3673	**< 0.01**
Size x sex	1	0.0004	0.0004	0.0197	1.4453	0.8921	0.1830
Size x species	1	0.0003	0.0003	0.0158	1.1627	0.4879	0.3108
Sex x species	1	0.0004	0.0004	0.0187	1.3774	0.7943	0.2130
Size x sex x species	1	0.0008	0.0008	0.0355	2.6104	1.9593	**0.0268**
Residuals	59	0.0171	0.0003	0.8029			
Total	66	0.0213					
(D) LATERAL MANDIBLE							
Size	1	0.0022	0.0022	0.0257	2.384	2.326	**< 0.01**
Sex	1	0.0031	0.0031	0.0351	3.256	2.940	**< 0.01**
Species	1	0.0161	0.0161	0.1854	17.179	5.674	**< 0.001**
Size x sex	1	0.0007	0.0007	0.0079	0.735	-0.496	0.691
Size x species	1	0.0008	0.0008	0.0096	0.888	-0.101	0.538
Sex x species	1	0.0013	0.0013	0.0145	1.342	0.837	0.200
Size x sex x species	1	0.0010	0.0010	0.0118	1.092	0.389	0.347
Residuals	64	0.0600	0.0009	0.6906			
Total	71	0.0868					

CAPÍTULO III – Diversidade críptica no complexo *Lophostoma silvicola*

Artigo a ser submetido ao periódico Zoologica Scripta

Tackling the Linnean shortfall in *Lophostoma* (Chiroptera, Phyllostomidae): an approach with multiple lines of evidence

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Short running title: Tackling the Linnean shortfall in *Lophostoma*

ABSTRACT

The Linnean shortfall represents one of the main challenges for biodiversity, because it implies a gap in the taxonomic knowledge of the most basic units of study in ecology, evolution, and conservation: species. Here, we address the Linnean shortfall in the *Lophostoma silvicola* species complex using multiple lines of evidence through an integrative approach to clarify the species boundaries, distribution, and evolutionary relationships. Our results provide a comprehensive view on the taxonomic status of the complex and indicate three clearly differentiated genetic lineages, namely, *Lophostoma silvicola* (*sensu stricto*), in Paraguay, Bolivia, southern Peru and central Brazil; *Lophostoma laephotis* in Guyana, French Guiana, Suriname and northern Brazil; and *Lophostoma amblyotis*, in central/northern Peru, western Brazil, Ecuador, Venezuela and Panama. We suggest raising *L. laephotis* and revalidate *L. amblyotis* to the species level. Our data suggest that these species diverged from a recent speciation probably driven by ecological factors.

KEYWORDS

bats, cryptic species, integrative taxonomy, mammals, species delimitation, systematics, taxonomy, round eared bat.

1 | INTRODUCTION

The discovery and description of new species in a world under accelerated change due to anthropogenic pressures is task against the clock for science. Despite describing hundreds of new species each year, it is unknown how many species may be disappearing or how many species are yet to be discovered (Yap et al. 2015; Malcom et al. 2019). Conservative estimates suggest that the total number of species is approximately 5–9 million (Mora et al., 2011; Costello et al., 2012), but only about 1.9 million have been named (Mace et al. 2005; Roskov et al. 2019). This lack of taxonomic resolution has been called the Linnean shortfall (Lomolino 2004; Hortal et al. 2015), and has profound implications, as species represent the fundamental units of biological studies (Bianchi & Gonçalves, 2021). In ecological and evolutionary studies, species are the cornerstone for exploring broad-scale patterns of biodiversity organization and the processes behind them (Scheffers et al. 2012).

Tackling the Linnean shortfall has become a priority, since we are living the sixth mass extinction due to accelerated human–induced species losses (Wake & Vredenburg 2008; Ceballos et al. 2015, 2020) with a rate estimated to be as high as those of the five previous mass extinctions of Earth's history (Pimm et al. 1995; Barnosky et al. 2011). One of the main challenges to face this shortfall is the presence of cryptic species. These species are commonly defined as the occurrence of different evolutionary lineages of phenotypically similar organisms classified as a single species (Bickford et al. 2007). Even those species understood as well-studied may hold cryptic species; some are virtually invisible and overlooked in conservation plans, aggravating the possibility to reduce the Linnean shortfall. However, new approaches have paved the way for the development of new techniques and methods to understand the taxonomic units. Side by side with these practical advances, new species concepts (see de Queiroz 2007), have helped taxonomists to identify and separate cryptic species. Several taxonomists have been using different lines of evidence (e.g. molecular, morphological, acoustic, ecological, climatic and geographic data), looking to recognize patterns across the data to identify and delimitate species (DeSalle et al., 2005; Bickford et al., 2007). Species boundaries are drawn from a set of evidence pointing out a separation of lineages (de Queiroz 2007), so that the taxonomist must decide which and how much evidence is enough to infer when two evolutionary lineages represent separate species. Under this approach, called "integrative

taxonomy" (Padial et al. 2010, but see Yeates et al 2010), multiples lines of evidence provide a stronger hypothesis considering different evolutionary lineages allowing the establishment of more robust boundaries (de Queiroz 2007).

Bats comprise one of the most diverse mammalian orders with approximately 1430 described species (Simmons & Cirranello 2020). But, due to its habits, behaviors (e.g. nocturnal and high-flying animals), and the difficulty of assessing them in the wild, the knowledge of its richness is incipient (Solari et al. 2019). This taxonomic group has experienced an elevated number of new species described in recent years. In the past two decades, more than 300 species were described as the result of discoveries based on fieldwork or taxonomic reviews using integrative taxonomy (Burgin et al. 2018). Although Chiroptera is considered a well-known group among the mammals, studies have unveiled a high cryptic diversity in many species, demanding a deeper revision (Clare 2007, 2011; Loureiro et al. 2019; Calahorra-Oliart et al. 2021). Considering the rapid and widespread habitat destruction worldwide, and particularly in the megadiverse Neotropics, this region should receive urgent attention as there is a risk of losing biodiversity before species are even described by science (Aguiar et al. 2020).

Lophostoma d'Orbigny, 1836 bats (Phyllostomidae: Phyllostominae) are gleaning insectivores common in the Neotropical region, where occupy a wide variety of habitats across their range, from semiarid regions to tropical humid forests (Williams and Genoways 2008). This genus, currently, comprises eight valid species, but recent studies using morphological and genetic data suggested that its diversity is underestimated, especially in species as *Lophostoma brasiliense* and *Lophostoma silvicola* which represent cryptic species (Velazco & Cadenillas 2011; Lim & Lee 2018).

Lophostoma silvicola is recognized as a cryptic species complex distributed continuously in the Neotropic from southern Mexico to southwestern Paraguay (Simmons 2005; Williams and Genoways 2008). Three subspecies are valid: *Lophostoma s. centralis* Davis and Carter, 1978, in Central America; *L. s. laephotis* (Thomas, 1910), from the Guianas to the lower Amazon basin of Brazil; and *L. s. silvicola* d'Orbigny, 1836, the nominal subspecies located in Paraguay, Bolivia, east of the Andes in Peru, Ecuador, and Colombia, and with records in Venezuela and Brazil (Simmons 2005; Williams and Genoways 2008). Moreover, molecular studies indicate possible additional lineages, suggesting an underestimated diversity within the *L. silvicola* complex (Clare et al. 2007, 2011; Velazco & Cadenillas 2011; Lim & Lee 2018). An integrative approach may furnish

robust evidence for taking taxonomic decisions and making precise taxa delimitation of *L. silvicola.* In this study, we employed multiple lines of evidence including multilocus molecular data, morphological, morphometric, and geometric morphometric data, and geographic data to clarify the species boundaries and diversity within the *Lophostoma silvicola* complex. Specifically, we aim to: (a) clarify the taxonomic status of the subspecies of *Lophostoma silvicola* based on extensive geographic sampling along the Neotropical region, (b) assess unrecognized cryptic taxa using comprehensive analysis, and (c) apply different algorithms of molecular species delimitation to infer the validity of species and subspecies. We hypothesize that *L. silvicola* is a complex of species with several independent evolutionary lineages given its wide distribution across the Neotropics, where a wide range of environmental conditions and geographical barriers possibly promoted genetic isolation and morphological diversification.

2 | METHODS AND MATERIALS

2.1 | Samples, DNA extraction and sequencing

We obtained samples for the molecular analysis from tissue samples as wing membrane (c. 1 mm^2), liver or muscle that had been frozen or preserved in either ethanol or lysis buffer. We generated new sequences from individuals in different geographic regions targeting two gene fragments: Cytochrome Oxidase subunit I (COI) and Cytochrome-*b* (Cyt-*b*). These sequences were complemented with an additional 174 COI and 16 Cyt-*b* sequences of *Lophostoma,* which were downloaded from GenBank. In total, we analyzed 210 sequences (see Supplementary Data 1 for museum ID, voucher numbers, locality data, and GenBank accession numbers).

We extracted the DNA using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions, eluting to a final volume of 100 µL. We stored the total genomic DNAs at -20 °C before amplification. We used the pairs of primers designed by Folmer et al. (1994) targeting the mitochondrial gene COI, LCOI 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'), and HCOI 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'), and by Irwin *et al.* (1991) targeting the mitochondrial gene Cyt-*b*, L14724 (5'-CGAAGCTTGATATGAAAAACCATCGTT-3') and H15915 (5'-AACTGCAGTCATCTCCGGTTTACAAGAC-3'). The PCR conditions for COI consisted in an initial denaturation step at 92ºC for 10 min, followed by 35 cycles of 95ºC for 30 s (denaturation), 49–51ºC for 40 s (annealing), 72ºC for 90 s

(polymerization), 72ºC at 10 min (final extension), and an infinite hold at 4ºC. For Cyt-*b* the same conditions were followed, except for 48–50ºC for 45 s (annealing). We purified the PCR products using Exonuclease I and shrimp alkaline phosphatase (Affymetrix, Inc. USB Products, Cleveland, OH, U.S.A.). The two DNA strands for the PCR products were sequenced by Macrogen, Inc. (Seoul, South Korea). We visually inspected, verified and manually edited the sequence chromatograms using the Staden package (Staden et al., 2000). We verified the sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), confirming the high similarity of our submitted sequences to *Lophostoma* species.

2.2 | Phylogenetic analysis and molecular species delimitation

We used PhyloSuite (Zhang et al., 2020) for the workflow of the analyses. We aligned the sequences of each gene with MAFFT (Katoh & Standley, 2013) using '--auto' strategy and normal alignment mode. We removed ambiguously aligned fragments using Gblocks (Talavera & Castresana, 2007) with the following parameter settings: minimum number of sequences for a conserved/flank position (21/21), maximum number of contiguous non-conserved positions (8), minimum length of a block (10), allowed gap positions (with half). We selected the best partitioning schemes and evolutionary models for pre-defined partitions for each genetic marker using PartitionFinder2 (Lanfear et al., 2017), with all algorithms and AICc criterion. Each gene was analyzed independently using Bayesian methods (MB). Bayesian Inference phylogenies were inferred using MrBayes 3.2.6 (Ronquist et al., 2012) under partition model (2 parallel runs, 50 million generations), discarding the initial 20% of sampled data as burn-in. For the GMYC analysis (see below), we built an ultrametric tree using the BEAUti2.5/BEAST v2.5 (Bouckaert et al., 2019) with the same substitution models of the previous analyses. We assumed a strict clock model and a coalescent tree prior with constant population size. We did two independent runs for each dataset with 50 million generations, sampling the parameters every 5000 generations. We used Tracer v.1.7 (Rambaut et al., 2018) to inspect the convergence to the stationary distribution of the chains. The first 20% of the generations were discarded as 'burn-in' and then combined the chains: the combined ESS for each parameter was higher than 200. We visualized and edited the trees using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

For delimiting species using the genetic data, we applied four DNA-based single-locus species delimitation approaches, two distance-based and two tree-based. The methods used were: (a) the automatic barcode gap discovery method (ABGD; Puillandre et al.,

2012), (b) the assemble species by automatic partitioning (ASAP; Puillandre et al., 2021), (c) the Generalized Mixed Yule Coalescent approach (GMYC; Pons et al., 2006), and (d) a Bayesian version of the Poisson Tree Processes model approach (bPTP; Zhang et al., 2013). These analyses were performed using the ABGD web server (ABGD – https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) setting the parameters Simple Distance (p-distances) with relative gap width (X = 1.5); the ASAP web (https://bioinfo.mnhn.fr/abi/public/asap/) also setting Simple Distance (p-distances); and the Exelixis Lab's web server (bPTP – http://species.h-its.org/ptp/) setting unrooted, 500 000 MCMC generations, burn-in of 0.2; (GMYC – http://species.h-its.org/gmyc/) setting "single threshold" method. For distance methods the uncorrected p-distances yield more accurate (or at least similar) results when compared to other models of nucleotide evolution (e.g., K2P; see Srivathsan & Meier, 2012; Collins & Cruickshank, 2012).

2.3 | Morphological and morphometric analysis

We analyzed the morphology of 428 adult specimens of *Lophostoma silvicola* (215 females, 205 males and eight specimens of undetermined sex) coming from different localities throughout its entire distribution in the Neotropic (Fig. 1). Reviewed specimens are housed in 14 natural history museums: Museu Nacional do Rio de Janeiro, Rio de Janeiro, Brazil (MNRJ); Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil (MZUSP); Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Perú (MUSM); Colección Zoológica Universidad del Tolima, Ibagué, Colombia (CZUT); Instituto de Investigaciones Biológicas Alexander Von Humboldt, Villa de Leyva, Colombia (IAvH); Museo de Historia Natural Universidad de Caldas, Manizales, Colombia (MHN-UCa); Museo de La Salle, Bogotá, Colombia (MLS-BOG); Museo de Historia Natural Universidad Distrital Francisco José de Caldas, Bogotá, Colombia (MUD); American Museum of Natural History, New York, USA (AMNH); Field Museum of Natural History, Chicago, USA (FMNH); Louisiana State University Museum of Natural Science, Louisiana, USA (LSUMZ); National Museum of Natural History (U.S. National Museum), Smithsonian Institution, Washington, USA (USNM); Museum of Texas Tech University, Lubbock, USA (TTU) and Biodiversity Research and Teaching Collections, Texas A&M University, College Station, USA (TCWC). Using previously published measurements, our analyses included the holotype of *Lophostoma silvicola laephotis* and *Lophostoma evotis*. A list of specimens included in this study, with their respective localities is presented in the Supplementary Data 2.

From these specimens we examined the qualitative and quantitative variation of a dataset of 18 morphological characters that include two externals and 16 craniodental. Craniodental and mandibular measurements were recorded following Velazco and Cadenillas (2011) and are described in the Supplementary Data 3. These measurements were taken using a digital caliper with 0.01 mm resolution on each specimen and were $\log_{10}$ transformed for statistical analyses.

2.4 | Species delimitation using phenotypic data

We conducted two types of analyses to determine whether morphometric data can diagnose distinct phenotypic groups. First, we grouped the specimens according to the results of the genetic analyses, considering clusters present in both gene-trees (see Results). Then, we performed a principal component analysis (PCA) and discriminant function analysis (DFA) to investigate whether these previously established groups could be distinguished based on external and craniodental morphology using the package 'MASS' (Ripley et al., 2013) in R version 4.1.0 (R Core Team 2021). Differences between females and males, and between the candidate species in univariate trait measurements were visually assessed using box and density plots. We used Student's t-test or the nonparametric Mann-Whitney U-test to test for sexual dimorphism and one-way ANOVA with post-hoc Tukey's honest significant differences (HSD) to test differences between the candidate species. Differences in multivariate space were calculated using multivariate analysis of variance (MANOVA) with the multivariate measurements as a response variable, and sex and species as predictors. Second, we conducted normal mixture model analyses (NMMs) to estimate the number of distinct normal distributions that best fit the pooled morphological data following procedures described by Cadena et al. (2018). These procedures use the R packages 'clustvarsel' and 'mclust' (Scrucca et al. 2016; Scrucca and Raftery 2018), involving series of steps in order to find the variables that most effectively delimit morphological clusters and with which to fit different normal mixture models for selecting the best model determining the optimal number of clusters. In this sense, we conducted a principal component analysis (PCA) on the covariance matrix and selected the set of principal components most useful for group discrimination in NMMs using the R package 'clustvarsel' (Scrucca and Raftery 2018). Afterwards, we used different models to test the existence of two to four morphological groups. Two morphological clusters represent the current taxonomic treatment of *Lophostoma silvicola* and *Lophostoma evotis* as independent species, three clusters the oldest taxonomy that considered up to three

species (*silvicola*, *evotis,* and *laephotis*) and four clusters as suggested by the genetic results. Due to the small number of specimens, *L. s. centralis* was not included. Models were fitted using the R package ‘mclust’ (Scrucca et al., 2016) and ranked according to values from Bayesian Information Criterion (BIC).

2.5 | Geometric morphometric Analysis

Two-dimensional images of skull and mandible were obtained using a digital camera (Nikon Coolpix P900, Tokyo, Japan) and following a standardized protocol where skulls and mandibles were in the same position and perpendicular to the axis of the camera. From these images, we digitized landmark and semilandmarks configurations using tpsDig version 2.3 (Rohlf 2017). To determine how many anatomical points could appropriately capture the shape and size information, we used the *lasec* function in the R package ‘LaMDBA’ (Watanabe 2018). This function performs a Landmark Evaluation Curve analysis and produces a sampling curve and a table with fit values that allows it to recognize the number of anatomical points necessary to characterize the shape variation and size. We determined the number of landmarks and semilandmarks for each region considering a required fit of 0.9, 0.95, and 0.99 (Supplementary Data 4). Then, the shape and size of the skull were obtained through 22 landmarks in ventral view and 9 landmarks with 16 semilandmarks in dorsal view of 116 adult specimens, while the shape and size of the mandible were obtained from 9 landmarks in lateral view of 124 adult specimens. Detailed descriptions of the landmarks and semilandmarks and a figure with details about these anatomical points on each view can be found in the Supplementary Data 5A.

Coordinates were superimposed using a Generalized Procrustes Analysis (GPA) that removes differences unrelated to the shape (scale, position, and orientation; Rohlf and Slice 1990) using the *gpagen* function in the R package ‘geomorph’ (Adams et al. 2019). We symmetrized both sides (left and right) of the landmarks in the dorsal and ventral views of the skull to avoid redundancy, and only the symmetric part of the variation was analyzed (Klingenberg et al. 2002). We obtained procrustes shape coordinates, and a size estimator called centroid size (CS) as the square root of the sum of squares of the distance of each landmark to the centroid (mean of all coordinates) of the configuration (Bookstein 1997).

Analysis were conducted to assess the effect of sex (sexual dimorphism), species and their interaction on cranial and mandibular size by evaluating the fit of models using the

randomized residual permutation procedure (RRPP) with the *lm.rrpp* function in the R package 'RRPP' (Collyer & Adams, 2018, 2020). RRPP was also used to test the effect of (1) size, (2) sex, and (3) species on skull and mandible shape and its interactions. All models were fit using the type-II (hierarchical) sum of squares, and its significance was based on 10 000 permutations of residual randomization. We used the *anova.lm.rrpp* function to compute analysis of variance (ANOVA) tables for each model, which use distributions of random statistics and use the F distribution to calculate effect sizes. Pairwise comparisons were conducted on significant factors using the *pairwise* function. These comparisons calculate distances among species pairs, effect sizes and P-values based on distances between means (Collyer & Adams, 2020).

Differences in skull and mandible shape among candidate species were also explored using ordination methods. First, we performed a principal component analysis (PCA) on the procrustes aligned data using the *gm.prcomp* function in the R package 'geomorph' (Adams et al. 2019). Among the PCs produced, we choose those that contained a significant cumulative of shape variance on each view (~90%). After, we generated the deformation grids with the extremes (maximum and minimum) of shape variation along the principal components 1 and 2 (PC1 and PC2). Then, we used a discriminant function analysis (DFA) to determine whether the groups could be reliably distinguished. Procrustes distances between groups were tested for significance with a 10 000 permutations procedure. The probability of a specimen belonging to any of the predefined groups was estimated via jackknife cross-validation of the scores.

2.6 | Estimating missing landmarks and error measurement

A common issue in geometric morphometric studies is missing data due to incompleteness of samples. A widely used but not recommended solution is to remove those incomplete specimens or, preferably, estimate them (Arbour & Brown 2014). When we had specimens with missing data in the dorsal/ventral skull (structures with bilateral symmetry), we first used the *reflectMissingLandmarks* function in the R package 'StereoMorph' (Olsen and Westneat 2015). This function permits imputing missing points from the mirrored side (reflecting labelling). For those missing landmarks that could not be estimated taking advantage of bilateral symmetry, then, we conducted a rigorous evaluation that included simulations of missing data, testing for the impact of missing data estimation and analyses about performance of different estimation techniques (Supplementary Data 5B). Among the evaluated techniques, we used the Bayesian PCA

(BPCA), least-squares regression (REG), mean substitution (MS) and the geometric-morphometric-specific method Thin-plate spline interpolation (TPS). From our analyses we concluded to use REG, and TPS in the ventral cranium and lateral mandible respectively to estimate missing values in our datasets.

Finally, we tested the error of digitization of the landmarks through a Procrustes ANOVA, which measures and compares random errors (Klingenberg & McIntyre, 1998; Klingenberg et al., 2002). In this sense, we digitized all skulls and mandibles twice and performed a Procrustes ANOVA between these two replicates, which yielded very high replicability (> 94 % all views).

2.8 | Taxonomic decision

We defined the candidate species according to the results of the following lines of evidence: (1) monophyletic lineages using the gene trees; (2) genetic distance-based approaches; (3) tree-based methods; (4) delimitation methods using morphometric linear distances; (5) normal mixture models (NMMs) and (6) geometric morphometric analyses. We evaluated the accumulation of evidence from each method recognizing candidate species to support a “new” species hypothesis. Here, we assumed the unified species concept, understanding species as a lineage evolving separately from other lineages (De Queiroz, 2007).

3 | **RESULTS**

3.1 | Phylogenetic analyses and molecular species delimitation

All the gene trees recovered the same results concerning the reciprocal monophyly of the *Lophostoma* species. For both Bayesian algorithms, most species were monophyletic with the higher support values (PPs=1): *L. carrikeri*, *L. schulzi*, *L. evotis*, *L. occidentale*, *L. brasiliense*, and *L. nicaraguae*. *Lophostoma silvicola* was the only non-monophyletic species, recovered as paraphyletic (PP = 1) including *L. evotis* (Fig. 2).

The gene tree topologies were incongruent among markers and algorithms, producing three scenarios. COI and Cyt-*b* makers produced the same topology under Beast algorithm, while under MrBayes recovered different relationships. Two clades were consistent among the analysis: (*L. carrikeri* (*L. brasiliense, L. nicaraguae*)) and (*L. occidentale* (*L. silvicola* (*L. evotis*, *L. silvicola*))). Both clades are sisters, while the position of *L. schulzi* is different in each scenario. *Lophostoma schulzi* appears as sister to

the remaining *Lophostoma* species (COI under MrBayes); sister to *L. occidentale* (Cyt-*b* under MrBayes); or sister to (*L. carrikeri* (*L. brasiliense, L. nicaraguae*)) (both makers under Beast; Fig. 2).

For the clade formed by the paraphyletic *L. silvicola* and *L. evotis*, the Cyt-*b* phylogenies (Fig. 3) recovered four main clades, one of them corresponding to *L. evotis.* Besides *L. evotis*, the remaining groups seemingly matching current subspecies taxonomy and are recognized henceforth as candidate species: candidate species A (samples from Bolivia, Paraguay and central Brazil); candidate species B (samples from Guyana, French Guiana and Suriname); and candidate species C (samples from Peru, Ecuador, Venezuela and Panama). The COI phylogenies recovered the same groups, and two additional lineages. One clade clustering the specimens from southern Peru (Manu) and eastern Brazil (Rondonia), candidate species D; and a single lineage sequence from Cerrado in Brazil, candidate species E (Fig. 3). The average Cyt-*b* pairwise distance among the candidate species (A, B, C) ranged from 4.75% to 5.94% (Table 1), while the pairwise distance using COI ranged from 4.48% to 8.43% (Table 2).

Tree- and distance-based methods of species delimitation did not produce congruent results, inferring different numbers of species for the complex depending on the method applied. Distance-based algorithms recognized between four (Cyt-*b*) to six (COI) species, while tree-based algorithms between seven (Cyt-*b*) to 36 (COI) species (Table 3). However, the four DNA-based single-locus species delimitation approaches (ABGD, ASAP, bPTP, and GMYC) were consistent in recognizing to *L. evotis* and most recognized the same three main clades mentioned above in *L. silvicola*, regardless of the genetic marker used.

3.2 | Phenotypic species delimitation

The paired tests showed significant differences between the sexes, but the density and PCA plots lacked evidence of sexual dimorphism. Thus, the following analyses were carried out considering the pooled sexes (Supplementary Data 6). One-way ANOVA recovered differences among candidate species for all 18 characters which continued statistically significant after adjustment with the Benjamini–Hochberg procedure (all $P < 0.001$) (Benjamini & Hochberg, 1995). Post-hoc Tukey's HSD tests also identified significant differences in mean values for some traits of candidate species. Candidate species A differed from candidate species B in 14 traits related to shorter lengths of skull (GLS, CIL, CCL, BB, ZB, PL, PWC, MSTW, MTRL, MLTRL, PWM2) and mandible

(DENL, MANDL, COH). Candidate species A also differed from candidate species C in six traits, which indicated a shorter length of skull (CCL, MTRL, PWM2) and mandible (DENL, MANDL, COH). Candidate species B differed from candidate species C in all 18 morphological traits, by having larger external and craniodental measurements. *Lophostoma evotis* showed significant differences regarding all other species. In summary, significant differences were found in size between the candidate species, where B is the largest species, while A and *L. evotis* are the smallest (see graphical summaries and test results in Supplementary Data 7). These differences were also confirmed when we analyzed the multivariate space, where MANOVA pointed out significant differences between each of the candidate species ($p < 0.001$). The variation in the values of individual traits observed between species is described in Table 4.

The PCA analysis based on 18 morphometric traits (two external and 16 craniodental) showed the first principal component (PC1) accounting for 68.97% of the variation, PC2 for 6.06% and PC3 for 4.47%, together accounting for ~80% of the variation (Fig. 4A). PCA confirms size-related differences: PC1 describes variation associated with cranial and mandibular lengths (DENL, CCL, CIL), while PC2 relates to the variation in forearm and metacarpal III lengths. Although there is overlap between species values, the DFA suggests that at least 83, 71 and 84% of the individuals of the candidate species A, B and C respectively may be distinguished based on the external and craniodental measurements analyzed. DFA indicated low values in the classification using only cranial characters (Table 5; Fig. 4B). DFA results on morphological characters were similar considering both PCs or log-transformed data.

The normal mixtures analysis, with no *a priori* species definition, provided maximum support for models specifying two morphological groups (i.e., two distinct phenotypic distributions). However, the clusters estimated by this model included one very large (255 specimens) and one very small (24 specimens) group, each of which included representatives scattered across the Neotropic. Model support for the presence of two, three or four morphological groups according to different taxonomic proposals was larger for two groups (BIC = -3155.074; Fig. 5).

Overall, our results pointed to the existence of two or four phenotypic groups defined by morphological variation in our sample of *L. silvicola*, considering both NMMs and distance-based approaches respectively.

3.3 | Geometric morphometric

Variation in cranium and mandible size

The centroid size in the cranium and mandible was significantly different between some pairs of species, supporting the differences in size described using linear measurements. Candidate species B is the largest species in the complex while *L. evotis* is the smallest. Candidate species C showed significant differences respecting candidate species A and candidate species B ($P < 0.05$ in all views, see pairwise comparison and graphical summaries in Supplementary Data 8A). We found evidence of sexual dimorphism in size in ventral skull and lateral mandible views, where males were larger than females (Supplementary Data 8B), however, a separate analysis by sex did not alter the overall patterns; therefore, we present the results with both sexes included. The variance of the factors tested, represented by mean squares value and the R^2, showed that most of the variance in skull and mandible size is found among the species (Table 6).

Variation in cranium and mandible shape

Models did not show sexual dimorphism in the shape of the skull but a significant sexual dimorphism in the shape of the mandible (Table 7). When we tested for cranial and mandibular shape variation in the entire procrustes shape space, we found significant differences among species in all views tested ($P < 0.05$ in all cases). Even when we evaluated the differences between females and males separately in the mandible (due to sexual dimorphism) the differences in shape between species remained (results not shown: $P < 0.05$ in all cases). The interaction between size and species was significant just for the ventral skull (Table 7). Fitted linear models found significant effect of size on shape variation in all the examined views, however the morphological variation explained by size was very low (< 8% in all cases; Table 7). So, the allometric effect was ruled out, and the analyzes and graphs were carried out on the original shape coordinates.

The PCA showed an ordination for dorsal and ventral skull, but not for lateral mandible. The first three PC scores accounted for ~65 and ~60% of the skull and mandible total shape variation respectively. We only show the results from the first two PCs, which accounted for ~50% of the variation (Fig. 6). In the skull, specimens with negative scores on PC1 had a rostrum more elongated and robust at the level of the canines, a larger distance between the anteriormost point of premaxilla to nasal, a thicker postorbital constriction, a smaller braincase, a larger distance between the molars with a wider palatal, a greatest breadth across the zygomatic arches and a shorter distance between

basion and opisthion. In the PC2 individuals with negative scores had a shorter and smaller braincase. With respect to mandible, the PC1 positive scores were associated with shorter, thicker, and more robust jaws, with a larger perpendicular height from the ventral margin of mandible to the tip of the coronoid process. Negative scores with more elongated, thin, and slender jaws. Most individuals of candidate species B had positive scores on PC1 in the skull and mandible, while most individuals of candidate species C negative scores, showing some useful characteristics that can differentiate these species (Fig. 6).

The Discriminant Function Analysis (DFA) also showed that species were significantly different in the shape of both the skull and the mandible (Fig. 7). The percentage of correct classification using DFA shows high values for candidate species C in all views (dorsal skull – 89.02 % –, ventral skull – 87.84 % – and lateral mandible – 92.85 %), and acceptable values for candidate species B in the skull, but not lateral mandible (dorsal skull – 73.33 % –, ventral skull – 69.57 % – and lateral mandible – 43.47 %), in the same way as for candidate species A (dorsal skull – 75 % –, ventral skull – 77.77 % – and lateral mandible – 11.11 %). Moreover, the morphological structures with the higher percentage of correct classification and probably the most useful to identify each species were the ventral (79.12 %) and dorsal skull (78.39 %), whereas the lateral mandible had a smaller percentage (49 %).

4 | DISCUSSION

The number of species within the genus *Lophostoma* has been shown to be higher than is currently valid (Velazco & Cadenillas 2011; Lim & Lee 2018; Esquivel et al. in. prep). Without a clear understanding of the taxonomic units, boundaries, and relationships within the genus, any analyses about evolutionary and ecological processes could be under severe bias. Under an integrative approach, we recognized at least four independent evolutionary lineages within the *L. silvicola* species complex.

The molecular results suggested *L. silvicola* as a paraphyletic species, grouping *L. evotis* and multiple independent evolutionary lineages. We opted for the most parsimonious scenario of molecular species delimitation to run the subsequent analyses. Thus, four lineages were explored within the L. *silvicola* complex: *L. evotis* and the candidate species A, B, and C. This split of lineages was corroborated by phenotypic analyses, and their geographical distribution is parapatric. The distribution of these putative species hinted at reevaluating the status of current synonyms and subspecies of *L. silvicola*. Thus, the

candidate species A from Paraguay, Bolivia, southern Peru and central Brazil, corresponds to *L. silvicola sensu stricto*; the candidate species B from Guyana, French Guiana, Suriname and northern Brazil, corresponds to *L. laephotis;* and candidate species C from central/northern Peru, western Brazil, Ecuador, Venezuela and Panama, corresponds to *L. amblyotis*. Currently, *L. amblyotis* is a junior synonym of *L. silvicola*. Thus, we raising *L. laephotis* to the species level and revalidate *L. amblyotis*. Our results are consistent in recognizing these species as independent evolutionary lineages. The morphology, cranial/mandibular shape and genetics of these species are distinct (Fig. 8). These species increase the number of valid taxonomic units in *Lophostoma* from eight to ten (Esquivel et al. in prep), with nine of them occurring in South America.

The use of morphological, morphometric, geographic and molecular evidence under an integrative approach (Dayrat 2005; DeSalle et al., 2005) became a powerful tool to the species discovery and delimitation, allowing taxonomists tackling the Linnean shortfall especially in cryptic species. This approach assumes that a greater amount of evidence from different datasets represents a more robust species hypothesis and decreases the probability of false identifications (Damm et al., 2010). Here, the use of multiple lines of evidence allowed the recognition of additional taxonomic entities within the *silvicola* complex. Individual approaches indicated the existence of the complex (Velazco & Cadenillas 2011; Lim & Lee 2018), but were unable to establish limits to each lineage.

Molecular delimitation methods are a powerful tool for recognizing cryptic species in bats (Salicini et al., 2011; Demos et al., 2018, 2019). Due to the limitations of single-locus species delimitations, we applied conservative criteria to establish the number of species, where convergent results from different algorithms were considered a robust primary species hypothesis (Puillandre et al., 2021). The distance-based and tree-based approaches used here, which are grounded on distinct species criteria, converged to recognize *L. laephotis* and *L. amblyotis* as taxonomic entities distinct from *L. silvicola* supporting our main conclusions. GMYC identified more groups than the other methods, but this algorithm tends to overestimate the number of species (Damm et al., 2010).

The current study provides further evidence for strong genetic differentiation within *L. silvicola*. mtDNA divergence among internal lineages is equivalent to interspecific genetic distances with other well-recognized sister species (e.g., *L. evotis,* see Table 1-2) and is greater than distances between many other sister pairs in Phyllostomidae as for example between *Sturnira hondurensis* Goodwin, 1940 and *S. ludovici* Anthony, 1924: 5.74%,

Sturnira burtonlimi Velazco & Patterson, 2014 and *S. adrianae* Molinari, Bustos, Burneo, Camacho, Moreno & Fermin, 2017: 3.93% (Molinari et al. 2017), *Micronycteris buriri* Larsen, Siles, Pedersen & Kwiecinski, 2011 and *M. megalotis* (Gray, 1842): 1.9% (Larsen et al. 2011) among others. Furthermore, the genetic divergence values are greater than the threshold values proposed by the methods (Cyt-*b* 3.3%; COI 3%), supporting the recognition of candidate species as valid species.

Diversification of *Lophostoma* occurred during the Mid-Miocene, approximately 15.4 mya ago (Hoffmann et al., 2008). Specifically, the divergence of *L. silvicola* began about 7.8 mya ago in the Late-Miocene, with an explosive diversification towards the current lineages less than 3 mya ago, in the Plio-Pleistocene (Hoffmann et al., 2008). This rapid and recent diversification may partly explain the high genetic but low morphological differentiation present between these lineages. Distributional data on the species of the *L. silvicola* complex provides additional insights about this rapid diversification. The current known distribution of each species in the complex suggests constraints in the elevational range —none of these species has been recorded above 2000 m.a.s.l —and type of forest. Andes is the main terrestrial biogeographic barrier South American, and triggered the diversification of several species of bats (Patterson et al., 2012), including *Lophostoma* species (e.g., *L. nicaraguae*, *L. brasiliense*, *L. occidentale*) (Esquivel et al. in prep). Under this scenario, it was to be expected for the cis-Andean and trans-Andean populations to present large enough genetic distances to be consider different species. However, we found that *L. amblyotis* occurs on both sides of the Andes and with, apparently, low genetic divergence. This could confirm a recent separation of these populations which, although separated by the Andes (allopatric populations), probably have not had enough time to accumulate genetic or morphological differences. We hypothesize that distinct environmental conditions related to forest type (the biogeographic gradient hypothesis, Moritz et al. 2000) may better explain the diversification within the complex. *Lophostoma laephotis* seems to be restricted to the Amazonian forests of northeastern South America; *L. silvicola* to the forests in southern Peru, savannas in Bolivia and Brazil, specifically the Cerrado, while *L. amblyotis* to sub-Andean forests. However, more geographic and genetic data are needed to test these distributional predictions.

We also found a high phenotypic similarity between all the species of the *silvicola* complex. Although we reviewed more than 420 specimens, and the analyzes revealed significant differences in the mean values of individual traits between species, we were

unable to find non-overlapping traits allowing a reliable diagnosis of each of the species. Finding diagnostic morphological features between "cryptic" species is a complex task (Fišer & Zagmajster, 2009; Schlick-Steiner et al., 2010) due to high intraspecific variation and low interspecific variation (Jugovic et al., 2012). In our case, the variation within the populations is quite high, in contrast to the differences between species, so that univariate morphological characters alone are not enough to discriminate between species of the *silvicola* complex. The morphological distinction between these species is reduced to variations in size, differences in multivariate space, and differences in the shape of the skull and jaw.

How many species are there in *Lophostoma*?

Our findings help to improve the knowledge about the systematics and taxonomy of this group and to reduce its Linnean shortfall. However, unanswered questions remain within *Lophostoma*. Given the limitations of the data set analyzed here, we cannot infer the status of the subspecies *L. s. centralis.* We could not evaluate its state due to low number of samples from museum collections, as well as the impossibility of obtaining further tissue samples from the field. We highlight the relevance of continuing to collect specimens in unsampled areas in Costa Rica, Nicaragua and specially Honduras, needed to clarify the taxonomic status of *L. s. centralis* and the distribution limits of *L. amblyotis* and *L. evotis* in Central America. We were also unable to establish the phylogenetic position of *L. kalkoe* and of a new species that appears within *L. occidentale* in our molecular analyzes, which deserves additional studies. Then, the results of this work should be considered an additional step that raises more questions than it answers. We recognize ten species of *Lophostoma*, but the high number of putative species within *L. silvicola stricto sensu* must be explored using other approaches such as the use of nuclear markers or ecological niche models.

ACKNOWLEDGEMENTS

We are especially thankful to museum curators for allowing access to the specimens under their care, as well as the loan of tissue samples. We thank particularly Adam Ferguson and Bruce Patterson (FMNH), Heath Garner (TTU), Jessica Light (TCWC), Ludmilla Aguiar (UnB), Marcelo Weksler and João Oliveira (UFRJ), Ciro Líbio (UFMA), Thiago Bernardi Vieira (UFPA), Leonora Pires Costa and Monique Nascimento (UFES), and Octavio Saldaña (Programa para la Conservación de los Murciélagos de Nicaragua - PCMN). We would like to thank Juan Díaz-Nieto and Juan Martinez-Ceron (Universidad EAFIT) for kindly sharing their sequences with us, and Jessica Light and Toby Hibbitts at TCWC for photos of holotypes. This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES - Finance Codes 001 and 88882.439397/2019-01), given in the form of a MSc scholarship to DAE and a post-doctoral fellowship to FBM. MJRP was supported by a National Council for Scientific and Technological Development (CNPq) productivity grant.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Data 1. Specimens used for phylogenetic analyses. Species, vouchers numbers, GenBank/BolSystems accession numbers and geographic information are given for the *Lophostoma* samples used in the phylogenetic analyses.

Supplementary Data 2. List of specimens included in this study.

Supplementary Data 3. Detailed descriptions of external and craniodental measurements used in this study.

Supplementary Data 4. Results from performing LaSEC with 1000 iterations.

Supplementary Data 5. A- Detailed descriptions of the landmarks and semilandmarks used in this study and a figure with details about these anatomical points on each view. **B-** Selection of the best missing data estimator to our dataset using simulations with incomplete specimens.

Supplementary Data 6. Paired test and density plots showing differences between sex.

Supplementary Data 7. Density plots of individual traits between species.

Supplementary Data 8. A- Plot centroid size by species. **B** – Plot centroid size by species/sex.

Figures

Figure 1. Sampling localities of *Lophostoma silvicola* specimens analyzed in this study. Dark dots represent localities from specimens in museums, the red star indicates the type locality for *L. silvicola*, while the green and yellow star point out type localities for subspecies (*laephotis* and *centralis*, respectively). A list of revised specimens with their respective localities is presented in the Supplementary Data 1.

Figure 2. Phylogenetic gene-trees showing relationships between *Lophostoma* species.

Figure 3. Majority rule (50%) consensus tree of *Lophostoma* based on Bayesian analyses using: (A) Cytochrome oxidase I; (B) Cytochrome-*b*. Values above branches are Bayesian posterior probabilities.

Figure 4. Morphometric variation in the *Lophostoma silvicola* species complex. **A**- Principal Components Analysis (PCA) on 18 externals, craniodental and mandibular variables performed for all samples of the *Lophostoma silvicola* complex. Each individual is represented by a dot, painted according to the group to which it belongs (candidate species A: black; candidate species B: red; candidate species C: light blue; *L. evotis*: yellow). Outer solid-line ellipses delimit the area enclosing 95% of the individual points in each group, whereas inner broken-line ellipses encompass 50% of those points. **B**- Linear Discriminant Function after cross-validation tests. Even though there is overlap between two putative species showing morphological similarity, individuals tend to cluster together with their respective group.

Figure 5. Support for species delimitation scenarios without *a priori* information generated from normal mixture models. Plot shows results for normal mixture models specifying one to nine morphological clusters, as well as models fitting both previous and current hypotheses of subspecies.

Figure 6. Principal Component Analysis (PCA) of *Lophostoma silvicola* species complex obtained from three different views. Each individual is represented by a dot, painted according to the group to which it belongs (candidate species A: black; candidate species B: red; candidate species C: light blue; *L. evotis*: yellow).

Figure 7. Linear Discriminant Analysis (LDA) of *Lophostoma silvicola* species complex obtained from three different views. Each individual is represented by a dot, painted according to the group to which it belongs (candidate species A: black; candidate species B: red; candidate species C: light blue; *L. evotis*: yellow).

Figure 8. Cyt-*b* phylogram showing results of species delimitation methods. Numbers indicate support values of the adjacent node retrieved in the Bayesian inference. Titles of

columns indicate the current treatment of *L. silvicola* as one group (Current), and the results from species delimitation methods employed (see Taxonomic decision section). Boxes in different colours indicate species inferred with each method.

Tables

Table 1. Pairwise cytochrome-*b* sequence divergence (%) among *Lophostoma* candidate species (average in black; standard error in blue).

Table 2. Pairwise cytochrome oxidase I sequence divergence (%) among *Lophostoma* candidate species (average ± standard error).

Table 3. Results from genetic algorithms. Numbers in parentheses represent the number of species estimated by each algorithm within the *silvicola* complex.

Table 4. External and craniodental measurements (mm), including mean, standard deviation, range, and sample size of the candidate species. Measurement acronyms follow Table 1.

Table 5. DFA classification results without (DFA) and with (DFA-CVs) leave-one-out cross-validation for all morphometrics analyses and datasets. MANOVA statistically significant differences between the candidate species.

Table 6. ANOVA results regarding effects of sex, species and their interaction on centroid size (log CS).

Table 7. ANOVA results regarding effects of size (allometry), sex (sexual dimorphism), species and their interactions on shape.

Figure 1

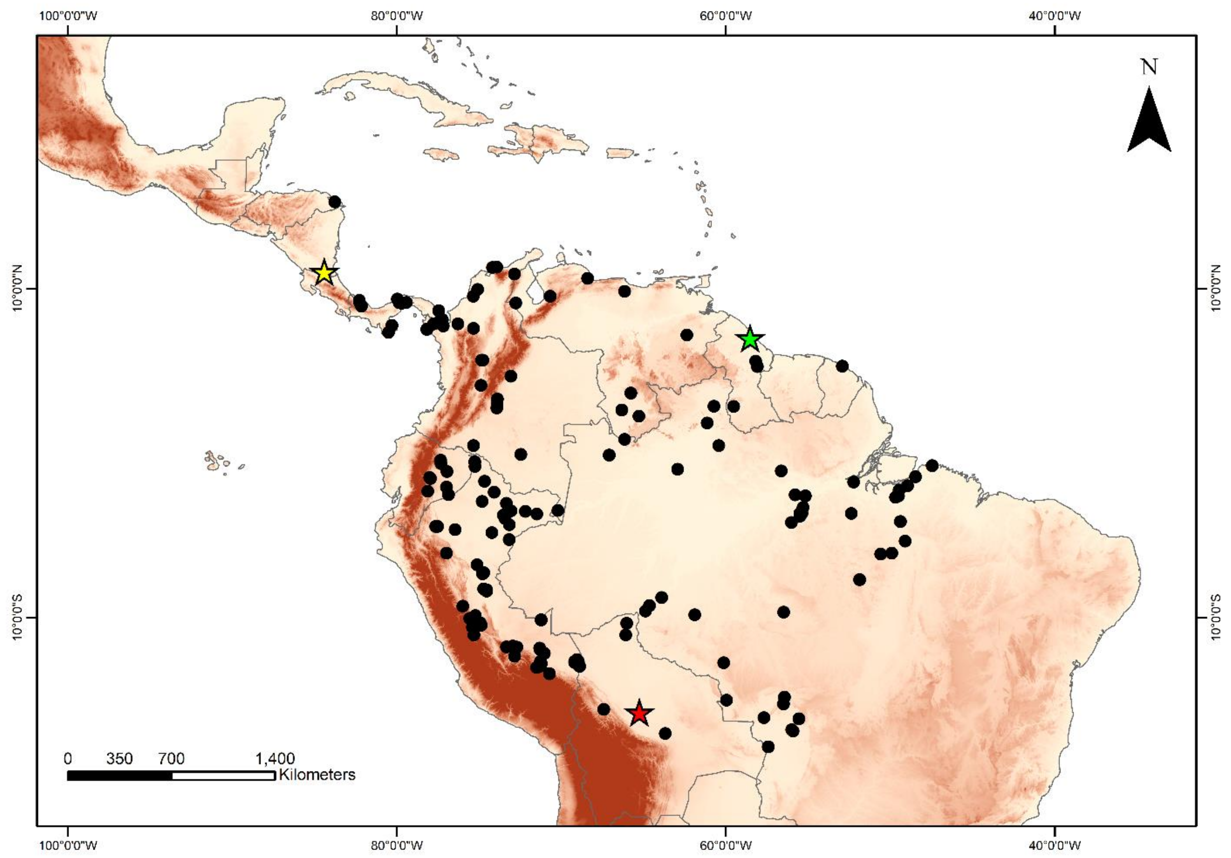

Figure 2

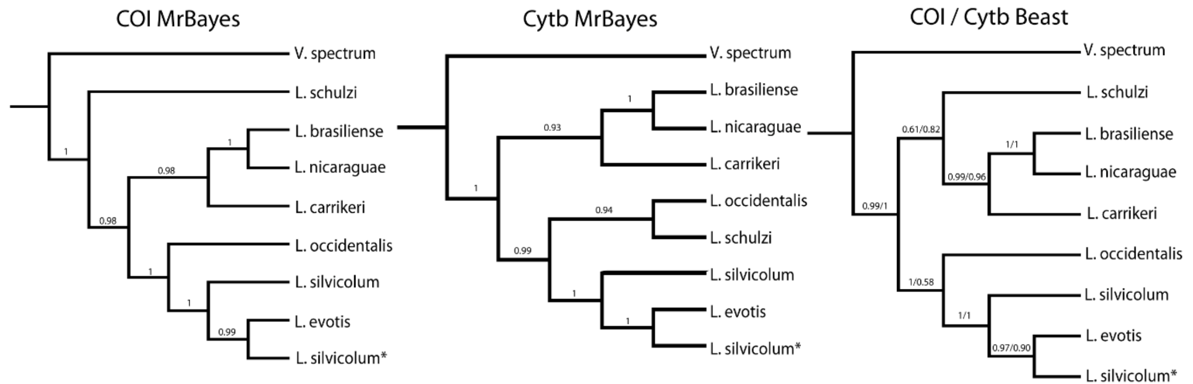

Figure 3

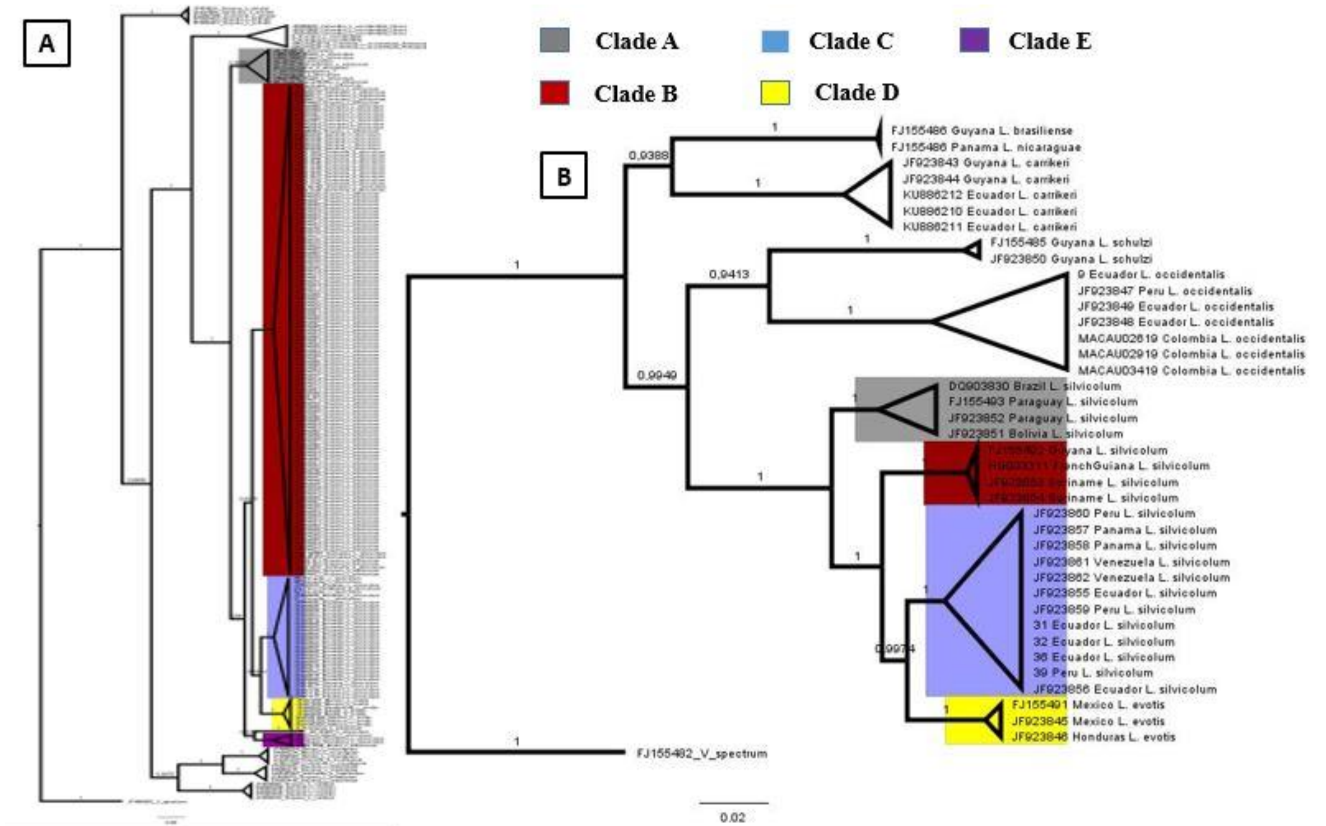

Figure 4

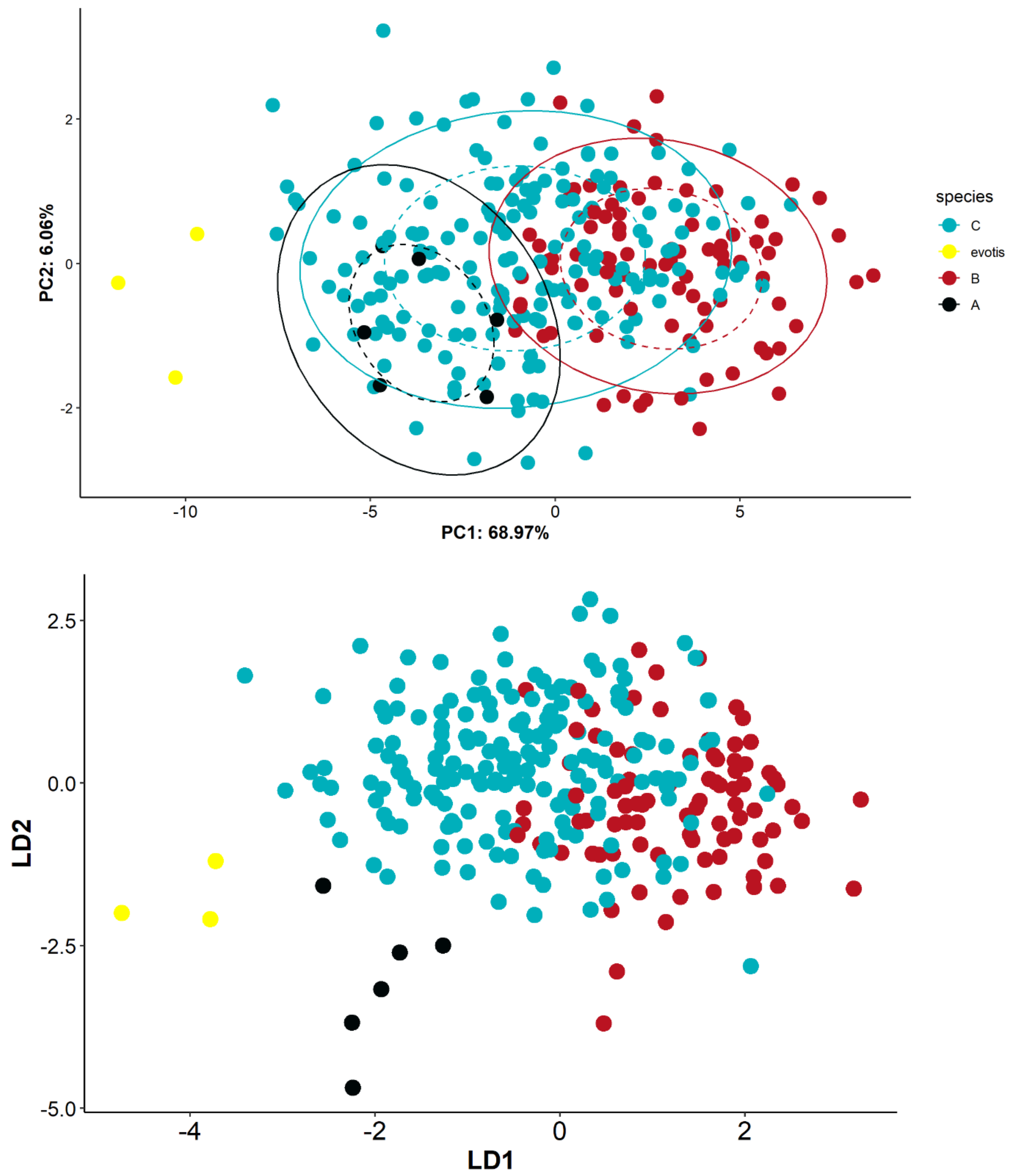

Figure 5

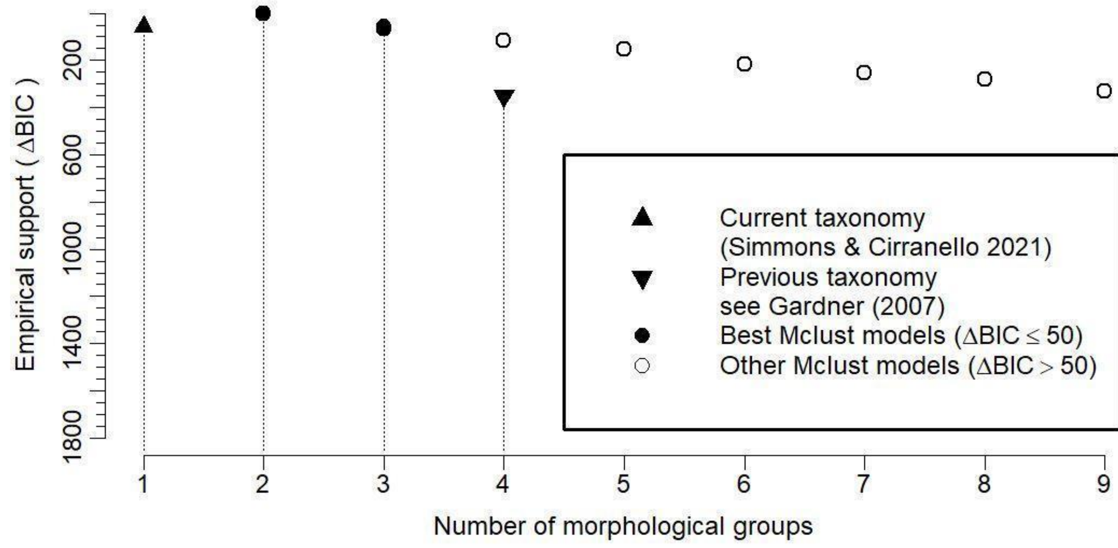

Figure 6

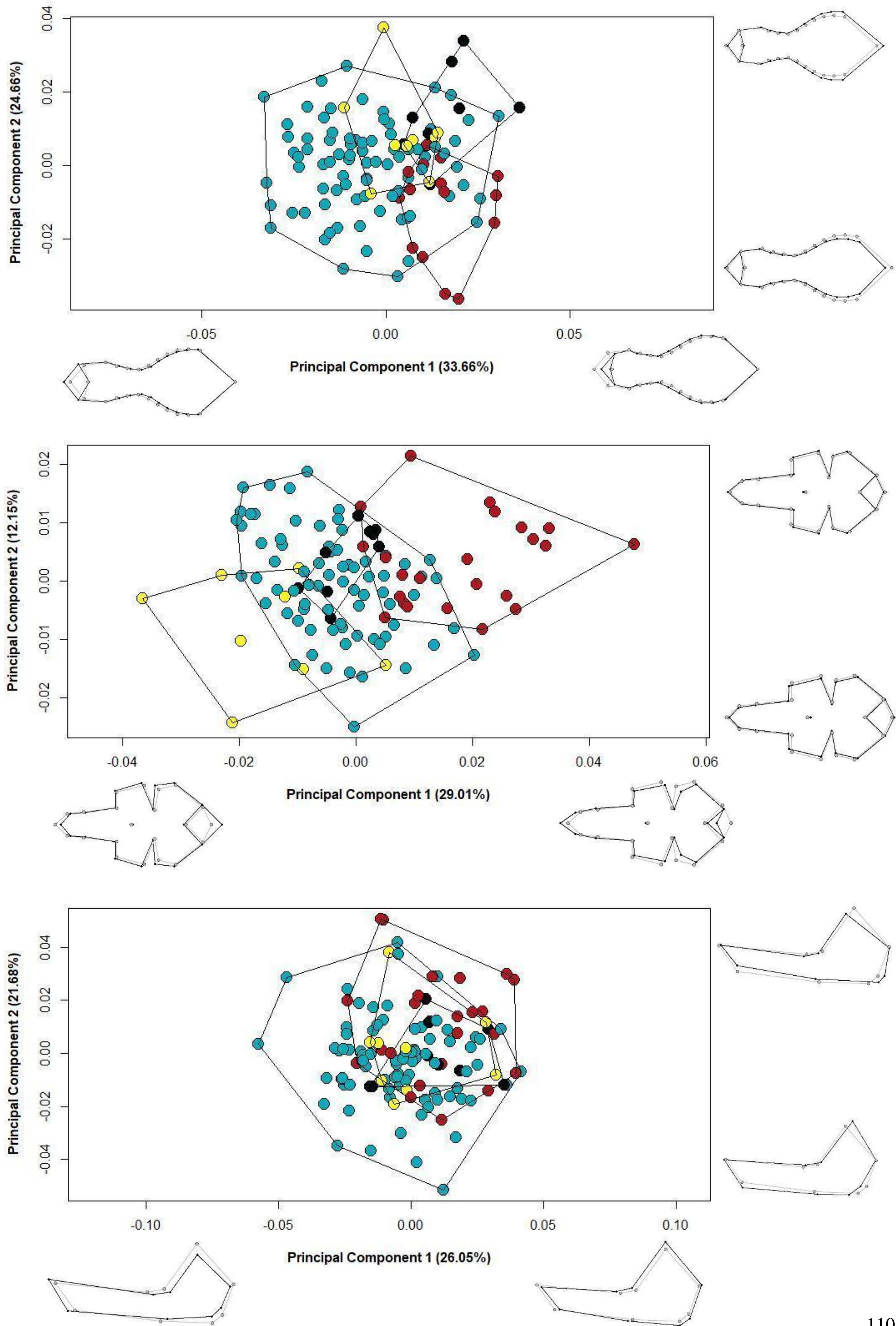

Figure 7

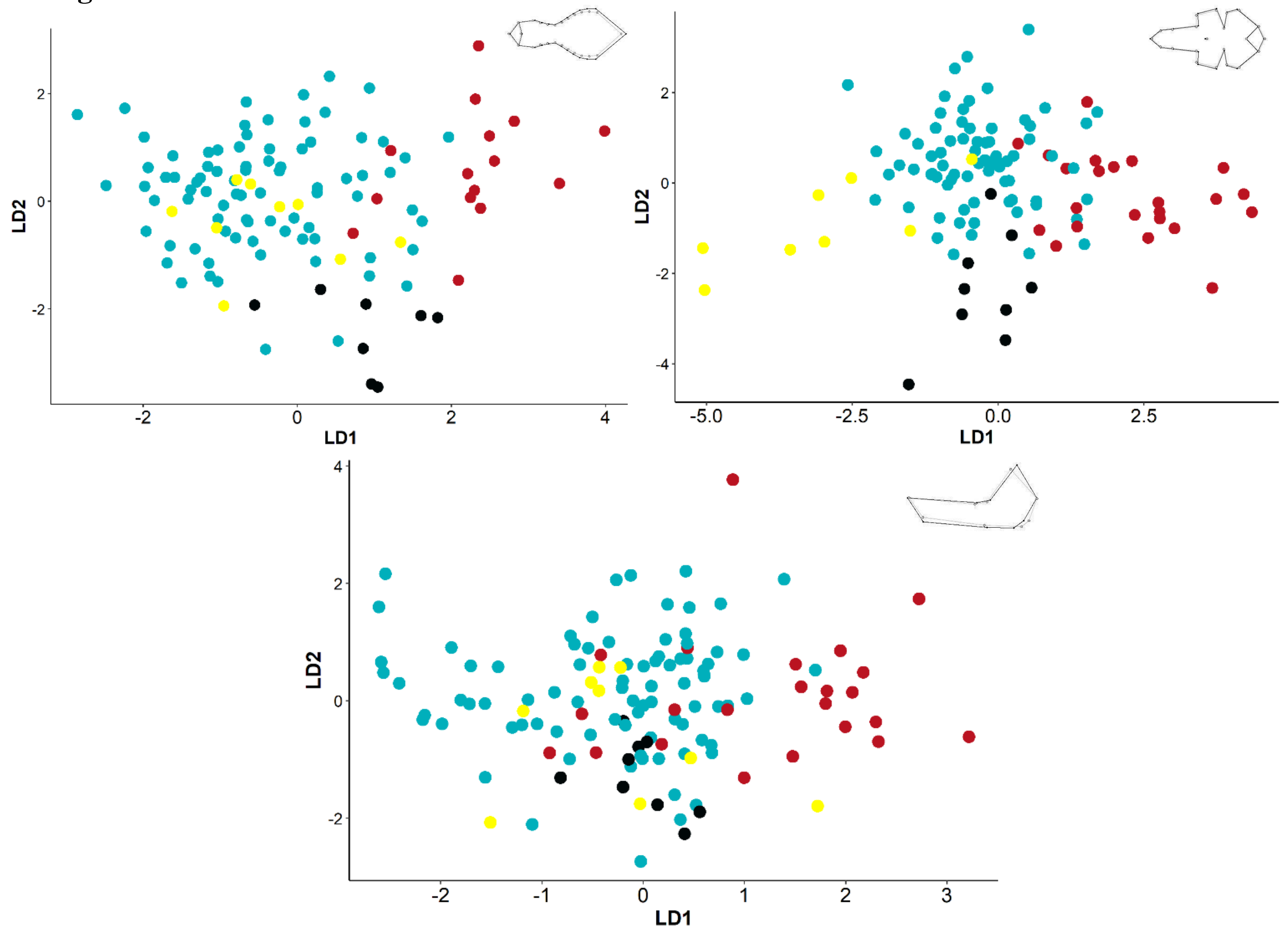

Figure 8

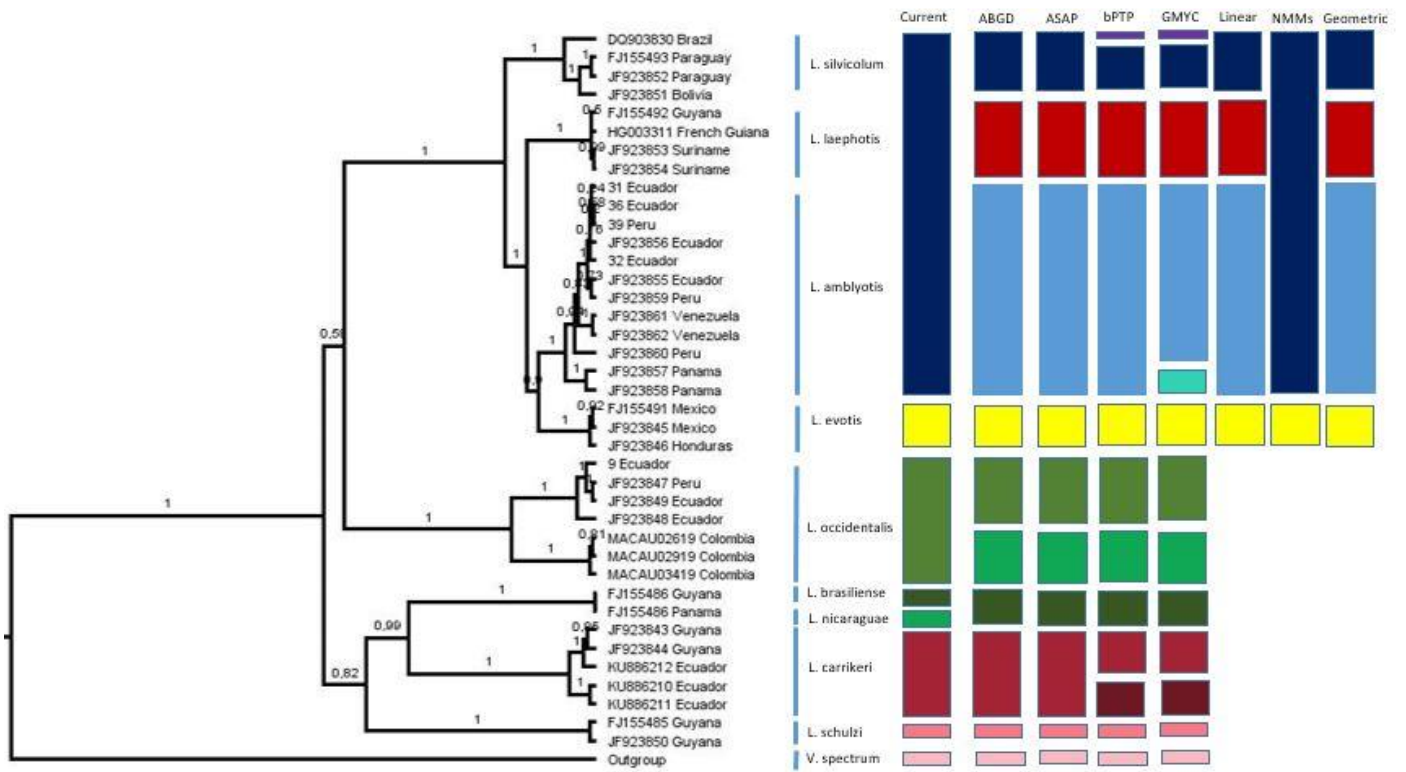

Table 1

	1	2	3	4	5	6	7	8	9
1– L. brasiliense		0.000	0.009	0.009	0.009	0.009	0.010	0.010	0.009
2– *L. nicaraguae*	0		0.009	0.009	0.009	0.009	0.010	0.010	0.009
3– *L. carrikeri*	10.91	10.91		0.009	0.009	0.009	0.010	0.009	0.009
4– *L. evotis*	12.87	12.87	12.13		0.008	0.009	0.007	0.006	0.005
5– L. occidentale	12.95	12.95	13.54	12.32		0.008	0.009	0.009	0.008
6– L. schulzi	12.68	12.68	11.31	12.21	11.80		0.009	0.009	0.009
7– Candidate species A	13.45	13.45	12.65	6.04	12.18	12.38		0.006	0.006
8– Candidate species B	13.07	13.07	11.87	5.48	12.77	12.06	**5.94**		0.005
9– Candidate species C	13.28	13.28	12.67	4.11	12.42	12.70	**5.55**	**4.75**	

Table 2

	1	2	3	4	5	6	7	8	9	10	11
1– L. brasiliense		0.0094	0.0124	0.0127	0.0129	0.0131	0.0132	0.0132	0.0130	0.0133	0.0132
2– L. nicaraguae	6.91		0.0119	0.0129	0.0127	0.0132	0.0128	0.0126	0.0130	0.0128	0.0137
3– L. carrikeri	12.26	11.53		0.0131	0.0133	0.0133	0.0138	0.0135	0.0139	0.0134	0.0141
4– L. evotis	13.33	13.17	13.86		0.0128	0.0132	0.0097	0.0084	0.0073	0.0087	0.0102
5– L. occidentale	13.49	13.91	14.85	13.47		0.0140	0.0111	0.0118	0.0119	0.0115	0.0123
6– L. schulzi	13.46	14.23	13.64	12.84	16.36		0.0129	0.0131	0.0131	0.0133	0.0142
7– Candidate species A	13.29	12.82	15.20	6.80	11.20	12.36		0.0092	0.0086	0.0082	0.0114
8– Candidate species B	12.92	12.13	14.20	5.02	11.94	12.60	**6.17**		0.0079	0.0091	0.0104
9– Candidate species C	12.90	13.01	14.76	3.95	11.92	12.88	**5.61**	**4.48**		0.0081	0.0104
10– Candidate species D	14.44	13.84	14.26	5.80	11.75	13.38	**5.35**	**6.29**	**5.44**		0.0100
11– Candidate species E	13.80	13.37	14.37	7.24	12.12	14.30	**8.43**	**7.30**	**7.63**	**6.85**	

Table 3

	Distance-based		**Tree-based**	
	ABGD	**ASAP**	**bPTP**	**GMYC**
COI	**13 (6)**	**13 (6)**	**21 (9)**	**54 (36)**
Threshold dist.	0.030	0.028		
CYTB	**10 (4)**	**10 (4)**	**12 (5)**	**15 (7)**
Threshold dist.	0.033	0.028		

Table 4

	Candidate species A	*Candidate species B*	*Candidate species C*	*L. evotis*
FA	53.15 ± 0.92	55.34 ± 1.94	53.54 ± 2.34	49.73 ± 1.46
	(51.98–54.10) 7	(51.45–59.90) 90	(45.39–60.39) 195	(48.66–51.39) 3
MET-III	42.01 ± 1.22	44.03 ± 1.90	42.97 ± 2.11	38.78 ± 1.41
	(40.10–43.70) 7	(38.30–48.82) 90	(37.34–50.24) 195	(37.46–40.26) 3
GLS	26.58 ± 0.64	28.48 ± 0.79	27.43 ± 0.98	25.35 ± 0.39
	(25.54–27.45) 10	(27.01–30.50) 91	(24.98–30.45) 211	(24.88–25.96) 7
CIL	23.32 ± 0.39	24.90 ± 0.62	23.93 ± 0.83	22.17 ± 0.40
	(22.62–23.80) 10	(23.60–26.24) 91	(22.29–27.38) 211	(21.63–22.58) 7
CCL	22.51 ± 0.40	24.07 ± 0.61	23.13 ± 0.77	21.21 ± 0.31
	(21.70–23.15) 10	(22.75–25.52) 91	(21.38–25.28) 211	(20.85–21.64) 7
BB	10.35 ± 0.28	10.85 ± 0.27	10.57 ± 0.32	10.00 ± 0.21
	(9.95–10.71) 10	(10.20–11.66) 91	(9.62–11.40) 211	(9.72–10.33) 7
ZB	13.10 ± 0.29	13.84 ± 0.48	13.22 ± 0.49	12.20 ± 0.34
	(12.65–13.52) 10	(12.86–15.16) 91	(11.45–14.52) 211	(11.62–12.67) 7
PB	4.27 ± 0.09	4.22 ± 0.14	4.10 ± 0.17	4.08 ± 0.17
	(4.18–4.40) 10	(3.94–4.62) 91	(3.70–4.53) 211	(3.77–4.27) 7
PL	12.21 ± 0.41	13.42 ± 0.43	12.78 ± 0.59	11.32 ± 0.24
	(11.79–12.95) 10	(12.00–14.57) 91	(11.00–14.61) 211	(10.99–11.59) 7
PWC	5.71 ± 0.30	6.08 ± 0.34	5.82 ± 0.33	5.13 ± 0.15
	(5.30–6.16) 10	(5.25–6.72) 91	(4.91–6.60) 211	(4.93–5.30) 7
MSTW	10.92 ± 0.26	11.41 ± 0.34	10.91 ± 0.37	9.94 ± 0.40
	(10.52–11.40) 10	(10.61–12.65) 91	(10.20–11.97) 211	(9.43–10.54) 7
MPW	13.67 ± 0.40	13.87 ± 0.51	13.36 ± 0.49	12.25 ± 0.10
	(13.20–14.50) 10	(12.71–15.01) 91	(12.07–14.75) 211	(12.07–12.33) 7
MTRL	9.44 ± 0.21	10.14 ± 0.24	9.71 ± 0.33	8.77 ± 0.20
	(9.13–9.75) 10	(9.60–10.84) 91	(8.80–10.58) 211	(8.47–9.03) 7
MLTRL	7.59 ± 0.17	8.18 ± 0.23	7.81 ± 0.33	6.93 ± 0.32
	(7.37–7.95) 10	(7.52–8.79) 91	(7.04–9.66) 211	(6.45–7.23) 7
PWM2	8.43 ± 0.19	9.11 ± 0.29	8.67 ± 0.33	7.96 ± 0.18
	(8.17–8.74) 10	(8.40–9.74) 91	(7.73–9.68) 211	(7.75–8.31) 7
DENL	16.93 ± 0.21	18.42 ± 0.55	17.61 ± 0.64	16.16 ± 0.47
	(16.66–17.20) 10	(17.20–19.88) 91	(15.85–19.17) 211	(15.56–16.82) 7
MANDL	10.57 ± 0.17	11.39 ± 0.33	10.87 ± 0.37	10.30 ± 0.43
	(10.17–10.74) 10	(10.24–12.07) 91	(9.96–11.80) 211	(9.72–10.99) 7
COH	6.58 ± 0.16	7.37 ± 0.40	7.01 ± 0.40	6.53 ± 0.56
	(6.35–6.80) 10	(6.60–8.39) 91	(6.08–8.31) 211	(5.86–7.35) 7

Table 5

Dataset	N	DFA % correct			DFA-CVs % Correct			MANOVA
		A	*B*	*C*	*A*	*B*	*C*	
Log-Transformed Data								
Cranial	320	60.00	69.57	85.72	40.00	67.39	83.89	< 0.001*
Cranial and External	276	83.33	75.00	87.36	83.33	71.59	84.07	< 0.001*
Principal Components (~90%)								
Cranial	320	40.00	70.65	85.31	40.00	67.39	84.36	< 0.001*
Cranial and External	276	83.33	68.18	87.36	66.67	65.91	85.16	< 0.001*

Table 6

	Df	SS	MS	R^2	F	Z	P
Centroid Size (CS)							
(A) DORSAL SKULL							
Sex	1	0.0097	0.0097	0.0178	3.2657	1.4290	0.0726
Species	4	0.2067	0.0517	0.3788	17.3367	6.5090	**< 0.001**
Sex x species	4	0.0127	0.0032	0.0233	1.0643	0.3883	0.3448
Residuals	107	0.3190	0.0030	0.5845			
Total	116	0.5457					
(B) VENTRAL SKULL							
Sex	1	0.0323	0.0323	0.0251	4.2022	1.6508	**0.0427**
Species	4	0.4441	0.1110	0.3449	14.4575	5.5683	**< 0.001**
Sex x species	4	0.0166	0.0041	0.0129	0.5403	-0.4072	0.6545
Residuals	103	0.7909	0.0077	0.6143			
Total	112	1.2875					
(D) LATERAL MANDIBLE							
Sex	1	0.0588	0.0588	0.0444	8.6443	2.3308	**0.0037**
Species	4	0.4930	0.1233	0.3721	18.1226	6.4189	**< 0.001**
Sex x species	4	0.0078	0.0020	0.0059	0.2870	-1.0924	0.8602
Residuals	115	0.7821	0.0068	0.5903			
Total	124	1.3248					

Table 7

	Df	SS	MS	R^2	F	Z	P
Shape							
(A) DORSAL SKULL							
Size	1	0.0047	0.0047	0.0534	8.0157	4.0002	**< 0.001**
Sex	1	0.0013	0.0013	0.0145	2.1809	1.6100	0.0539
Species	4	0.0128	0.0032	0.1471	5.5225	5.9479	**< 0.001**
Size x sex	1	0.0007	0.0007	0.0082	1.2322	0.6368	0.2620
Size x species	4	0.0029	0.0007	0.0334	1.2530	0.8382	0.2055
Sex x species	4	0.0019	0.0005	0.0220	0.8256	-0.4631	0.6766
Size x sex x species	2	0.0016	0.0008	0.0179	1.3473	0.8760	0.1912
Residuals	99	0.0575	0.0006	0.6591			
Total	116	0.0873					
(B) VENTRAL SKULL							
Size	1	0.0045	0.0045	0.0600	9.2070	6.0339	**< 0.001**
Sex	1	0.0009	0.0009	0.0114	1.7470	1.5886	0.0578
Species	4	0.0105	0.0026	0.1389	5.3250	6.9117	**< 0.001**
Size x sex	1	0.0009	0.0009	0.0117	1.7967	1.7044	**0.0452**
Size x species	4	0.0036	0.0009	0.0476	1.8266	2.8451	**0.0024**
Sex x species	4	0.0026	0.0007	0.0349	1.3374	1.4079	0.0795
Size x sex x species	2	0.0007	0.0004	0.0099	0.7613	-0.7990	0.7866
Residuals	95	0.0466	0.0005	0.6195			
Total	112	0.0753					
(D) LATERAL MANDIBLE							
Size	1	0.0142	0.0142	0.0815	11.700	5.728	**< 0.001**
Sex	1	0.0025	0.0025	0.0141	2.027	1.722	**0.0446**
Species	4	0.0091	0.0023	0.0525	1.882	2.631	**0.0043**
Size x sex	1	0.0004	0.0004	0.0026	0.369	-1.660	0.9509
Size x species	4	0.0038	0.0010	0.0220	0.790	-0.754	0.7785
Sex x species	4	0.0043	0.0011	0.0249	0.892	-0.285	0.609
Size x sex x species	2	0.0014	0.0007	0.0078	0.559	-1.388	0.9136
Residuals	107	0.1298	0.0012	0.7456			
Total	124	0.1740					

CAPÍTULO IV – Conclusões e considerações finais

Com este trabalho aprofundamos o conhecimento taxonômico dos morcegos neotropicais do gênero *Lophostoma*, revelando e identificando a diversidade críptica presente em dois complexos de espécies indicados para o gênero, *Lophostoma brasiliense* e *Lophostoma silvicola*. Especificamente, foi provida evidência para a revalidação de *Lophostoma nicaraguae* como uma linhagem separada de *Lophostoma brasiliense* e foi proposta a categorização ao nível de espécie para *L. laephotis* e *L. amblyotis*. Todas essas espécies muito semelhantes morfologicamente. O trabalho também mostrou que o número de espécies dentro desse gênero está atualmente subestimado e melhora o conhecimento sobre a diversidade, os limites entre as espécies e a distribuição desse fascinante grupo de organismos. Os dados taxonômicos e moleculares gerados nesta dissertação servirão como base para futuros estudos evolutivos, filogenéticos e biogeográficos, além de contribuir para o estabelecimento de futuras atividades de manejo e conservação das espécies.

Os métodos de delimitação de espécies foram eficientes para revelar a diversidade críptica e delimitar as espécies. No entanto, o uso do Generalized Mixed Yule-Coalescent (GMYC), apresentou discordâncias relacionadas a uma sobre estimação no número de entidades taxonômicas. Os métodos de delimitação de espécies fenotípicos baseados em modelos mistos normais mostraram uma eficiência muito restrita para delimitar espécies crípticas neste trabalho. Apesar de delimitar adequadamente as espécies no complexo *Lophostoma brasiliense*, sua utilidade foi limitada no complexo *silvicola*. Possivelmente, um maior número de amostras por grupo pode melhorar sua eficiência, ou talvez a escolha de outros caráteres fenotípicos.

Lophostoma ocupa um grande número de habitats na região neotropical com a maioria das suas espécies presentes na América do Sul. Diferentes processos e barreiras geográficas têm influenciado a diversificação desse grupo de morcegos. Os Andes têm sido uma barreira fundamental que parece explicar a diversificação de *L. brasiliense* e *L. nicaraguae,* assim como delimitar a distribuição de *L. occidentale* e *L. carrikeri*. No entanto, as barreiras não são apenas físicas, mas também ecológicas, como as associadas ao tipo de floresta ou biomas. Diferentes coberturas florestais parecem ter influenciado a diversificação do complexo *L. silvicola* como foi discutido no capítulo II.

Estudos futuros devem incluir a análise com marcadores nucleares para elucidar ainda mais as relações filogenéticas das espécies dentro do gênero e dentro de cada complexo de espécies. Da mesma forma, seria interessante poder obter mais dados de áreas pouco amostradas neste estudo, como a região nordeste e centro do Brasil, as florestas subandinas na Bolívia e as planícies do Leste e Caribe na Colômbia. Questões que precisam de ser abordadas, incluem o estado taxonômico da subespécie *L. s. centralis,* para o qual são necessários tecidos e dados provenientes de Costa Rica e Honduras; a descrição de uma linhagem não conhecida de *L. occidentale* que poderia representar uma nova espécie revelada aqui nesse trabalho; e a posição filogenética de *L. kalkoe*, espécie que não foi possível obter tecidos.