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What is for dinner? First report of human blood in the diet of the hairy-legged vampire bat *Diphylla ecaudata*

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Blood-feeding is one of the most specialized foraging habits, as it demands extreme morphological, physiological, and behavioral adaptations. Three species of vampire bats (*Desmodus rotundus*, *Diaemus youngi*, and *Diphylla ecaudata*) rely on blood as their only food. The first two are considered less specialized, whereas *D. ecaudata* is frequently pointed out as a bird-specialist. We assessed what prey *D. ecaudata* consumes in the Caatinga dry forests of northeastern Brazil, a highly modified biome. How the species would behave in a situation of scarcity of wild birds and increase in the availability of domestic animals? Could *Diphylla* have been induced to include also mammals in its diet? Using PCR-amplification of DNA fragments in the feces of *D. ecaudata*, we detected the regular consumption of chicken blood and human blood — a novel prey for this species. Our results suggest that the diet of *D. ecaudata* is more flexible than expected. The record of humans as prey and the absence of blood from native species may reflect a low availability of wild birds in the study site, reinforcing the impact of human activities on local ecological processes. This also opens a range of research possibilities on vampire bats in the Caatinga, both on the species' biology and the consequences for public health, considering the potential increase in the transmission of rabies in the region.

Key words: blood-feeding, Brazil, Caatinga, Chiroptera, Desmodontinae, faecal DNA

INTRODUCTION

Bats of the family Phyllostomidae comprise one of the most ecologically diverse mammal groups, which includes insectivorous, nectarivorous, frugivorous, carnivorous, and hematophagous species (Fenton *et al.*, 1992). Among those habits, hematophagy is the most specialized diet, as it requires highly specific morphological, physiological, and behavioral adaptations for blood consumption (Coen, 2002). Only three monospecific genera of phyllostomids are able to use blood as their only food: *Desmodus rotundus* (Geoffroy, 1810), *Diaemus youngi* (Jentink, 1893), and *Diphylla ecaudata* Spix, 1823.

Known as vampire bats, these three species of the subfamily Desmodontinae occur only in the Americas (Fenton, 1992). They have several morphological and physiological adaptations that allow consuming and digesting blood: sharp incisors and canines used to make a small wound in vascularized regions of the prey's body, saliva with components that avoid blood coagulation and favor bleeding, and organs of the digestive system that show maximum efficiency in the elimination of the excess of water and urea present in the blood of their prey (Greenhall and Schmidt, 1988; Coen, 2002). These species are not able to store fat (Coen, 2002), do not survive fasting for more than two days (Uieda, 1994), and due to urea processing they are extremely sensitive to dehydration (Breindenstein, 1982). *Desmodus rotundus* and *D. youngi* seem to be less specialized in terms of their prey (Greenhall *et al.*, 1983, Bobrowiec *et al.*, 2015), whereas *D. ecaudata* is frequently considered a specialist, which feeds preferably on birds (Greenhall *et al.*, 1984; Uieda, 1994).

Many studies on the diet of insectivorous and frugivorous bats are based on morphological analyses of food remains found in feces (e.g., Siemers and Swift, 2006; Vleut *et al.*, 2013). In the case of vampire bats, feces are formed only by soft parts, which make the morphological identification of the prey impossible (Carter *et al.*, 2006). In those cases,

molecular tools applied to the study of diet gain attention, as through the PCR technique DNA fragments of the prey can be isolated and identified even in case of degraded samples, such as those found in feces (King *et al.*, 2008). Hence, based on this method, several knowledge gaps about bat diet have been filled, especially for insectivorous species (Clare *et al.*, 2009; Dodd *et al.*, 2012; Long *et al.*, 2013). However, there were few studies that used molecular techniques to determine the diet of hematophagous bats (Carter *et al.*, 2006; Bobrowiec *et al.*, 2015), and none assessed *D. ecaudata*.

Understanding how prey availability affects predator behavior is useful, for example, to predict responses to habitat loss or degradation (Meyer *et al.*, 2008). A very specialized diet can make a species more vulnerable to human impacts, due to the decrease in the availability of its natural prey (Layman *et al.*, 2007). However, in the case of hematophagous bats, at the same time that habitat fragmentation and loss can negatively impact their natural prey, those processes can also result from the conversion of a natural landscape for livestock farming, which greatly increases prey availability (Bobrowiec *et al.*, 2015). Hence, vampire bats can be favored by some degree of environmental disturbance, especially when this disturbance comes with an increase in the availability of domesticated animals, such as chickens, goats, and cows (Fenton *et al.*, 1992; Gomes *et al*., 2010).

Considering that vampire bats are unable to feed on food other than blood (Uieda, 1994), in a human-disturbed area we can expect a very low availability of large wild prey, which makes domestic animals a more accessible food for vampire bats (Fenton *et al.*, 1992). Our study used fecal DNA as a methodological strategy to investigate the diet of the hematophagous bat *D. ecaudata* in a Caatinga area of Pernambuco, northeastern Brazil, in a region of dry forests strongly affected by human action. In natural conditions, the diet of *D. ecaudata* is assumed to be composed of wild bird blood only (Greenhaal *et al.*, 1984). How that species would behave in a situation of scarcity of wild birds and increase in the availability of domestic animals? Could *Diphylla* have been induced to include also domestic mammals in its diet? This is exactly the scenario in the Caatinga drylands of north-eastern Brazil, where we accessed the degree of specialization in the diet of *D. ecaudata* using molecular tools.

Materials and Methods

Study Area and Sampling

We collected fecal samples from a colony of *D. ecaudata* in a cave (08°29'12.0"S, 37°16'48.0"W) in Catimbau National Park, state of Pernambuco, northeastern Brazil. Annual rainfall varies between 650 and 1,100 mm. There is irregular variation in rainfall between years (SNE, 2002) and the rainy period extends from March to July. Average annual temperature is 23ºC; July is the coldest month (ca. 21ºC), and December the warmest (ca. 25ºC). The landscape is marked by valleys, ranges, and hills and the predominant vegetation is shrubby-arboreal Caatinga (SNE, 2002). Although an IUCN Category II protected area, Catimbau National Park did not went through the legal expropriation process since its creation in 2002, and about 230 families live inside the park limits. Overall, nearly 1,100 families live in and immediately around the park. Most of those families practice subsistence agriculture and cattle ranching. There is an estimate of around 2,800 goats in the park (2–100 animals/family), with an average density of 4.6 goats/km^2 (Santos, 2015). Goats are maintained free or released for grazing during the day, and kept in corrals during the night. Grazing is usually restricted within a 2 km-radius around houses (Santos, 2015). Most of the wild animals in the park area are locally extinct due to years of hunting. Reports from local hunters and tourism guides confirm that wildlife is scarce and hard to find.

Considered a hot cave (Ladle *et al.*, 2012), the studied cave harbors a population of eight bat species of four different families, mostly insectivorous species. *Pteronotus gymnonotus* (Mormoopidae) is the most abundant species, with concentrations that may reach over 128,000 individuals. No other species of vampire bats (e.g. *D. rotundus* or *D. youngi*) were found in the cave during sampling periods, discarding the possibility of contamination of the samples. The studied colony of *D. ecaudata* is composed of approximately 30 individuals, and includes juveniles and pregnant females. The colony is located in the first gallery of the cave, and is organized in small agglomerations formed mainly by females and juveniles, but also has solitary individuals. The presence of fresh guano deposits right below the bats facilitated the collection of fecal samples for the study, which was carried out monthly from May 2014 to January 2015. We placed a sterilized plastic container below the colony to collect fresh samples. Twenty-four hours later, we collected the samples from the container using sterile filter paper, placed them in sterile 2-ml tubes filled with 96% ethanol, and stored them at -20°C no later than eight hours after collections. To avoid possible contaminations, the entire collection and molecular procedures was performed by a single person (the first author) using sterile materials, gloves and surgical masks, within a biosafety cabinet.

Molecular Procedures

Seventy samples were collected, and DNA extraction was conducted in 61 of them, applying the CTAB DNA extraction procedure (cetyltrimethylammonium bromide 2% — Doyle and Doyle, 1987) modified by Bobrowiec *et al.* (2015). We used 50 mg of each fecal sample. The material was macerated in 2-ml tubes, which received 500 µl of CTAB extraction buffer, previously heated at 60°C, and 10 µl of Proteinase K (10 ng/ml). After incubation at 60°C for 30 min, samples were

mixed with 1 ml of chloroform and centrifuged for 10 min at 13,000 rpm to precipitate impurities and separate the DNA. The aqueous phase containing the DNA was transferred to a new tube (1.5 ml), to which 750 µl of cold isopropanol were added, and then stored at -20°C for two hours to precipitate the DNA. Next, samples were centrifuged for 30 min at 13,000 rpm and the supernatant discarded. The precipitated DNA was washed with 70% ethanol, re-suspended in 50 µl of ultrapure water, and stored at -20°C.

The quantitative and qualitative evaluation of the extracted DNAs was made with integrity gel and by the comparison with patterns of known molecular weight (25 and 50 ng — Fago Lambda, Invitrogen) in 1% agarose gel, respectively. The contents of the extracted DNA were confirmed in nanophotometry (Nanodrop 2000, Thermo Scientific).

From the 61 samples for which DNA was assessed, 39 had a fragment section of the mitochondrial cytochrome *b* gene (*Cytb*) amplified with the universal primers L14841 (5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3') and H15149 (5'-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3) (Kocher *et al.*, 1989). These primers comprise a broad variety of possible bat prey and their use is possible in DNA samples that were possibly degraded after the digestion process (Kocher *et al.*, 1989; Bobrowiec *et al.*, 2015).

Amplifications occurred through PCR reactions in a final volume of 25 µl containing 12.5 µl of 2X Taq Master Mix (Vivantis Technologies), 0.5 µl of $MgCl_2$ (50 mM), 1.0 µl of each primer (2.0 mM), and 10 ng of extracted DNA. To optimize PCR reactions, we used a temperature gradient with 2°C variation, comprising a minimum temperature of 56°C and a maximum temperature of 64°C in a Veriti® thermal cycler (Applied Biosystems). Next, the reactions were optimized in a 2720 thermal cycler (Applied Biosystems), according to the following temperature conditions: 94°C for 4 min, followed by 40 cycles of 92°C for 45 sec, 56°C for 30 sec, and 72°C for 1 min and 30 sec. At last, 72°C for 10 min for the final extension step (Bobrowiec *et al.*, 2015). We observed the PCR products under ultraviolet light in 1.8% agarose gel after electrophoresis at 60V for 30 min, and quantified them by comparison to the 1 kb marker ladder (Amresco). Negative controls containing ultrapure water were included when performing DNA extractions and PCR reactions to certify that no contamination occurred.

We purified the PCR products using the enzyme complex ExoSAP IT (Affymetrix — USB Corporation), according to the protocol provided by the producer, and quantified them by nanophotometry (Nanodrop 2000, Thermo Scientific). Sequencing reactions were carried out for 20 of the previous 39 PCR-positive samples with the sequencing kit Big Dye® Terminator v3.1 (Life Technologies), according to the protocol provided, in both forward and reverse directions, in ABI 3500 automatic sequencer.

Data Analysis

To obtain a consensus sequence, we aligned and edited the sequences obtained in the program BioEdit v. 7.2.5 (Hall, 1999). Then, we compared the sequences to homologous sequences of *Cytb* deposited in GenBank. In addition, we aligned the consensus sequences with the sequences of *Homo sapiens*, *Gallus gallus*, *D. ecaudata*, and *Tropidurus hispidus* obtained from the GenBank. Sequences were aligned using a gap-opening penalty of 15 and a gap-extension penalty of 0.3 (Hall, 2001). After alignment, the initial and terminal ends of the sequence were cut off to obtain a homogeneous block of comparable sites and avoid controversial results. Alignment was tested for the best-fit model of sequence evolution in the program jModelTest (Posada, 2008). Based on the model indicated, the data matrix was analyzed by Bayesian inference (BI) in the program MrBayes v.3.1.2. (Huelsenbeck and Ronquist, 2001) and maximum parsimony (MP), using the interface PaupUp in Paup v.4.0b10 (Calendini and Martin, 2005). These two approaches were used in order to get a statistical analysis of the similarity between sequences, and to certify that the obtained DNA did not came from bats. In those analyses, we used the species *T. hispidus* as the outgroup. To obtain a standard deviation smaller than 0.01, the analysis by Bayesian inference was computed in 1,000,000 generations and the support for branches was estimated through posterior probabilities. Maximum parsimony analysis was carried out through a heuristic search, in which the characters were treated as 'non-ordered' and received equal weights. The maximum number of trees (MaxTrees) analyzed was 100,000 with 5,000 random replications by the random addition of terminals. The permutation of branches was carried out using the algorithm tree-bisection-reconnection (TBR). A majority-rule consensus topology was computed and the support for branches was tested with 1,000 bootstrap pseudoreplicates.

Results

We extracted DNA in good quality and quantity from 61 samples with the CTAB method, with values above 100 ng/µl (Supplementary Fig. S1). We obtained 39 amplifications positive for the region of the gene *Cytb*, in a total of 64% of the samples extracted (Fig. S2). Among those, 10 were obtained when the annealing temperature was 64°C and the other 10 when the annealing temperature was 56°C.

From the 39 amplifications, 20 were positive PCRs, and we obtained 15 sequences. Among those, 12 showed homology with *Cytb* sequences of chicken (*G. gallus*) deposited in GenBank, with similarity degrees between 75 and 100% (Supplementary Table S1). Other three sequences showed a similarity between 86 and 91% with the sequences of *H. sapiens* (Supplementary Table S2). Samples containing human blood were recorded in May, June and August 2014. The bat DNA was not found in any of those samples.

After the alignment and edition of sequences, we obtained a homologous block with 260 bp. The SYM model (Symmetrical model) + gamma was selected as the best model to explain the evolution of the *Cytb* sites analyzed. The parameters of this model were: -lnL = 2418.7381; K = 56; freqA = 0.2449; freqC = 0.2676; freqG = 0.2344; freqT = 0.2532; Γ shape = 1.4330.

The topology obtained by Bayesian inference and maximum parsimony (Fig. 1) showed that the fecal samples of *D. ecaudata* contained DNA from

G. gallus (2, 9, 10, 12, 19, 27, and 59) and humans (*H. sapiens* — 15, 20, and 39), as they grouped with the sequences of those species coming from the GenBank with good supports for branches. The tree obtained showed no DNA contamination of the *D. ecaudata* specimens studied, as no sequence obtained grouped with those vouchered *Cytb* deposited in GenBank.

Discussion

The isolation of DNA from the prey of *D. ecaudata* pointed to the consumption of blood from two sources in natural conditions: chickens and humans. This is the first record of human blood in the diet of this vampire bat species in natural conditions. This finding contradicts several previous studies

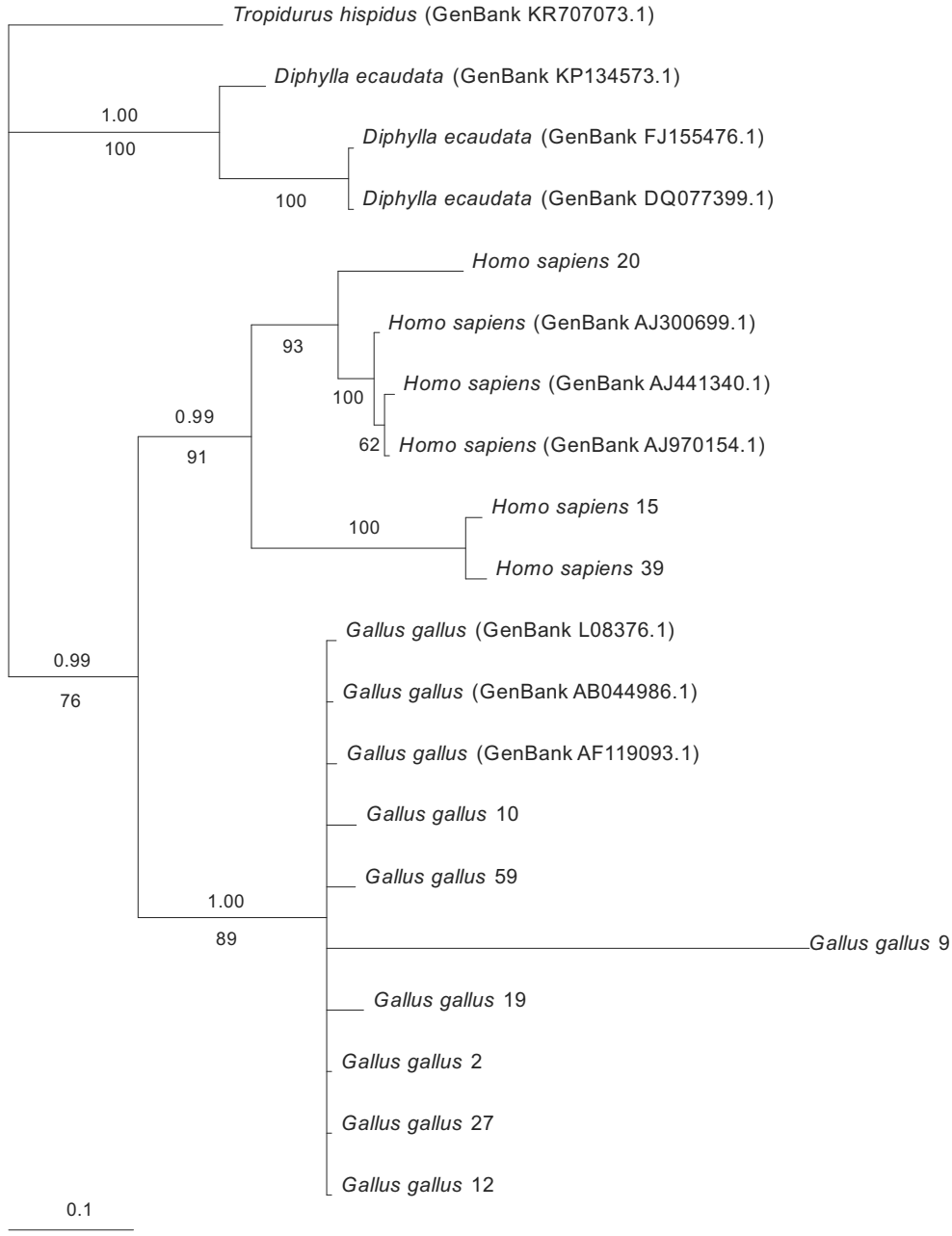

Fig. 1. Topology of Bayesian inference (SYM + gamma distribution) and maximum parsimony based on 260 bp of *Cytb* between sequences obtained from fecal samples of *D. ecaudata* in the Caatinga drylands of Northeastern Brazil and sequences of *H. sapiens*, *G. gallus*, and *D. ecaudata* originating from GenBank. The number above and below the branches indicate the statistic support for branches of posterior probabilities and bootstrap, respectively. Sequences from GenBank show their respective access codes

that considered *D. ecaudata* the species with the most specialized diet among vampire bats, feeding exclusively on bird blood in natural conditions (Villa-R., 1966; Villa-R. *et al.*, 1969; Greenhall *et al.*, 1984; Uieda, 1992). Studies on vampire bats in captivity suggest that, in spite of cases in which individual *D. ecaudata* consumed mammal blood (Ruschi, 1951; Piccinini *et al*., 1991), there is a preference for bird blood (Uieda, 1994). In their experiments, Villa-R. (1966) and Hoyt and Altenbach (1981) tried without success to feed individual *D. ecaudata* with defibrinated bovine blood, and blood from rats and rabbits kept alive in the laboratory. The refusal of this blood type was also observed in a more systematic study carried out by Uieda (1994), in which the animals opted for fasting when only pig and goat blood was offered, with cases of death of individuals by starvation.

Animals such as vampire bats, which have a very specialized diet, present behavioral, morphological, and physiological adaptations to the type of food they consume (Uieda, 1994; Ferrarezzi and Gimenez, 1996). Mammal and bird blood differ in their composition, mainly in terms of nutrient composition. Bird blood, for example, has higher amount of water and fat, whereas mammal blood is rich in dry matter, mainly proteins (Coen, 2002). Studies on the feeding physiology of the common vampire bat *D. rotundus* showed that this species has physiological characteristics that allow higher efficiency in protein processing (Coen, 2002). On the other hand, species with a preference for bird blood, such as *D. youngi* and *D. ecaudata*, have higher ability to process and use large amounts of fat found in the blood of their prey (Coen, 2002). Our results corroborate this hypothesis, as the most frequent DNA detected in the molecular analysis belonged to *G. gallus*. However, the detection of human DNA shows that *Diphylla* is able to, even though unusual, use a mixed diet composed of bird and mammal blood. As in *D. youngi*, *D. ecaudata* does not seem able to efficiently process the large amount of dry matter found in mammal blood, and so it needs to mix those two food types (Coen, 2002). Besides the physiological reasons previously explained, the conditions in which prey are maintained (free versus kept hen houses, for example) may influence the food available to vampire bats (Bobrowiec *et al*., 2015). House conditions in Catimbau are usually poor, and domestic animals are usually in close contact with humans, what may explain the occurrence of both chicken and human blood in our samples. However, food availability and the reasons behind preferences must be further investigated in detail.

Environmental Conditions and Sietary Plasticity

Our results suggest that the diet of *D. ecaudata* is more flexible than expected and understanding what factors would lead this species to use a food that is not ideal is an interesting ecological exercise. A reduction of wild birds that could be prey of *Diphylla* is a common situation in the Caatinga, which harbors approximately 22.6 million people, one of the largest human populations in semi-arid regions of the world (INSA, 2012). Caatinga is considered one of the most anthropized biomes in Brazil (Leal *et al.*, 2003) and heavy exploitation of natural resources, use of timber as a main fuel source, and broad dissemination of invasive exotic species, such as goats, resulted in intense habitat fragmentation and defaunation (Leal *et al.*, 2005; MMA/IBAMA, 2011). Among the 510 species of resident birds in the Caatinga, 251 show medium to high sensitivity to human disturbance (Silva *et al.*, 2003). Moreover, as hematophagous bats feed exclusively on blood and attack a single prey each night, they need large-sized prey to survive (Fenton, 2001). However, many large-sized birds are considered game in the region, such as the white-browed guan (*Penelope jacucaca*), the yellow-legged tinamou (*Crypturellus noctivagus*), and the picazuro pigeon (*Patagioenas picazuro*) (Olmos *et al.*, 2005). With the transformation of the landscape into impoverished forest remnants (Castelletti *et al.*, 2003), the natural wild prey availability to *D. ecaudata* has been severely reduced by humans, contributing to the disruption of important ecological processes (Leal *et al.*, 2003). In this context, domesticated birds seem to become more accessible preys to vampire bats, as they are more abundant and easier to access (Uieda, 1994). The consumption of human blood by *D. ecaudata* in natural conditions is new and can also result from such intensive human occupation. So two factors may have contributed to the consumption of domestic animals in the Caatinga: greater availability and ease of consumption of domestic prey and anthropogenic disturbances that decrease the offer of wildlife. Like *D. rotundus*, *D. ecaudata* is probably being attracted to more anthropized areas and attacking larger, domesticated prey (Fenton *et al*., 1992).

Human blood consumption by *D. ecaudata* opens a range of research possibilities on vampire bats in the Caatinga, both on the species' biology

and the consequences for public health, considering the potential increase in the transmission of rabies in the region (Ito, 2001; Schneider *et al.*, 2009). Among potential topics for study are the physiological capacity of *D. ecaudata* for processing other types of food and the fact that one more species of vampire bat is now feeding on human blood in the area. The partition of resources and possible competition with *D. rotundus*, as they share the same trophic niche (i.e. sanguinivores), must now be assessed. The assessment of the impact of hu-man occupation on the use of space by vampire bats also deserves attention, especially when determining the food source and the displacement capacity of those bats. In addition, those topics allow inferring on the social and economic impact of the predation of domesticated animals and creating preventive actions focused on the transmission of rabies. These research opportunities remain open.

Supporting Information

Contents: Fig. S1. Scan of the agarose gel (1%) electrophoresis with DNA extracted from fecal samples of the vampire bat *D. ecaudata* from Northeastern Brazil. C+ = DNA Fago Lambda (25 ng/µl); 1 to 12 = DNA extracted from the fecal samples; Fig. S2. Scan of the agarose gel (1.8%) electrophoresis with PCR outputs from fecal samples of the vampire bat *Diphylla ecaudata* from Northeastern Brazil. L = DNA marker 1 kb ladder; 1 to 6 = samples analyzed; Table S1. Significant alignements using the Basic Local Alignment Search Tool (BLAST) between DNA sequences obtained from fecal samples of the vampire bat *D. ecaudata* and sequences from chicken *Gallus gallus* deposited in the GenBank; Table S2. Significant alignements using the Basic Local Alignment Search Tool (BLAST) between protein-DNA sequences obtained from fecal samples of the vampire bat *D. ecaudata* and sequences from *Homo sapiens* deposited in the GenBank. Supplementary Information is available exclusively on BioOne.

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