Chromosome Painting in *Lonchorhina aurita* Sheds Light onto the Controversial Phylogenetic Position of Sword-Nosed Bats (Chiroptera, Phyllostomidae)

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Abstract

The subfamily Lonchorhininae encompasses 6 species of sword-nosed bats (*Lonchorhina*) and is one of the most problematic lineages in the Neotropical leaf-nosed bats (Phyllostomidae) phylogeny. There are at least 5 different hypotheses to explain when the subfamily diverged from the remaining phyllostomids, but none with robust statistical support. Here, we generated a chromosome painting homology map of *Lonchorhina aurita* karyotype (2n = 32 and FN = 60) using whole-chromosome probes of *Macrotus californicus* (MCA; 2n = 40 and FN = 60). We placed the karyotype changes of *L. aurita* in a phylogenetic context to discuss the most likely branching position of Lonchorhininae based on karyotypic evolution. We show that *L. aurita* has a derived karyotype with 24 segments homologous to the 20 MCA chromosomes used as probes. Comparative analyses between 7 published painted bats species across 4 phyllostomid subfamilies (Macrotinae, Phyllostominae, Glossophaginae, and Lonchophyllinae) revealed that one inversion (MCA 4inv) and one fusion (MCA 17 + 18) are shared derived features between the karyotypes of *L. aurita* and species of Phyllostominae not yet

observed in other bats. Our data show that chromosomal homology maps may contribute with new insights into a long-standing phylogenetic debate that has endured for decades.

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Introduction

The insectivorous sword-nosed bats of the genus *Lonchorhina* Tomes, 1863 are currently classified into 6 species (*L. aurita*, *L. fernandezi*, *L. inusitata*, *L. mankomara*, *L. marinkellei*, and *L. orinocensis*), characterized by the largest nose leaves in the Neotropical family Phyllostomidae [Mantilla-Meluk et al., 2019; Simmons and Cirranello, 2020]. Morphological data traditionally placed *Lonchorhina* within the subfamily Phyllostominae [Wetters et al., 2000], but earlier molecular phylogenies supported the recognition of a new monotypic subfamily, Lonchorhininae [Baker et al., 2003a]. In this classification, based on mtDNA and nuclear RAG2 sequence data, Lonchorhininae appears as a basal branch relative to Phyllostominae that diverged after Macrotinae, Micronycterinae, and Desmodontinae. Thenceforth, studies based on larger molecular datasets and different methods have displayed alternative branching positions for Lon-

Table 1. Summary of syntenic associations and conserved chromosomes of *Macrotus californicus* (MCA) detected by chromosome painting in Phyllostomidae

MCA associations	Taxon						
	LAU (2n = 32)	LOC (2n = 34)	GCR (2n = 32)	TSA (2n = 16)	GSO (2n = 32)	ACU (2n = 30)	LCO (2n = 28)
10/5q	+	+	+		+		
12/2q ^a	+	Inv(+)	Inv(+)	+	+		
13/3q	+	+	+	+	+	+	+
14/1q	+	+	+		+		
3p/11	+	+	+		+		
2p/1p	+	+	+		+		
4 ^a	Inv(+)	Inv(+)	Inv(+)		+		
5p		+	+				+
6	+	+	+		+		+
7	+	+	+		+	+	
8	+	+	+		+	+	+
9	+	+	+				+
9/17					+	+	
15	+	+	+		+		
16	+	+			+		
16/19			+				+
17/18	+	+	+				
18/5p					+	+	
19/5p	+						
X	+	+	+	+	+	+	+

The abbreviations represent taxa analyzed sensu Baker et al. [2016]. Lonchorhininae: LAU, *Lonchorhina aurita* – data from the present report. Phyllostominae: LOC, *Lophostoma occidentale*; GCR, *Gardnerycteris crenulatum*; TSA, *Tonatia saurophila* – data from Sotero-Caio et al., [2015]. Glossophaginae: GSO, *Glossophaga soricina*; ACU, *Anoura cultrata*. Lonchophyllinae: LCO, *Lonchophylla concava* – data from Sotero-Caio et al. [2013]. ^a Additional rearrangement distinguishes karyotypes by inversion (inv) of MCA, 12inv+2q and MCA, 4inv, both submetacentric chromosomes.

chorhininae, but so far, no consensus has been reached and the phylogenetic position of the subfamily remains unclear [Rojas et al., 2011, 2016; Dumont et al., 2012; Dávalos et al., 2014; Baker et al., 2016; Amador et al., 2018].

In groups with such complex taxonomy and systematic issues, comparative cytogenetic approaches can be used in an integrative framework to refine available hypotheses of evolutionary relationships, given that chromosomal changes act as mutational events, and extant karyotypes can be traced back to ancestral states, recapturing the evolutionary process [Rokas and Holland, 2000]. Chromosome painting was proven very useful in the discussion of cytotaxonomic issues and understanding evolutionary processes in mammals [Ferguson-Smith and Trifonov, 2007]. In bats, this technique has allowed refinement of karyotypic evolution hypotheses [Sotero-Caio et al., 2017; Solari et al., 2019], taxa delimitation [Volleth et al., 2017], and validation of the use of chromosome rearrangements as phylogenetic markers [Volleth et al., 2002; Benathar et al., 2019].

Among the available chromosome-specific probes derived from bats, those from *Macrotus californicus* (2n = 40, FN = 60) have the benefit of representing a karyotype closest to the ancestral karyotype of Phyllostomidae (*M. waterhousii*, 2n = 46, FN = 60), which allows for straightforward inference of ancestral versus derived states in chromosome evolution studies within the family [Baker and Bickham, 1980]. Up to now, the karyotypes of 6 phyllostomid species have been painted with *M. californicus* probes (Table 1) [Sotero-Caio et al., 2013, 2015].

Herein, we generated a genome-wide homology map between *M. californicus* and *L. aurita* using *M. californicus* chromosome-specific probes. Our goals were to identify shared syntenic associations among *L. aurita* and published chromosome painting data from species encompassing 3 phyllostomid subfamilies (Phyllostominae, Glossophaginae, and Lonchophyllinae), as well as plotting the identified chromosome changes in contrasting phylogenetic hypotheses of Phyllostomidae and to discuss the most parsimonious phylogenetic position of the subfamily Lonchorhininae.

Fig. 1. Chromosome painting on *Lonchorhina aurita* (LAU) karyotype. **a** G-banding karyotype of a female LAU showing *Macrotus californicus* (MCA) chromosome homologies. **b–d** Mitotic metaphases showing examples of duo-color hybridizations of MCA probes. Biotin-labeled probes were detected with avidin-FITC (green) and digoxigenin-labeled probes with anti-digoxigenin-rhodamine (red). The slides were counterstained with DAPI. Scale bar, 5 μ m.

Materials and Methods

Sampling and Chromosome Banding

Karyotype analyses were carried out in 4 *L. aurita* (LAU; 1 male and 3 females) collected in Toritama, Pernambuco, Brazil (08°00'24" S and 36°03'24" W). Voucher specimens were deposited in the mammalian collection at the Department of Zoology, Universidade Federal de Pernambuco, Brazil. Mitotic chromosomes were obtained from bone marrow following the standard protocol by Baker et al. [2003b] and G-banding was carried out in one reference female specimen following Seabright [1971] methods.

Chromosome Painting and Comparative Analyses

FISH was performed using whole-chromosome probes of the phyllostomid bat *M. californicus* (MCA; 2n = 40, XY; FN = 60) prepared from flow-sorted chromosomes and DOP-PCR-labelled with biotin and digoxigenin [Sotero-Caio et al., 2013]. FISH was carried out on a female LAU sample as reference, following procedures previously described [Volleth et al., 2002; Sotero-Caio et al.,

2013]. The slides were denatured with 70% formamide/30% 2 $\times$ SSC at 75°C for 10 min. No hybridization with MCA Y chromosome paint was performed. FISH images were captured using a Leica DMLB 5500B epifluorescence microscope and the Leica Las-AF software and G-banding images using a Leica DM2500 and CytoVision 7.7 software. For image processing, brightness and contrast adjustments were performed using Adobe Photoshop CS5, and ideogram drawing was done on Inkscape (<https://inkscape.org>).

For comparative purposes, the chromosome painting data of the present study were integrated with those from Sotero-Caio et al. [2013, 2015], which used the same set of probes to 6 species of Phyllostomidae belonging to subfamilies Phyllostominae, Glossophaginae, and Lonchophyllinae (Table 1). Shared chromosome characters detected using MCA probes were parsimoniously plotted on respective ancestral nodes (across all taxonomic ranks) of phyllostomid phylogenetic trees. Under this method of comparative analysis, parsimony implies that when 2 or more species share a derived chromosome rearrangement, their most recent common

ancestor also displayed that rearrangement. This approach was repeated for multiple phylogenetic hypotheses displaying alternative branching positions for the subfamily Lonchorhininae [Baker et al., 2003a, 2016; Rojas et al., 2011, 2016; Dávalos et al., 2014; Amador et al., 2018]. Finally, assessment of the number of events of chromosome rearrangements, as well as the number of required reversals to explain convergent events, allowed us to discuss when Lonchorhininae likely branched out during the radiation of phyllostomid bats according to karyotype evolution.

Results

All specimens of *L. aurita* (LAU) had a karyotype with $2n = 32$ and a fundamental number of $FN = 60$ (Fig. 1a). Hybridization of *M. californicus* (MCA) chromosome-specific probes allowed us to provide a complete homology map of the *L. aurita* karyotype shown in Figure 1. FISH with the entire set of MCA autosomes + X chromo-

Fig. 2. G-banding comparison between *Macrotus californicus* (MCA) chromosome 4 and the correspondent syntenic homologues (pairs 7) in other phyllostomids: *Glossophaga soricina* (GSO; Glossophaginae); Phyllostominae: *Lophostoma occidentale* (LOC), and *Gardnerycteris crenulatum* (GCR) modified from Sotero-Caio et al. [2013, 2015]; *Phyllostomus hastatus* (PHA) and *Lophostoma silvicola* (LSI) modified from Pieczarka et al. [2005] and Ribas et al. [2015]; and *Lonchorhina aurita* (LAU; Lonchorhininae).

Fig. 3. Integration of chromosome painting data to alternative phylogenetic hypotheses for Phyllostomidae subfamilies. Putative synapomorphies for subfamilies are mapped in slightly different nodes according to the Lonchorhininae position (red). **a** Hypothesis 1 tree recovered by Baker et al. [2003a] and hypothesis 2 by Rojas et al. [2011], Dumont et al. [2012], and Dávalos et al. [2014] (distinct only by branch order – dashed grey arrows). **b** Hypothesis 3 by Baker et al. [2016]. **c** Hypothesis 4 according to Rojas et al. [2016]. **d** Hypothesis 5 recovered by Amador et al. [2018]. The occurrence steps of derived characters MCA 4inv and 17 + 18 are shown. **e** Ideograms indicate syntenic associations shared among lineages at the most basal family node (node A), also in the common ancestor of Lonchorhininae, Phyllostominae, Glossophaginae, and Loncho-

some as probes identified a total of 24 homologous segments on the *L. aurita* genome (Fig. 1a). Eight *L. aurita* chromosomes (LAU 7, 8–11, 14, 15, and X) had hybridization signal conservation, with their whole chromosome length corresponding to single MCA chromosomes (MCA 4, 6–9, 15, 16, and X); however, LAU 7 differs from its homologous (MCA 4) by a pericentric inversion, which was confirmed by G-band comparison with other Phyllostomidae (Fig. 2). Four MCA probes revealed 2 distinct signals on different autosomes of *L. aurita*: MCA 1 (LAU 3 and 6), MCA 2 (LAU 4 and 6; Fig. 1b), MCA 3 (LAU 2 and 5), and MCA 5 (LAU 1 and 13; Fig. 1d). Most signals corresponded to entire chromosome arms; however, MCA 13 signal encompassed the entire short arm (LAU 2p) and also the portion of the long arm (2q proximal) of the submetacentric LAU 2. In addition, 6 probes hybridized only to a single chromosome arm (MCA 11, 12, 14, 17, 18, and 19; Fig. 1b–d).

The comparative analysis showed several conserved chromosomes and syntenic associations in all phyllostomids analyzed (Table 1). Thirteen autosome associations were shared between *L. aurita* and species of the subfamily Phyllostominae, and 11 between *L. aurita* and *Glossophaga soricina* (GSO, Glossophaginae). Most of the conserved chromosome/chromosome associations were shared across investigated species and thus could be plotted at the most basal family node (node A; Fig. 3). Other conserved chromosomal associations were likely present in the common ancestor of Lonchorhininae, Phyllostominae, Glossophaginae, and Lonchophyllinae (node B; Fig. 3).

Interestingly, the *L. aurita* karyotype, but no other analyzed species, presents 2 derived syntenies in common with Phyllostominae (inversion [inv] MCA 4inv and 17 + 18; Table 1, Fig. 1c–d). After placing the chromosome associations in a phylogenetic context, these syntenies either occurred independently after radiation of each sub-

phyllinae (node B), which are based on comparative analyses. The abbreviations indicate proposed rearrangements. Fis, fission; Fu, fusion; Inv, inversion. Abbreviations in parentheses correspond to chromosomal morphology or taxa analyzed by chromosome painting with MCA probes. A, acrocentric; B, biarmed (used to describe the linkage unit when the differences in morphology have not been confirmed with G-banding data); M, metacentric; SM, submetacentric; LAU, *Lonchorhina aurita*; LOC, *Lophostoma occidentale*; GCR, *Gardnerycteris crenulatum*; TSA, *Tonatia saurophila*; GSO, *Glossophaga soricina*; ACU, *Anoura cultrata*; LCO, *Lonchophylla concava*. Letters beside a chromosome segment represent the ancestral short (p) or long (q) chromosome arm. **Insert** on bottom-right shows MCA chromosome color key.

(For figure see next page.)

family (Fig. 3a–c) or as synapomorphies for a putative sister clade Lonchorhininae + Phyllostominae (Fig. 3d). Alternatively, though unlikely, these rearrangements occurred in the common ancestor after radiation of vampire bats (Desmodontinae) and were reversed to the original state after radiation of Phyllostominae and Lonchorhininae. The association of MCA 19+5p until now was detected only in *L. aurita* (Table 1, Fig. 1d).

Discussion

Unlike other *Lonchorhina* species with relatively restricted occurrences, *L. aurita* has an extensive distribution in Central and South America [Mantilla-Meluk et al., 2019]. Despite its wide distributional range, cytogenetic studies of *L. aurita* from different regions (e.g., Brazil and Trinidad) revealed identical karyotypes with $2n = 32$ and $FN = 60$, composed of 8 metacentric (M), 4 submetacentric (SM), and 3 subtelocentric (ST) chromosomes [Baker, 1979; Barros et al., 2009]. The specimens investigated herein presented identical karyotypic features.

G-banding comparison between *L. aurita* and *Trachops cirrhosus* ($2n = 30$, $FN = 56$; Phyllostominae) was provided by Barros et al. [2009], using *M. waterhousii* G-banded chromosome arms as reference and showed considerable divergence of the *L. aurita* karyotype from that of *Macrotus* but a high degree of similarities to that of *T. cirrhosus*. However, technique limitations prevented them to identify the rearrangements detected here. At least 3 conserved chromosome pairs between *M. waterhousii* and *L. aurita* were identified by Barros et al. [2009], all of which were present in our MCA/LAU mapping (MWA 6/7 = LAU 8 = MCA 6; MWA 25/26 = LAU 9 = MCA 15; and MWA 15/16 = LAU 11 = MCA 7; the last 2 correspond to pairs LAU 15 and 10 in our analysis, respectively). Our chromosome painting analysis refined the identification of homology between the 2 genera and revealed several chromosome and chromosome arm homologies (Fig. 1).

The integration of our data with comparative maps of other phyllostomid species [Sotero-Caio et al., 2013, 2015] enabled us to identify shared chromosome associations between taxa (Table 1). Karyotypic conservation was observed among *L. aurita* and species from the genera *Lophostoma* and *Gardnerycteris* (Phyllostominae), as well as *Glossophaga* (Glossophaginae), which retained most of the syntenic associations found in *L. aurita* and differ by rearrangements involving chromosomes MCA 16–19. The different syntenies and homoplasmy of chro-

mosomes MCA 16–19 were highlighted in an evolutionary framework by Sotero-Caio et al. [2015].

On the other hand, *L. aurita* karyotype contrasts with the extreme chromosomal reshuffling already observed in *Tonatia* and *Anoura* (Phyllostominae and Glossophaginae, respectively). Comparative analysis has shown that 2 out of the 3 rearrangements proposed as Phyllostominae synapomorphies by Sotero-Caio et al. [2015], MCA 4inv and the association 17 + 18, were also identified in the karyotype of *L. aurita* (Table 1). G-band comparisons confirmed the sharing of MCA 4inv with others Phyllostominae, as *Lophostoma silvicola* and *Phyllostomus hastatus* (Fig. 2); this rearrangement is also a Phyllostominae synapomorphy in Benathar et al. [2019]. Furthermore, the association MCA 19+5p, so far present only in *L. aurita*, suggests a probable synapomorphy for the genus *Lonchorhina*, pending confirmation of presence in other species.

Alternative Karyotype Evolution Scenarios for *L. aurita* in a Phylogenetic Perspective

In an attempt to provide new insights into the chromosome changes that shaped the karyotype of Lonchorhininae, we plotted our chromosome data onto distinct phylogenetic hypotheses, corresponding to 5 alternative branching positions for Lonchorhininae (Fig. 3a–d; hypotheses 1–5). The genus *Lonchorhina* was originally included in the subfamily Phyllostominae based on morphological data [Wetterer et al., 2000]. However, since earlier molecular phylogenies [Baker et al., 2003a] supported its elevation to subfamily rank (Lonchorhininae; Fig. 3a; hypothesis 1), multiple alternative branching positions have been recovered in studies using distinct approaches, with variable levels of support [Rojas et al., 2011, 2016; Dumont et al., 2012; Dávalos et al., 2014; Baker et al., 2016; Amador et al., 2018]. In some phylogenies, Lonchorhininae appears as a lineage that diverged before nectarivorous bats but after Phyllostominae (Fig. 3a; hypothesis 2). These studies incorporated diverse datasets such as mtDNA including *cyt b* [Rojas et al., 2011] and a combined analysis of molecular and morphological data [Dávalos et al., 2014]. Meanwhile, in other phylogenetic trees, such as in Baker et al. [2016], Lonchorhininae was recovered as an independent clade that diverged after Phyllostominae and Glossophaginae, but before Lonchophyllinae (Fig. 3b; hypothesis 3).

A sister relationship between Lonchorhininae and nectar-feeders of the subfamily Glossophaginae has been also one of the tree topology alternatives (Fig. 3c; hypothesis 4); these included analyses of mitochondrial *COI* incorporation with previous mtDNA data [Dumont et al., 2012], as

well as larger mtDNA and nuclear gene samples [Rojas et al., 2016]. Amador et al. [2018], with focus on the chiropteran phylogeny, recovered a congruent Phyllostomidae tree in which Lonchorhininae appears as sister to Phyllostominae (Fig. 3d; hypothesis 5). Furthermore, the same clade was also observed in the well-supported maximum likelihood tree of Dávalos et al. [2014] and by Botero-Castro et al. [2018], which recovered a tree of entire mitochondrial genomes but including only 21 species of the 11 subfamilies recognized by Baker et al. [2016]. It is noteworthy that there is no robust support for the position of Lonchorhininae in any of the available phylogenies, regardless of methods and sampling effort made. In such controversial cases, using integrative taxonomic approaches with different data types collating independent observations in support of a major hypothesis is essential [Solari et al., 2019]. Current collections of data, especially echolocation and morphological, but also genetic, have shown that *Lonchorhina* displays unique patterns and trends relative to other phyllostomids, which complicates inferences of relationships based on comparative analyses [Wetterer et al., 2000; Dávalos et al., 2014; Gessinger et al., 2019, 2021]. Thus, by estimating the branching position of *Lonchorhina* based on changes of chromosome architecture combined with all available phylogenetic hypotheses we hope to provide new information that might aid to the elucidation of relationships as new methods and approaches appear.

The simultaneous analysis of chromosomal changes along trees with alternative positions for the Lonchorhininae radiation demonstrates distinct number and order of chromosome change events, depending on branching order and position of Lonchorhininae on the Phyllostomidae tree. Assuming that shared syntenic associations between 2 taxa were also present in their common ancestor, several chromosomes and associations were consistently found in ancestral nodes and shown as ancestral Phyllostomidae characters (node A; Fig. 3e) or present in the common ancestor of phyllostomids that diverged after the radiation of vampire bats (node B; Fig. 3e). Similarly, some associations are unique to species within particular subfamilies and are shown in Figure 3 as synapomorphies on respective branches. These ancestral syntenies remained unchanged when compared to ancestral states proposed by Sotero-Caio et al. [2015] after the inclusion of the *L. aurita* dataset.

The major distinction across hypotheses is the emergence of the inversion MCA 4inv and fusion of MCA 17 + 18, which are shared only by *L. aurita* and Phyllostominae species. Analysis of the evolution of these associations, especially the inversion of chromosome homolo-

gous to MCA 4, results in 3 informative scenarios in the discussion of systematics of Lonchorhininae, as follows: (1) in hypothesis 1 and 2 (Fig. 3a), these rearrangements might have occurred in the ancestral B, were retained during divergence of Lonchorhininae and Phyllostominae, but then reversed back in the common ancestor of Glossophaginae and remaining Phyllostomid subfamilies (1 reversal), or alternatively arose independently twice (2 events for each chromosome rearrangement); (2) both hypothesis 3 and 4 (Fig. 3b–c) would require these characters to have appeared independently twice (in Phyllostominae and Lonchorhininae), or alternatively, emergence occurred in the ancestral B, followed by 2 independent reversals (in Glossophaginae and MCA of remaining phyllostomids); and (3) the most parsimonious evolutionary path of these derived chromosome states would be if Phyllostominae and Lonchorhininae were sister taxa (Fig. 3d), with the derived character states originating in their common ancestor. That would be the scenario with less steps (1 event for each chromosome change) and no character state reversal or independent origins.

Evolutionarily conserved chromosome associations shared between *L. aurita* and Phyllostominae might indicate common ancestry and support the phylogenetic hypothesis 5. However, Ferguson-Smith and Trifonov [2007] alert for de novo karyotypic changes (reuse of evolutionary breakpoints) and the need of careful breakpoint identification to prevent misleading phylogenetic interpretations and conclusions due to convergent events. This is especially true because homoplasy of chromosome characters can be less likely the effect of selection and more likely the result of underlying chromatin architecture of regions prone to breakage. Regional genomic landscapes can facilitate double-strand breaks, and some rearrangements are more prone to be fixed in populations, unless they confer selective disadvantages [Hedges and Deininger, 2007; Carbone et al., 2014]. It is important to keep in mind that chromosome painting was intensively applied to identified interchromosomal changes providing important information, and it is unable to accurately identify evolutionary breakpoint regions, but it has also been able to recover synapomorphic chromosome rearrangements in diverse mammalian families [Ferguson-Smith and Trifonov, 2007; Graphodatsky et al., 2012].

In the case of the present study, MCA 17 + 18 reversal is a likely scenario, especially in phyllostomids, which seem to have recurrent fusion and fission of the smallest chromosomes [Sotero-Caio et al., 2015]. In comparison, the reversal of chromosome 4 inversion is less likely, especially if it is shown to use the same breakpoints, and due

to constraints the rearrangement would impose in the fitness of heterozygous for the rearrangement. On the other hand, the inversion breakpoints might not be the same, and independent inversions of chromosome 4 remains a valid hypothesis. Even considering those scenarios, homology of rearrangements remains a valid hypothesis which, if confirmed, would imply a sister relationship between Phyllostominae and Lonchorhininae. In addition, the presence of 2 shared derived chromosome characters might be a further support for common ancestry of both subfamilies. Overall, to better understand the evolutionary process of the karyotypic changes, more robust molecular cytogenetic techniques, as BAC-FISH, and new "-omics" approaches using chromosome-level genome assemblies should be used to determine whether these chromosomes are homologous or homoplastic [Graphodatsky et al., 2012; Deakin et al., 2019].

In conclusion, our study showed that the *L. aurita* karyotype is derived when compared to that of *M. californicus*, but almost identical to other phyllostomid bats. We demonstrated alternative scenarios in the karyotype evolution of *L. aurita* in a phylogenetic context and, although we cannot exclude that these characters are a result of convergence, the associations MCA 4inv and 17 + 18 represent shared derived characters that parsimoniously support a sister-clade Lonchorhininae + Phyllostominae. Karyotyping the remaining *Lonchorhina* species will be helpful to confirm chromosomal signatures in the genus and to reinforce the hypothesis of a monophyletic Lonchorhininae + Phyllostominae. Nonetheless, data provided here will allow inference of a chromosome painting phylogeny with representatives of all 11 subfamilies shortly. Finally, our work provides insights into the value of integrative studies and inclusion of chromosome data to refine hypotheses of relationships of controversial groups.

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Statement of Ethics

All procedures performed in this study involving animals followed guidelines established by Animal Care and Use guidelines of ICMBio (Instituto Chico Mendes de Conservação da Biodiversidade) and using the collecting permit number (12,264-1) by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

C.G.S.-C. and N.S. coordinated the study. N.S. and C.G.S.-C. were part of the crew that collected the specimens. J.C.F. and D.P.B. performed the experiments. Data analysis, table and figures preparation was performed by J.C.F. and C.G.S.-C. The first draft was written by J.C.F. and later versions by J.C.F. and C.G.S.-C. All authors read, revised, and approved the final version.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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