Genetic Identification of Species of Bats that Act as Reservoirs or Hosts for Viral Diseases

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Authors' contributions

This work was carried out in collaboration between all authors, about design the study, write the protocol and interpret the data, anchor the field study, gather the initial data and perform preliminary data analysis, research the literature searches and produce the initial draft. All authors contributed at same way, read and approved the final manuscript.

Original Research Article

ABSTRACT

Introduction: Viruses have been identified as the main etiologic agents of both zoonoses and emerging infectious diseases (EIDs) and various species of wild fauna can be involved in the maintenance of these diseases. The very wide variety of bats, together with their ability to adapt to different environments and fly long distances, means that these animals are currently one of the main reservoirs for zoonoses and EIDs. For these reasons the correct identification of different bat species is essential.
Aims: This paper describes the genetic identification of 56 samples isolated from different bat species.
Methodology: Sequencing and phylogenetic analysis of the mitochondrial DNA cytochrome b (mtDNA cyt-b) gene.
Results: Four families (Molossidae, Vespertilionidae, Noctilionidae and Phyllostomidae), twelve

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genera and nineteen different species of bats were identified, and the Basic Local Alignment Search Tool (BLAST) was used to confirm species identity. The phylogenetic tree constructed revealed two main clusters (1 and 2), both consist in two subclusters.

Conclusions: Our results were concordant with those obtained by morphometric identification and genetic identification carried out by other authors, showing that the method described here can be used as an effective alternative to, or in combination with, morphometric identification of bats.

1. INTRODUCTION

Zoonoses are diseases that can be transmitted between humans and domestic or wild animals. According to World Health Organization (WHO) data, approximately 60% of infectious agents affecting humans originate in animals or products derived from them [1]. Zoonoses can be caused by bacteria, parasites, fungi, viruses or even unconventional agents. However, both classical and emerging viral zoonoses have been gaining attention because of recent episodes of diseases involving such agents around the world. Emerging infectious diseases (EIDs) are defined as diseases that have newly appeared in a population or have existed previously but are rapidly increasing in incidence or geographic range [2].

The viruses that cause zoonoses and/or EIDs belong to different families and cause a variety of diseases in humans. Rabies is one of the oldest known zoonoses and continues to cause serious public health problems. According to WHO estimates, 55,000 deaths are caused by this disease every year [1]. EIDs caused by viruses include Spanish flu, which became a pandemic and killed approximately 50 million people between 1918 and 1920, and Acquired Immunodeficiency Syndrome (AIDS) caused by the *human immunodeficiency virus* (HIV), which appeared in the '80s and is currently still a major public health problem34. The following viruses are also etiologic agents of emerging diseases: *Ebola virus*, *Nipah virus*, *SARS coronavirus* and *Influenza A virus*, as well as some viruses belonging to the genus *Lyssavirus*, such as *Aravan virus*, *Khujand virus*, *Irkut virus*, *West Caucasian bat virus*, *Shimoni bat virus* and *Bokeloh bat lyssavirus*.

Wild fauna play an important role in the emergence and maintenance of these diseases; indeed, the majority (71.8%) of zoonotic EIDs that have occurred since the '40s originated in wild fauna, and the number of such diseases continues to increase [3]. Identification of the reservoirs for these diseases can help clarify how the pathogens are maintained in nature, leading to more effective disease control and avoiding indiscriminate extermination of wild animals.

In recent years bats have been associated with a significant number of EIDs and have increasingly been recognized as potential reservoirs and/or hosts for viruses that can cross barriers and infect humans and other animals [4,5,6]. The very wide variety of bats and their great ability to adapt to different environments are reflected in the many different species already identified on all continents except polar regions and ocean islands, distant from continents and their different feeding habits, which can be insectivorous, frugivorous, polinivorous, nectarivorous, carnivorous, piscivorous, hematophagous and omnivorous [7]. This is reflected in the large number of viruses from different families identified recently in bats, such as *Bat adenovirus B*20, *Polyomavirus* [8], *Nipah virus* [9], *Menangle virus* [10] and *Hepatitis E-related virus* [11]. Bats are also important reservoirs for viruses belonging to the genus *Lyssavirus* in the family *Rhabdoviridae*. To date 14 species have been identified in this genus, 12 of which are included in the recent list published by the International Committee on Taxonomy of Viruses [12] and two of which are recently proposed species [13,14]. Twelve of these have already been identified in bats.

The great diversity of bats and the important role they play in the spread of diseases make the need for correct identification of different bat species particularly important. Morphometric species identification is an efficient method but requires specialized personnel. Furthermore, because many laboratories receive degraded carcasses this type of identification is often impossible.

Genetic identification can be a tool in such situations, and sequencing of mitochondrial DNA (mtDNA) has been used for the genetic identification of different mammal species. By

sequencing the hypervariable region of mtDNA, Carnieli et al. [15] identified which species of wild canid was involved in the maintenance of one of the rabies cycles in Brazil. The mtDNA cytochrome b (mtDNA cyt-b) gene has been used by various authors to study the ecology, evolution and systematics of bats [16,17,18,19,20], and in 2010 Larsen et al. [21], using the same gene, identified natural hybridization between various groups of bats.

The aim of the present study was to perform genetic sequencing of the mtDNA cyt-b gene of bats to genetically identify different Brazilian bat species, currently the main reservoirs in the Americas of the rabies virus and other species from the genus *Lyssavirus*, as well as other EID-causing viruses.

2. MATERIALS AND METHODS

2.1 Samples

For the genetic identification, 56 samples of livers from different species of bats that had previously been morphometrically identified were used. The samples came from different towns in the state of São Paulo, in the southeast of Brazil, and had been sent to the Rabies Diagnostic Laboratory at the Pasteur Institute of São Paulo.

2.2 DNA Extraction and Polymerase Chain Reaction (PCR)

Total DNA was extracted from the samples using the Wizard® Genomic DNA Purification kit #TM050 (Promega Corporation) in accordance with the manufacturer's instructions. Ultrapure DNase/RNase-free water was used as a negative control in all the steps from DNA extraction through PCR amplification.

Amplification of the mtDNA cyt-b gene from 5 µL of DNA was carried out in a final volume of 50 µL using Bat 05A (sense: 5'-CGACTAATGACATGAAAAATCACCGTTG-3') and Bat 14A (antisense: 5'-TATTCCCTTTGCCGGTTTACAAGACC-3') primers [22], 10 X PCR buffer (Invitrogen, Carlsbad, CA,USA), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 2 µM of each primer and 0.4 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA,USA). Cycling conditions were 1 cycle of 5 min at 94ºC and 35 cycles of 94ºC/45 s, 55ºC/45 s and 72ºC/2 min, followed by a final extension step of 10 min at 72ºC. After each PCR run the products were electrophoresed on 1% agarose gel in TBE buffer (1x 0.1 M Tris, 0.09 M boric acid and 0.001 M EDTA) containing 0.45mg/L ethidium bromide and visualized on a transilluminator under UV light, as previously described Carnieli et al. [15].

2.3 Purification of the PCR Products

The amplicons were purified using the GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare™) according to the manufacturer's instructions. After purification, the concentration of DNA samples was visually estimated by electrophoresis on 2% agarose gel using a Low Mass DNA Ladder (Invitrogen™) for comparison in accordance with the manufacturer's instructions.

2.4 DNA Sequencing

DNA sequencing was carried out with 4 µL of BigDye 3.1 (Applied Biosystems®), 4 µL of 5x sequencing buffer (Applied Biosystems®), 3.2 pmol of each primer (sense and antisense, as described in section 2.2), 30 to 50 ng of target DNA and DNAse/RNAse-free water to a final reaction volume of 10 µL. Reactions were carried out in a Mastercycler Gradient (Eppendorf®) under the following cycling conditions: 35 cycles at 96ºC/10 s, 50ºC/5 s and 60ºC/4 min with a ramp rate of 1ºC/s between each temperature. After each sequencing reaction the samples were purified using the HV MultiScreen 96-well filter plates and Sephadex™ G-50 fine (GE Healthcare™). After purification, the sequences were generated on an ABI 3130 genetic analyzer (Applied Biosystems™), as previously described Carnieli et al. [15].

2.5 Phylogenetic Analysis

The nucleotide sequences were edited with CHROMAS (version 2.23 Copyright 1998–2004 Technelysium Pty Ltd.), and the final sequences were aligned using CLUSTAL/W in BIOEDIT version 7.1.3.0[16]. The final sequences were used to confirm the identity of the species with BLAST. A phylogenetic tree was also constructed to determine and confirm the evolutionary relationships between the species. The tree was constructed with MEGA software (version 5.0) using the maximum likelihood method and the GTR (G + I) 5.0 substitution model[29], and the reliability was assessed by the bootstrap method with 1,000 replicates, as previously described Carnieli et al. [15].

3. RESULTS

The nucleotide sequence of the mtDNA cyt-b gene was determined for 4 families, 12 genera and 19 species of bat. The number of samples for each species analyzed is shown in Table 1. The sequences identified in the study were deposited in GenBank, and the GenBank numbers are shown in Table 2.

Based on the topology of the phylogenetic tree of the species studied, two main clusters (1 and 2) could be identified. Cluster 1 consists of subcluster 1a, corresponding to the family Molossidae, and subcluster 1b, corresponding to the family Vespertilionidae. Cluster 2 consists of subclusters 2a and 2b, corresponding to the

families Noctilionidae and Phyllostomidae, respectively (Fig. 1).

4. DISCUSSION

Bats are winged mammals that belong to the order Chiroptera. In the present study the mtDNA cyt-b gene from 18 species belonging to the three main bat families in Brazil (Phyllostomidae, Molossidae and Vespertilionidae) and one species (*Noctilio albiventris*) from family Noctilionidae was sequenced, corresponding to a total of 56 samples from 19 different species.

Rabies is one of the oldest recorded zoonoses, and the main reservoirs for the disease are

Table 1. Family, subfamily, genus, species and number of samples for which sequences of the mtDNA cyt-b gene were analyzed

Family	Subfamily	Genus	Species	No. of samples
Phyllostomidae	*Desmodontinae*	*Desmodus*	*Desmodus rotundus*	2
		Diphylla	*Diphylla ecaudata*	1
	Stenodermatinae	*Artibeus*	*Artibeus fimbriatus*	3
			Artibeus lituratus	7
			Artibeus obscurus	1
			Artibeus planirostris	4
		Platyrrhinus	*Plathyrrinus lineatus*	2
		Sturnira	*Sturnira lilium*	1
	Carolliinae	*Carollia*	*Carollia perspicillata*	3
Noctilionidae	-----	*Noctilio*	*Noctilio albiventris*	2
Molossidae	-----	*Tadarida*	*Tadarida brasiliensis*	3
		Molossus	*Molossus molossus*	9
		Cynomops	*Cynomops planirostris*	3
		Eumops	*Eumops perotis*	3
			Eumops glaucinus	2
			Eumops auripendulus	3
			Eumops bonariensis	1
Vespertilionidae	*Myotinae*	*Myotis*	*Myotis nigricans*	5
			Myotis albescens	1

Table 2. Details of the samples analyzed and deposited in GenBank, showing sample number, species name and GenBank number. All sequences obtained in this study have a total of 1140 nucleotides (nt)

Sample	Specie	GenBank number
SP1	*Artibeus obscurus*	KP134536
SP2	*Artibeus planirostris*	KP134537
SP3	*Artibeus planirostris*	KP134538
SP4	*Artibeus planirostris*	KP134539
SP5	*Artibeus planirostris*	KP134540
SP6	*Artibeus fimbriatus*	KP134541
SP7	*Artibeus fimbriatus*	KP134542
SP8	*Artibeus fimbriatus*	KT626651
SP9	*Cynomops planirostris*	KP134543
SP10	*Cynomops planirostris*	KP134544
SP11	*Cynomops planirostris*	KP134545

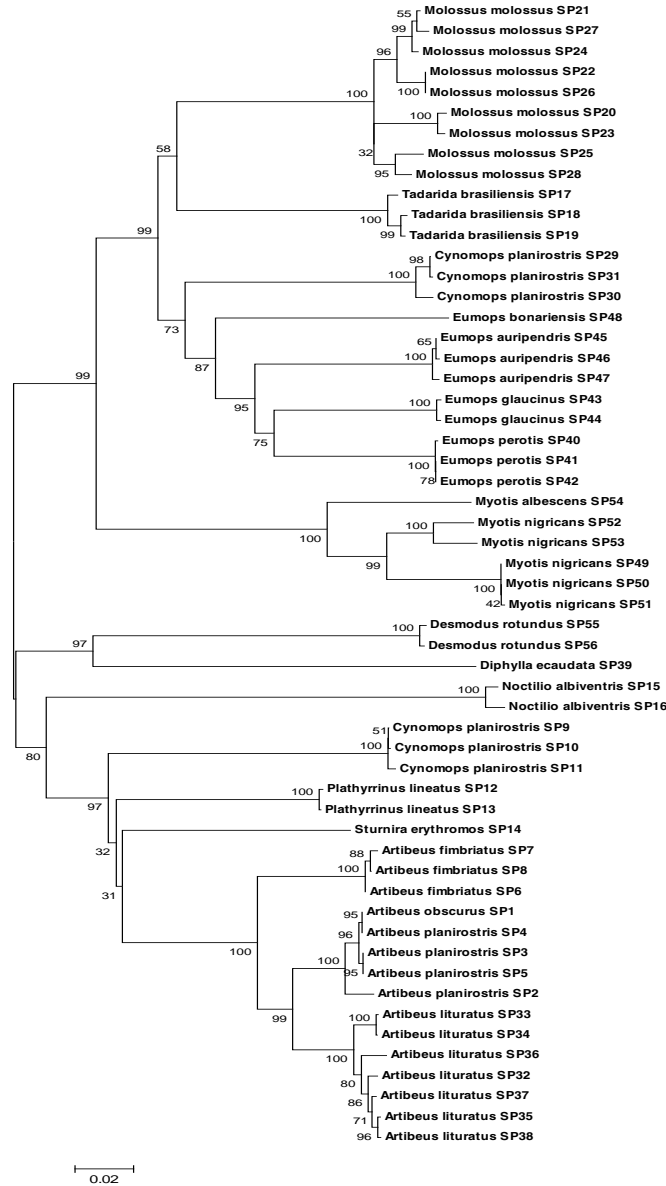| Sample	Specie	GenBank number
SP12	*Plathyrrinus lineatus*	KP134546
SP13	*Plathyrrinus lineatus*	KP134547
SP14	*Sturnira erythromos*	KP134548
SP15	*Noctilio albiventris*	KP134549
SP16	*Noctilio albiventris*	KP134550
SP17	*Tadarida brasiliensis*	KP134551
SP18	*Tadarida brasiliensis*	KP134552
SP19	*Tadarida brasiliensis*	KP134553
SP20	*Molossus molossus*	KP134554
SP21	*Molossus molossus*	KP134555
SP22	*Molossus molossus*	KP134556
SP23	*Molossus molossus*	KP134557
SP24	*Molossus molossus*	KP134558
SP25	*Molossus molossus*	KP134559
SP26	*Molossus molossus*	KP134560
SP27	*Molossus molossus*	KP134561
SP28	*Molossus molossus*	KP134562
SP29	*Cynomops planirostris*	KP134563
SP30	*Cynomops planirostris*	KP134564
SP31	*Cynomops planirostris*	KP134565
SP32	*Artibeus lituratus*	KP134566
SP33	*Artibeus lituratus*	KP134567
SP34	*Artibeus lituratus*	KP134568
SP35	*Artibeus lituratus*	KP134569
SP36	*Artibeus lituratus*	KP134570
SP37	*Artibeus lituratus*	KP134571
SP38	*Artibeus lituratus*	KP134572
SP39	*Diphylla ecaudata*	KP134573
SP40	*Eumops perotis*	KT626652
SP41	*Eumops perotis*	KT626653
SP42	*Eumops perotis*	KT626654
SP43	*Eumops glaucinus*	KP134574
SP44	*Eumops glaucinus*	KP134575
SP45	*Eumops auripendris*	KP134576
SP46	*Eumops auripendris*	KP134577
SP47	*Eumops auripendris*	KP134578
SP48	*Eumops bonariensis*	KP134579
SP49	*Myotis nigricans*	KP134580
SP50	*Myotis nigricans*	KP134581
SP51	*Myotis nigricans*	KP134582
SP52	*Myotis nigricans*	KP134583
SP53	*Myotis nigricans*	KP134584
SP54	*Myotis albescens*	KP134585
SP55	*Desmodus rotundus*	KP134586
SP56	*Desmodus rotundus*	KP134587

canids and bats. A recently published list of all bat species in Brazil in which the *rabies virus* (RABV) has been identified contains 41 species from 25 genera and three families[28]. RABV has also been identified in the bat *Artibeus obscurus* (Pasteur Institute of São Paulo, data not published), bringing this number to 42.

Because of its importance in Brazil and Latin America, the family Phyllostomidae accounted for the largest number of species analyzed here (47.37% of the 19 species analyzed). This family is very large and its members vary greatly in both their feeding habits and behavior. Species from the subfamilies Desmodontinae, Stenodermatinae and Carolliinae were analyzed; the first subfamily is made up of hematophagous bats while the others consist of fruit-eating bats that occasionally eat insects. Of the three species of hematophagous bats, two were

included in this study (*Desmodus rotundus* and *Diphylla ecaudata*). Of these three species, two feed off blood from birds and only *Desmodus rotundus*, the main species that transmits rabies in Latin America, feeds off the blood of mammals. *Desmodus rotundus* has also been found infected with *Venezuelan equine encephalitis virus*, from the family *Togaviridae* [23] and with a virus from the family *Coronaviridae* [24].

Fig. 1. Maximum-likelihood phylogenetic tree based on the 1140 nucleotides of the mtDNA cyt-b gene from the different bat species studied and bootstrap values, showing the clusters and subclusters of the respective families of bats. Samples are indicated by the initials of the species name in Table 2 with the respective sample number

Abbreviations: DR: Desmodus rotundus, DE: Diphylla ecaudata, AF: Artibeus fimbriatus, AL: Artibeus lituratus, AO: Artibeus obscurus, AP: Artibeus planirostris, PL: Plathyrrinus lineatus, SL: Sturnira lilium, CP: Carollia perspicillata, NA: Noctilio albiventris, TB: Tadarida brasiliensis, MM: Molossus molossus, CPL: Cynomops planirostris, EP: Eumops perotis, EG: Eumops glaucinus, EA: Eumops auripendulus, EB: Eumops bonariensis, MN: Myotis nigricans and MA: Myotis albescens

The subfamily Stenodermatinae includes bats from the genus *Artibeus*, which are fruit-eating bats with synanthropic habits. The following viruses have been identified in members of this subfamily: *Nepuyo virus*, from the family *Bunyaviridae*, and *Tacaribe virus*, from the *Arenaviridae* family, and *Mapuera virus*, from the family *Paramyxoviridae* [23] as well as RABV [28 and the Pasteur Institute of São Paulo, data not published]. *Agua Preta virus*, from the family *Herpesviridae*, and *Venezuelan equine encephalitis virus* have been identified in bats from the family *Carolliinae* [23,25]. These findings underscore the importance of the family *Phyllostomidae* as a reservoir for different viruses.

The family Noctilionidae is made up of only two species in the single genus *Noctilio*. One of these species (*Noctillio albiventris*) is insectivorous and the other (*Noctillio leporinus*) preferably piscivorous. Although this family is of little importance as a reservoir of diseases, two specimens of the species *N. albiventris* were included in this study.

The family Molossidae includes insectivorous bats that have evolved the ability to fly and maneuver at speed and whose capacity to adapt to different environments makes them the most common synanthropic bats. This family is the second most numerous in this study, accounting for 42.86% of the species analyzed. The genera from this family included in the study were *Tadarida*, *Molossus*, *Cynomops* and *Eumops*, in all of which the rabies virus has previously been identified [26]. The *Gossas virus*, also from the family *Rhabdoviridae*, and the *St. Louis encephalitis virus* and *Rio Bravo virus*, from the family *Flaviridae*, have been identified in bats from genus *Tadarida* [23], while the *Catu virus*, from the family *Bunyaviridae*, has been identified in bats from genus *Molossus* [23].

The family Vespertilionidae is the largest family of chiropterans in terms of both diversity and geographic distribution, 407 species belonging to 48 genera and 6 distinct subfamilies having been identified to date [27,7]. In Brazil, however, only the subfamilies Vespertioninae and Myotinae have been identified. This family was represented by two species from the genus *Myotis* (*Myotis nigricans* and *Myotis albescens*). In addition to RABV, the following viruses have also been identified in bats from the genus *Myotis*: *European bat lyssavirus* 2, *Aravan virus*,

Khujand virus and *Kern Canyon virus*, from the family *Rhabdoviridae*; *Montana myotis leucoencephalitis virus*, from the family *Flaviviridae*; *A Cytomegalovirus* and *Keterah virus* from the family *Herpesviridae* [23]; and other viruses from the genus *Gammaretroviru* [28] and *Alphacoronavirus* [29].

In a review of bats that act as viral reservoirs, Calisher et al. [23] reported that bats are frequently infected with viruses that cause diseases in humans and other mammals. Aguilar-Setien et al. [30] found *rabies virus* RNA in experimentally infected bats that did not develop clinical signs of the disease. These reports suggest the existence of unknown factors related to the immune system of bats and their ability to host and transmit different viruses.

The wide variety of EIDs, particularly viral ones, for which bats can act as reservoirs and/or hosts makes the need to study these animals all the more imperative. If the mechanism by which these viruses are maintained in nature and the role played by bats in the transmission and perpetuation of these pathogens is to be understood, correct identification of bats is fundamental. The mtDNA cyt-b gene was used in this study and allowed 19 species of bats in Brazil to be identified. The phylogenetic tree based on sequencing of this gene segregated the bats in the different families into clusters identical to those observed following morphometric identification and genetic identification described by Agnarsson et al. [31,32] and allowed identification to species level rather than just genus level.

5. CONCLUSION

In conclusion, our findings indicate that this genetic region is suitable for identification of bat species. Sequencing of the mtDNA cyt-b gene should therefore be used as an effective alternative to, or even in combination with, morphometric identification.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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