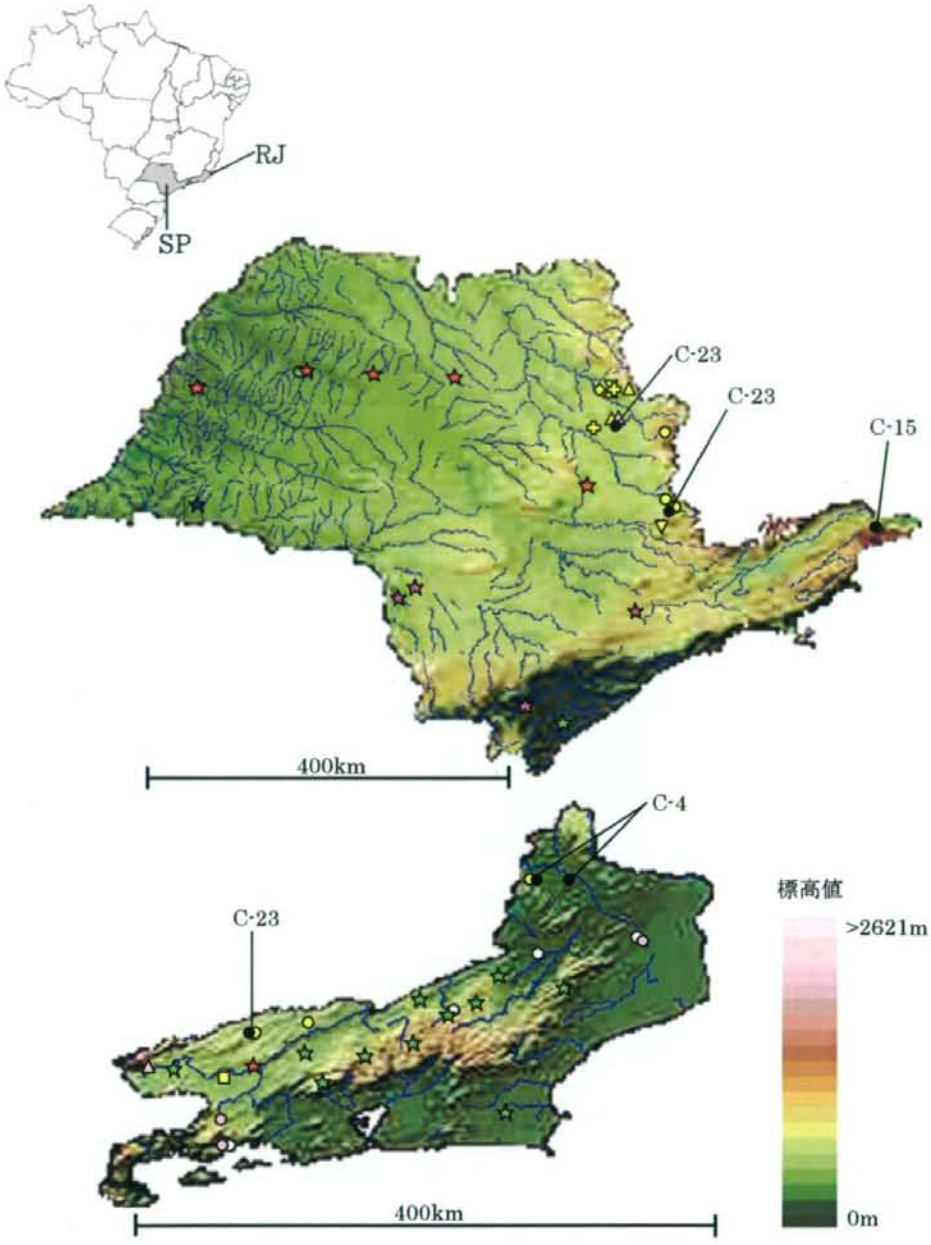


図6b　サン・パウロ州（SP）およびリオ・デ・ジャネイロ州（RJ）におけるウシおよび吸血コウモリ分離株の地理的分布
記号はFig. 3に対応し、黒丸は吸血コウモリ分離株の分離地域を示し、青いラインは川を示す。

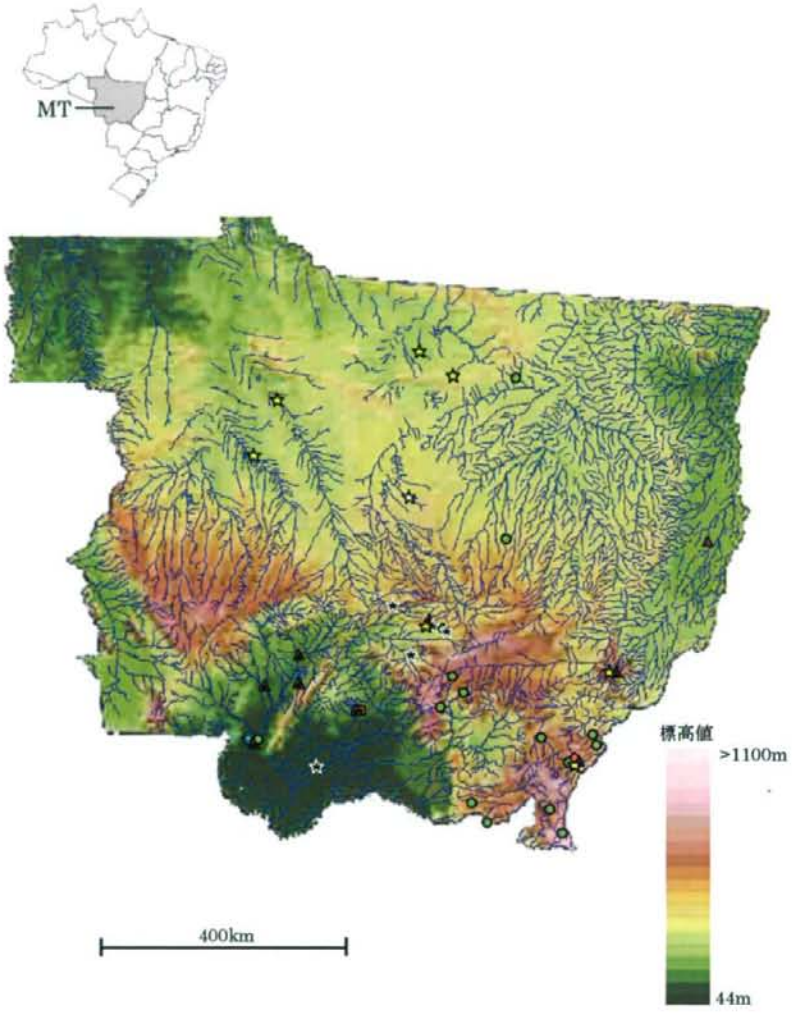


図６ｃ　マット・グロッソ州（MT）におけるウシ分離株の地理的分布
記号はFig.3に対応し、青いラインは川を示す。

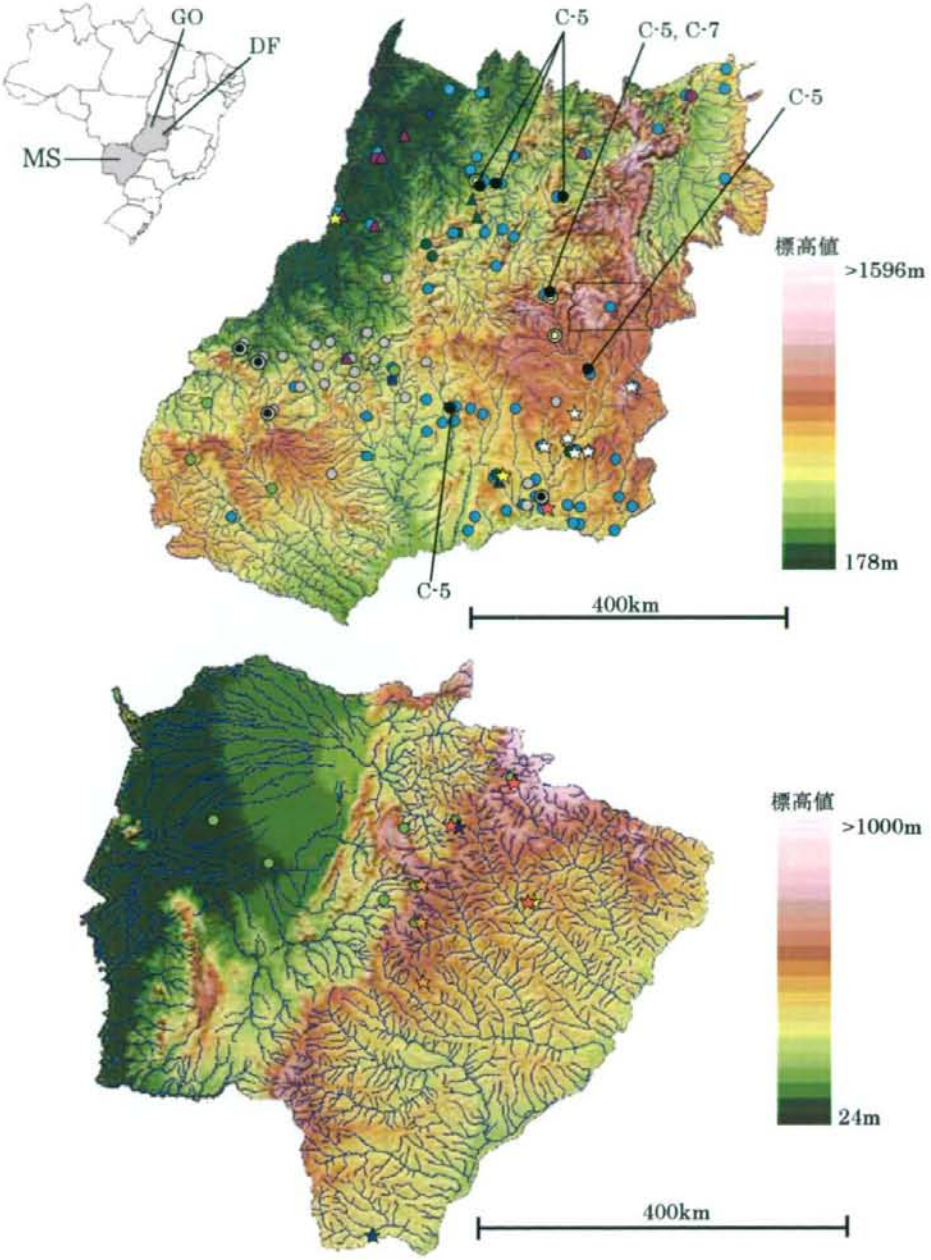


図6d　連邦自治区ブラジリア（DR）、ゴイアス州（GO）およびマット・グロッソ・ド・スール州（MS）におけるウシおよび吸血コウモリ分離株の地理的分布
記号はFig.3に対応し、黒丸は吸血コウモリ分離株の分離地域を、青いラインは川を示す。

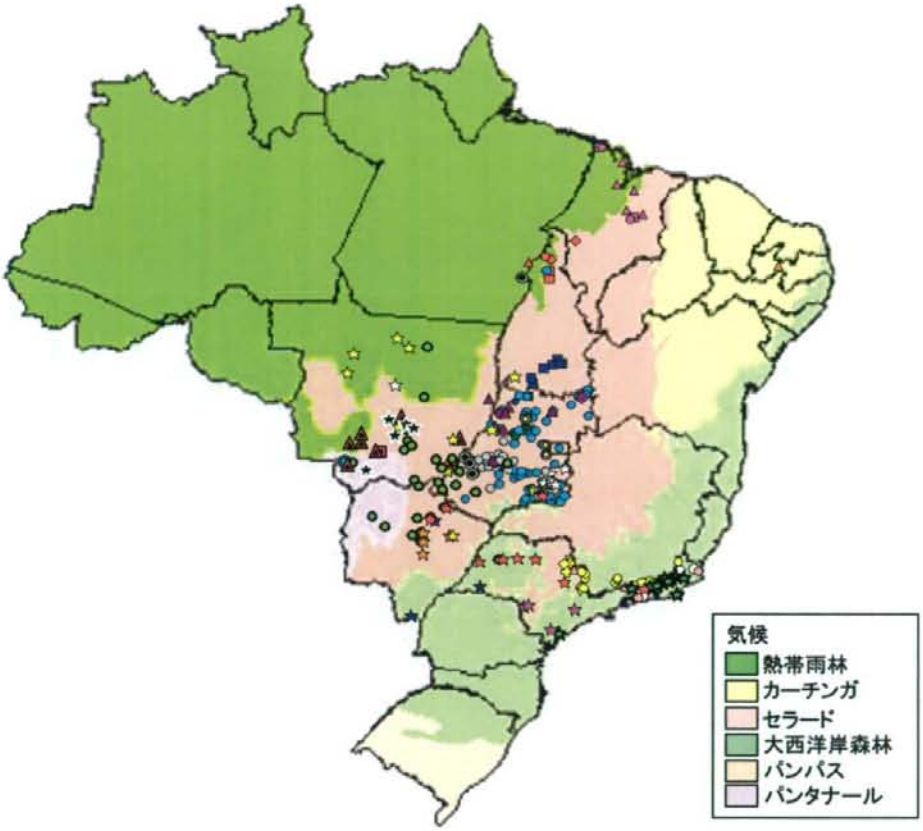


図７　気候要因による吸血コウモリ由来ウシ分離株の地理的分布
記号はFig. 3に対応している。

図８　ブラジル北東部における分離株の系統樹と分離地域

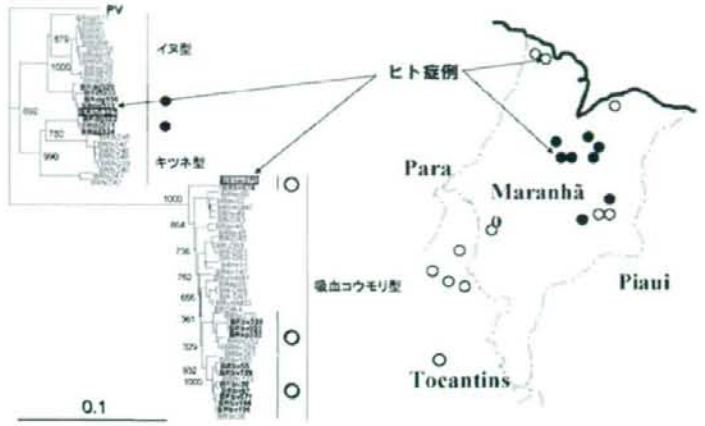


図９　ブラジル食肉目RVのN遺伝子領域に基づく系統樹

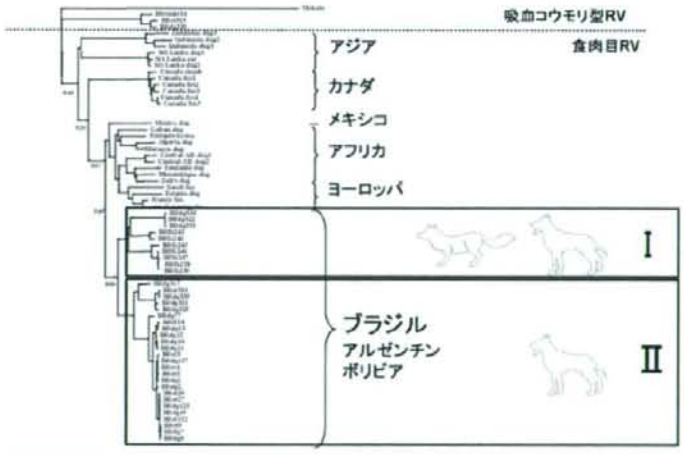
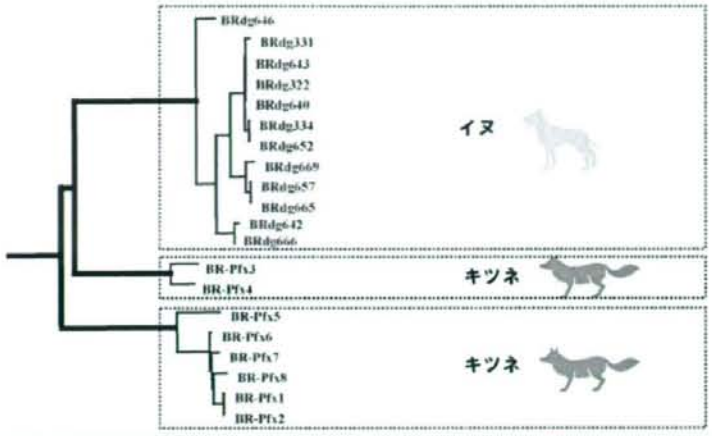


図１０　ブラジル食肉目RVの第Ｉ系統の系統樹



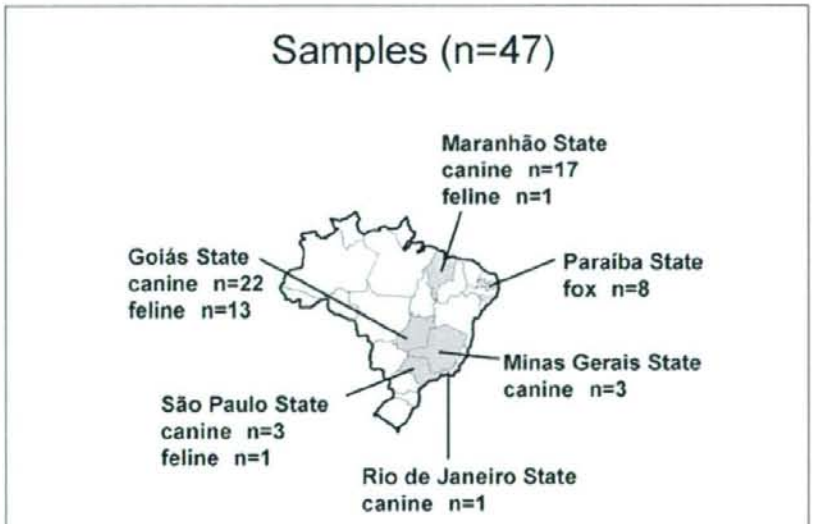


図１１

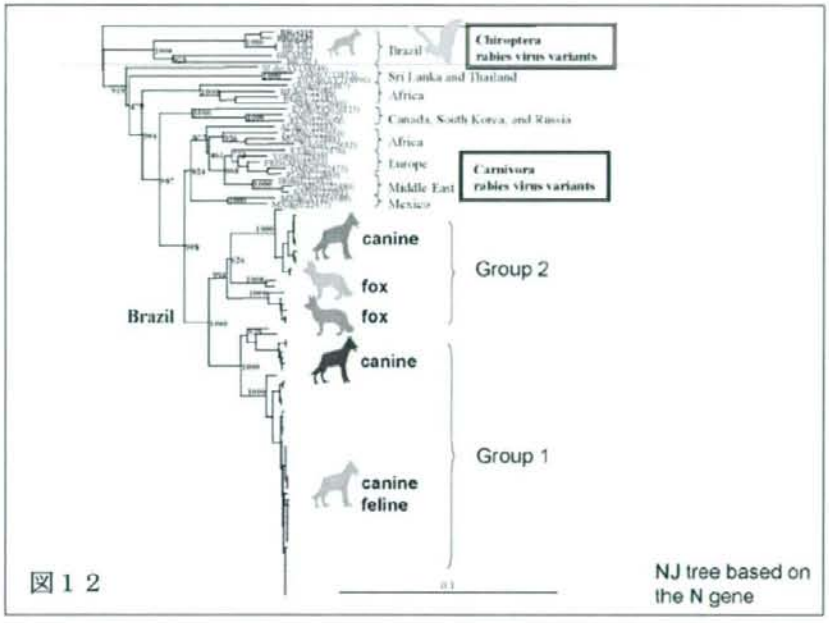

Brazil
Sri Lanka and Thailand
Africa
Canada, South Korea, and Russia
Africa
Europe
Middle East
Mexico
Chiroptera rabies virus variants
Carnivora rabies virus variants
canine
fox
fox
canine
canine feline
Group 2
Group 1
Brazil
0.1
NJ tree based on the N gene

図１２

Samples (n=56)

bat species	No.
Desmodus rotundus	20
Artibeus fimbriatus	1
A. lituratus	6
A. planirostris	1
Artibeus spp.	2
Eptesicus fulinaris	4
Molossus molossus	6
Nyctinomops laticaudatus	4
Tadarida laticaudata	2
Non-hematophagous bat	10

図１３

Bat rabies in Brazil

図１４

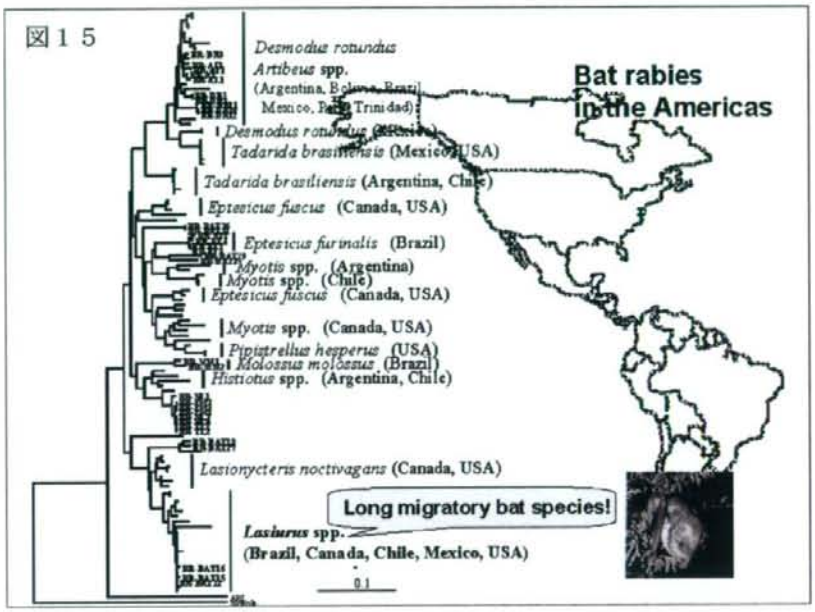
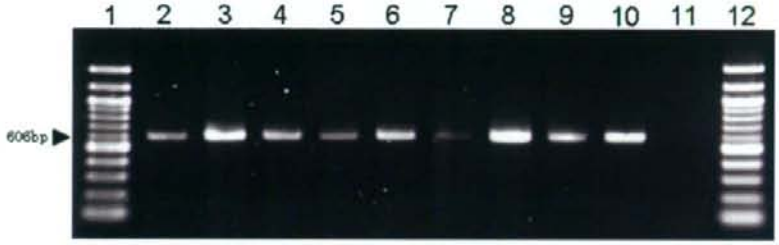


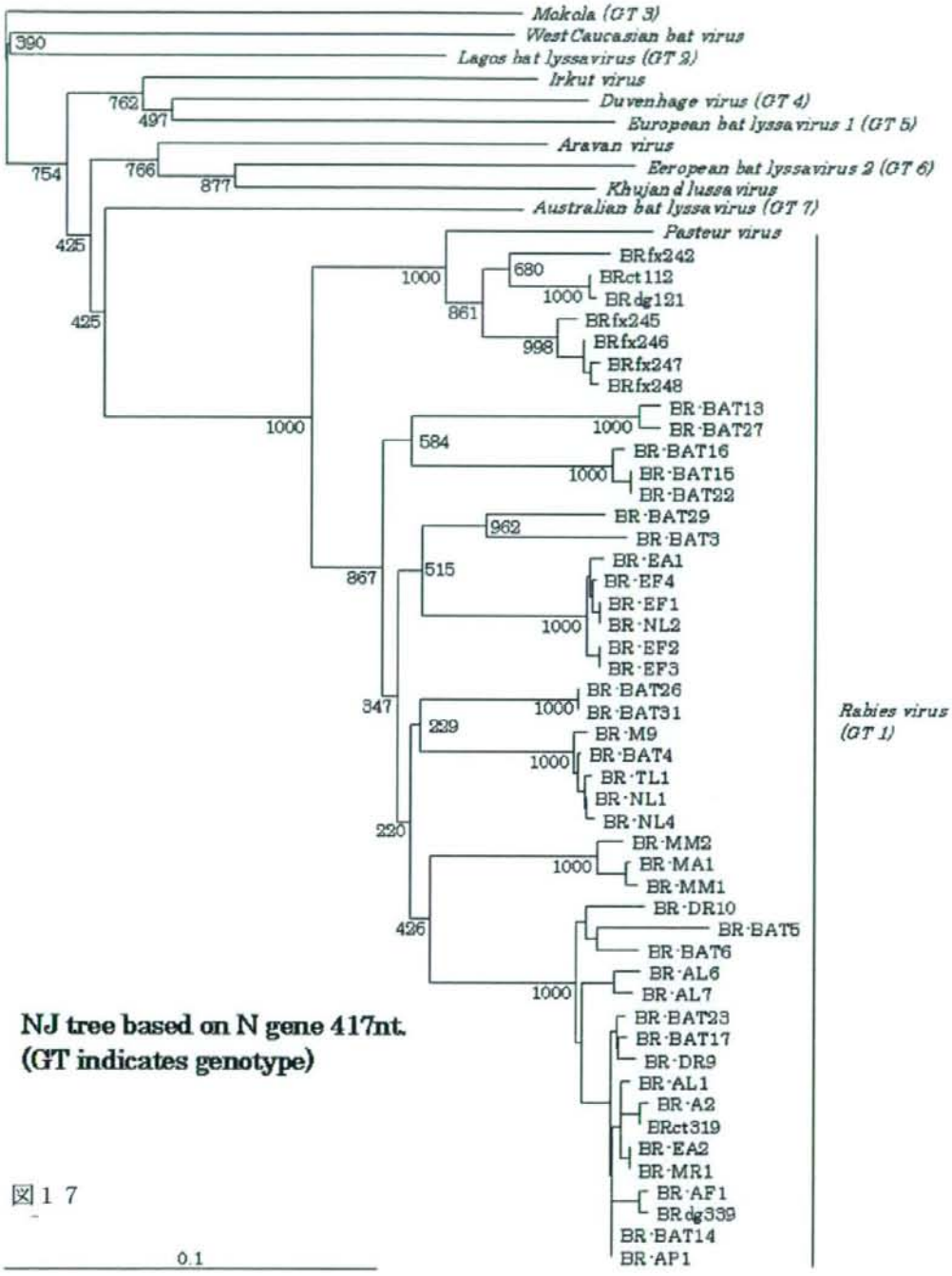
図１５
Desmodus rotundus
Artibeus spp.
(Argentina, Brazil, Mexico, Peru, Trinidad)
Desmodus rotundus (Chile)
Tadarida brasiliensis (Mexico, USA)
Tadarida brasiliensis (Argentina, Chile)
Eptesicus fuscus (Canada, USA)
Eptesicus furinalis (Brazil)
Myotis spp. (Argentina)
Myotis spp. (Chile)
Eptesicus fuscus (Canada, USA)
Myotis spp. (Canada, USA)
Pipistrellus hesperus (USA)
Molossus molossus (Brazil)
Histiotus spp. (Argentina, Chile)
Lasionycteris noctivagans (Canada, USA)
Lasiurus spp.
(Brazil, Canada, Chile, Mexico, USA)
0.1
Bat rabies in the Americas
Long migratory bat species!



RT-PCR using specific primers for detection of rabies virus (genotype 1) and other lyssa virus (genotype 2-7)

Lane 1, 12: 100bp DNA Ladder, Lane 2: BRfx248, Lane 3: BR-BAT27, Lane 4: BR-BAT15, Lane 5: BR-BAT29, Lane 6: BR-EF2, Lane 7: BR-BAT31, Lane 8: BR-NL1, Lane 9: BR-MM1, Lane 10: BR-AP1, Lane 11: negative control

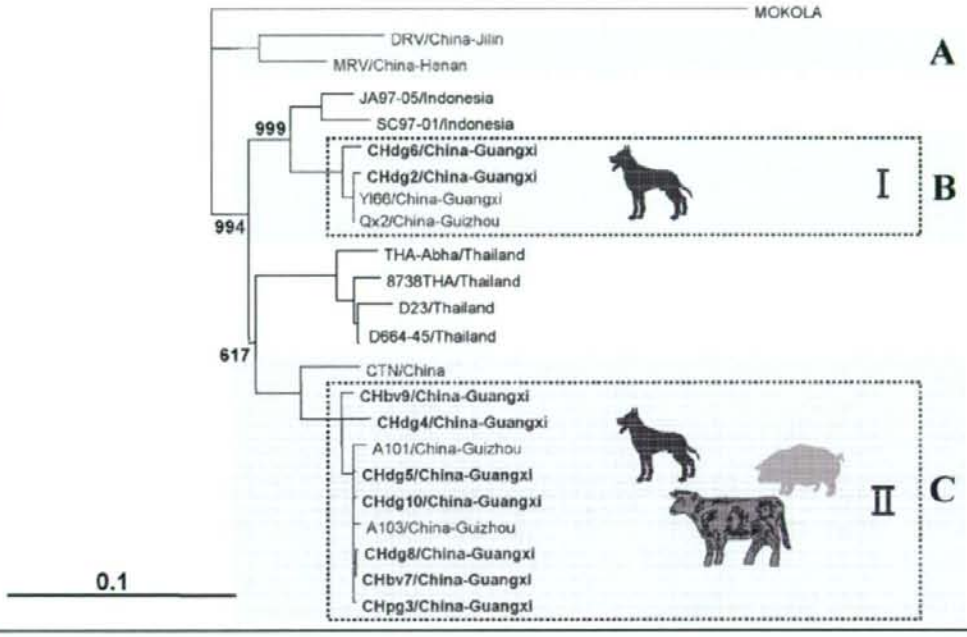
図１６



NJ tree based on N gene 417nt.
(GT indicates genotype)

図１７

図１８　中国広西自治区で分離されたRVの系統樹



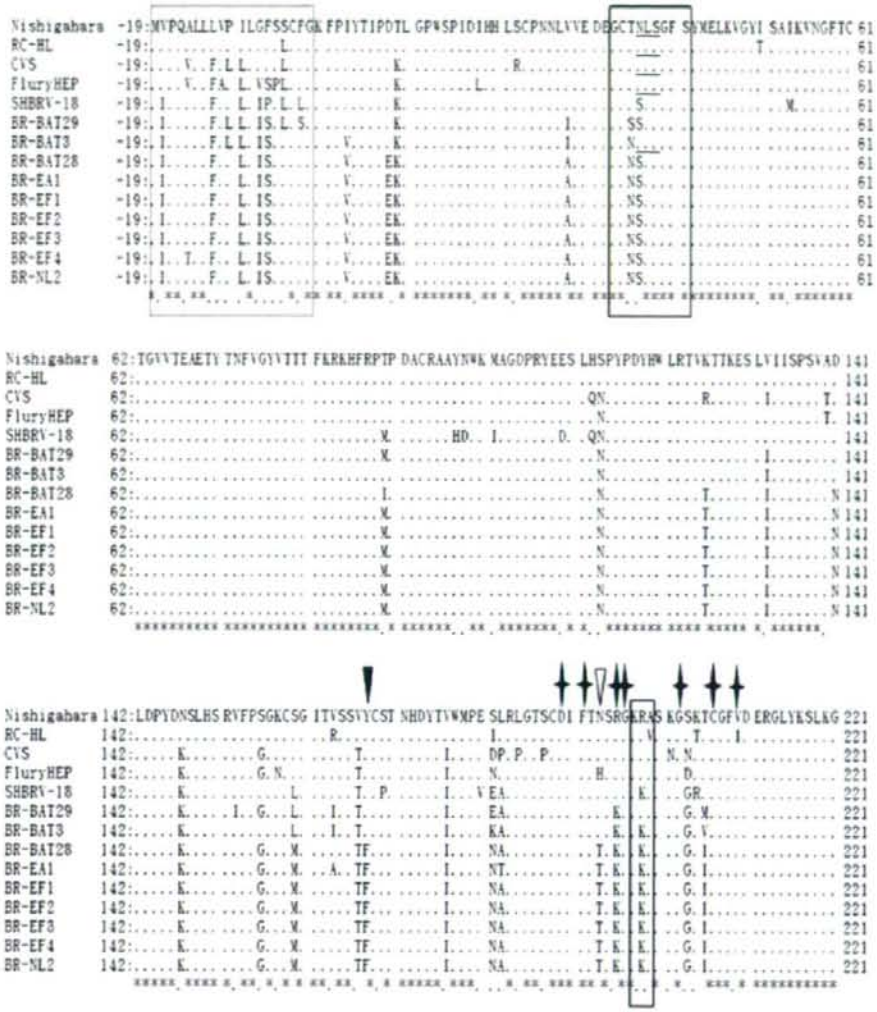


図１９a　G蛋白アミノ酸配列のマルチプルアラインメント 点線ボックスは（-19 to 0 and 440-461）シグナルペプチドおよび膜貫通ドメインを示す。ホックスは以下の順に抗原サイトを示す；抗原サイト II（34-42 および 198-200），抗原サイトI（231），VI（264），III（330-338），and “a”（342）．N-結合型グリコシレーションサイトは下線で示されている。333位は太字、▼は168F変異、▽は194T変異、★は蛇毒神経毒結合アセチルコリン受容体に類似のアミノ酸残基を示す。

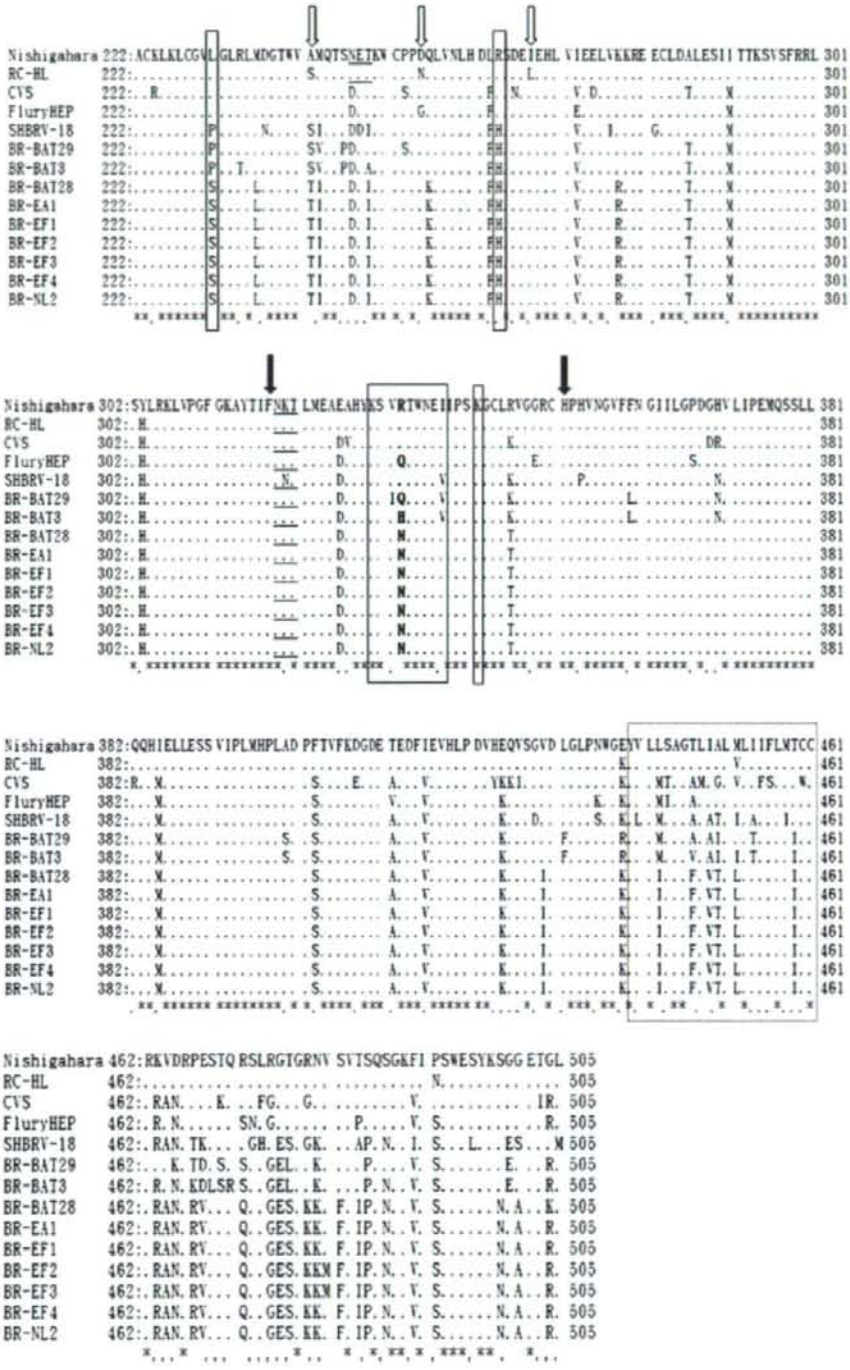


図１９b　G蛋白アミノ酸配列のマルチプルアラインメント 点線ボックス(440-461)は膜貫通ドメインを示す。ホックスは以下の順に抗原サイトを示す；抗原サイトI (231), VI (264), III (330-338), and “a” (342). N-結合型グリコシレーションサイトは下線、333位は太字、空矢印は西ヶ原株の病原性に重要な242, 255および268位を、黒矢印はp75NTRニューロトフィン受容体への結合に重要な318Fおよび352Hを示す。

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shoji Y, Kobayashi Y, Sato G, Gomes AA, Itou T, Ito FH, Sakai T.	Genetic and phylogenetic characterization of rabies virus isolates from wildlife and livestock in Paraiba, Brazil.	Acta Virology	50(1)	33-37	2006
Sato G, Kobayashi Y, Shoji Y, Sato T, Itou T, Ito FH, Santos HP, Brito CJ, Sakai T.	Molecular epidemiology of rabies from Maranhao and surrounding states in the northeastern region of Brazil.	Archives of Virology	151(11)	2243-2251	2006
Kobayashi Y, Ogawa A, Sato G, Sato T, Itou T, Samara SI, Carvalho AA, Nociti DP, Ito FH, Sakai T.	Geographical distribution of vampire bat-related cattle rabies in Brazil.	Journal of Veterinary Medical Sciences	68(10)	1097-1100	2006

Kobayashi Y, Inoue N, Sato G, Itou T, Santos HP, Brito CJ, Gomes AA, Santos MF, Silva MV, Mota CS, Ito FH, Sakai T	Phylogenetic characterization of rabies virus isolates from Carnivora in Brazil.	Journal of Veterinary Medical Sciences	69	691-696	2007
Kobayashi Y, Sato G, Kato M, Itou T, Cunha EM, Silva MV, Mota CS, Ito FH, Sakai T	Genetic diversity of bat rabies viruses in Brazil.	Archives of Virology	152	1995-2004	2007
Kobayashi Y, Okuda H, Nakamura K, Sato G, Itou T, Carvalho AA, Silva MV, Mota CS, Ito FH, Sakai T	Genetic analysis of phosphoprotein and matrix protein of rabies viruses isolated in Brazil.	Journal of Veterinary Medical Sciences	69	1145-1154	2007
Kobayashi Y, Sato G, Mochizuki N, Hirano S, Itou T, Carvalho AA, Albas A, Santos HP, Ito FH, Sakai T.	Molecular and geographic analyses of vampire bat-transmitted cattle rabies in Central Brazil.	BMC Veterinary Research	4	44	2008
Sato G, Kobayashi Y, Motizuki N, Hirano S, Itou T, Cunha EM, Ito FH, Sakai T.	A unique substitution at position 333 on the glycoprotein of rabies virus street strains isolated from non-hematophagous bats in Brazil.	Virus Genes	38	74-79	2009

GENETIC AND PHYLOGENETIC CHARACTERIZATION OF RABIES VIRUS ISOLATES FROM WILDLIFE AND LIVESTOCK IN PARAIBA, BRAZIL

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Received April 25, 2005; accepted December 20, 2005

Summary. – Thirty-four rabies virus (RV) isolates from foxes (8), insectivore bats (9), cattle (14), sheep (1), a goat (1) and a donkey (1) from Paraiba state, northeastern Brazil, were genetically characterized. Sequences of 890 nts of nucleoprotein (N) genes of these isolates were analyzed and compared with those of other Brazilian isolates characterized earlier. Phylogenetic analysis revealed three genetical lineages of RV co-existing in this region. Each lineage was found to be associated with particular host species and to circulate independently of each other. The first lineage was found in foxes (*Dusicyon* sp.) and could be discriminated from domestic carnivore isolates from Sao Paulo, Goias and Minas Gerais in the southern and central Brazil. The second lineage was associated with insectivorous bats (*Molossus* spp.) and differed from vampire bat-associated RV isolates. The third lineage was found in livestock and clustered with vampire bat-associated RV isolates from Sao Paulo, Tocantins, Goias and Matto Grosso. These results indicate that RV of these genetic lineages are co-circulating in the Paraiba state and that livestock in this region are infected with vampire bat-associated RV, suggesting that the vampire bat is the main reservoir of livestock rabies in this region.

Key words: Brazil; domestic animals; epidemiology; phylogenetic analysis; Rabies virus; wild animals

Introduction

Rabies is an enzootic disease caused by RV, a neurotropic virus of the genus *Lyssavirus*. Lyssaviruses (members of the *Lyssavirus* genus) are divided in 7 genotypes (GTs), among them GT1 (RV), GT3 (Mokola virus, MOKV) and GT7 (Australian bat lyssavirus, ABLV) (Fauquet *et al.*, 2005).

RV infects almost all kinds of mammalian species. RV infects the central nervous system, causing acute encephalitis, which is almost incurable after the clinical symptoms appear. However, there is a possibility of prevention of this disease by effective vaccine even when administered after exposure to the virus.

In Brazil, like most other South American countries, rabies occurs in two different epidemiological forms, sylvatic and urban. The main reservoir of urban rabies is the dogs, while that of sylvatic rabies is the vampire bat (Ito *et al.*, 2001a). In large cities of southern Brazil like Rio de Janeiro and Sao Paulo, dog rabies has been controlled by effective dog vaccination campaigns (Romijn *et al.*, 2003; Heinemann *et al.*, 2002). However, in rural areas, the dog remains the main reservoir of human rabies, and the rabies cases of humans and livestock contracted from wild animals, such as vampire bats (*Desmodus rotundus*) (Schneider *et al.*, 1996; Romjin *et al.*, 2003), marmosets (Favoretto *et al.*, 2001), non-human primates (Ramos *et al.*, 2002) and foxes have been reported as increasing (Almeida *et al.*, 2001), especially in the northern Brazil. In the Ceara state, the northeastern

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Abbreviations: ABLV = Australian bat lyssavirus; GT = genotype; MOKV = Mokola virus; N = nucleoprotein; RV = Rabies virus

Brazil, 13 human rabies cases originated from wild animals, such as bats, crab-eating raccoons (*Procyon cancrivorous*) and white-tufted-ear marmosets (*Calithrix jacchus jacchus*) during the past 7 years (1991–1998) (Favoretto *et al.*, 2001). The latter study revealed that these viruses represent a unique and independent rabies endemic cycle. In some other states surrounding Paraiba state, outbreaks of rabies in humans transmitted by vampire bats (Bahia, Amazonas and Para states) (Goncalves *et al.*, 2002; Schneider *et al.*, 2001; Pan American Health Organization, 2004) also occurred.

Although rabies cases in humans and livestock in the Paraiba state have occurred, there is so far no report on molecular analysis of the respective RV isolates. The aim of this study was to characterize genetically RV circulating in this area and to obtain epidemiological information for prediction and prevention of rabies outbreaks in both humans and livestock in this region.

Materials and Methods

Viruses. Thirty-four RV isolates obtained from brain specimens from foxes (8), cattle (14), insectivorous bats (9), sheep (1), a goat (1) and a donkey (1), collected in the Paraiba state, the northeastern Brazil, were examined.

RT-PCR. Total RNA was extracted from brain specimens using a QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacturer's instructions. To detect RV RNA in general, a RT-PCR specific for RV N gene (a sequence of 964 nts) was performed using the primer pair P1/P2. To detect carnivore-associated RV RNA in particular, the primer pair RHN1/RHNS3 yielding a product of 1,512 nts was used (Ito *et al.*, 1999, 2003).

Sequencing. The RT-PCR products of 964 nts were purified using a QIAquick PCR purification Kit (QIAGEN) and subjected to direct sequencing using the primers P1, P2, BRABN-S1, BRABN-S3 and BRABN-C3 (Shoji *et al.*, 2004), a Big Dye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems) and an automated sequencer (Applied Biosystems).

Phylogenetic analysis of RV isolates was based on the alignment of 890 nts-long sequences of N gene (nt 89–978) (Tordo *et al.*, 1986) using the CLUSTAL X program (Thompson *et al.*, 1994). A neighbor-joining tree was constructed using a method of Saitou and Nei (1987) and the bootstrap probabilities of each node were calculated using 1,000 replicates. A graphic output was obtained using the TREEVIEW program (Page *et al.*, 1996).

Results

Detection of RV RNA by RT-PCR

All the 34 RV isolates were positive by the RT-PCR with the primer pair P1/P2, while only 8 of them, originating exclusively from fox brains, were positive by the RT-PCR with the primer pair RHN1/RHNS3 (Fig. 1); the latter isolates thus represented carnivore-associated RV.

Sequencing and phylogenetic analysis of RV isolates

The sequences of 890 nt-long RT-PCR products (nt 89–978) corresponding to RV N gene were determined for all the 34 RV isolates and were deposited in the Gen Bank database (Acc. Nos. AB206407-AB206439 and AB207884).

The nucleotide sequence identities of all the isolates with RV (GT1), MOKV (GT3) and ABLV (GT7) were 81.7–99.4%, 72.2–72.7% and 75.9–78.9%), respectively, showing that these isolates belong to GT 1 of lyssaviruses. The nucleotide and deduced amino acid sequences exhibited different levels of identity with each other, 82.5–100% and 92.3–100%, respectively. Among all the fox isolates, the nucleotide sequences showed a 94.6–100% identity. The latter also showed the highest identity (93.2–94.6%) with those of domestic carnivore isolates from Sao Paulo, Minas Gerais and Goias in the southern and central Brazil. However, these levels of identity were lower than those (98.9–99.1%) within domestic carnivore isolates except for fox isolates.

The nucleotide sequence identity within the insectivorous bat isolates from Paraiba was 97.8–99.7%, while the nucleotide and deduced amino acid sequences identities of these isolates with other isolates described in this study were relatively low, 82.1–89.5% and 92.9–97.3%, respectively. The nucleotide sequence identity within the livestock isolates showed 98.2–100%. The nucleotide and amino acid identities of the livestock isolates from Paraiba with the isolates from vampire bats and livestock from other regions in Brazil (Goias, Mato Grosso, Sao Paulo and Tocantins) were the highest, 96.1–98.2% and 97.6–100%, respectively.

Multiple alignment of deduced amino acid sequences (298 aa) of representative RV isolates revealed 19 amino acid substitutions. Several of them appeared to be lineage-specific. Five substitutions were found in carnivore or bat isolates. Another seven substitutions resulted in an amino acid with changed chemical properties. Seven substitutions were found in insectivorous bat isolates (data not shown).

The phylogenetic analysis based on the sequences of 890 nts of N gene revealed that 55 isolates analyzed in this study clustered into four major genetic lineages: (A) the lineage of domestic carnivore isolates from southern and central Brazil; (B) the lineage of fox isolates from Paraiba; (C) the lineage of insectivorous bat isolates from Paraiba and Sao Paulo; (D) the lineage of livestock isolates from Paraiba and vampire bat and livestock isolates from southern and central Brazil (Fig. 2a). Insectivorous bat isolates from Paraiba together with BRNI-1 constituted a lineage that differed from other insectivorous bat isolates from Sao Paulo (BREF-4 and BRMA-1). Fox isolates from Paraiba formed two discrete sub-lineages (B-1 and B-2) and each sub-lineage

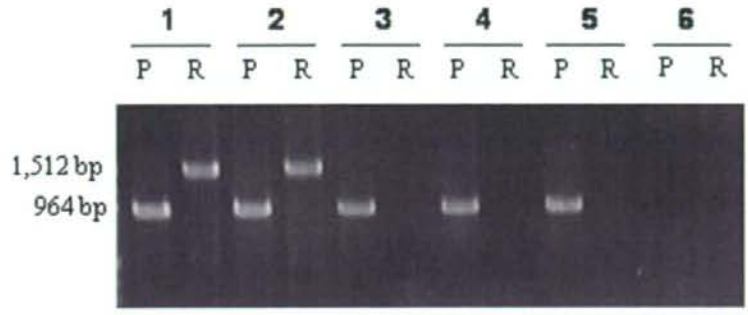

Fig. 1

Discrimination of carnivore-associated and non-carnivore-associated RV isolates by RT-PCR

Positive controls: BRdg 2 (Brazilian carnivore-associated RV, lanes 1) and BRvmbt 33 (Brazilian non-carnivore RV, lanes 3). Brazilian RV isolates BR-Pfx1 (lanes 2), BR-Pbt 1 (lanes 4) and BR-Pbv 1 (lanes 5). Negative control: RT-PCR without RNA template (lanes 6). P and R indicate P1/P2 and RHN1/RHNS3 primer pairs, respectively.

was distinguishable from domestic carnivore isolates from southern and central Brazil. Although the lineages A and B were categorized in carnivore rabies, they did not contain carnivore isolates from other countries (Fig. 2b). However, the lineages C and D were assigned to Chiropteran rabies together with other bat isolates. The insectivorous bat lineage C has closer relationship with North American and other Brazilian insectivorous bat isolates than the vampire bat lineage.

Discussion

In this study, viral RNA was detected by RT-PCR in 34 brain specimens from wild animals (foxes and insectivorous bats), livestock (cattle, sheep, a goat and a donkey) from Paraiba, northeastern Brazil. N gene sequences of these isolates and their phylogenetic analysis revealed that three genetic lineages of RV associated with specific host species occur in this area, indicating the presence of multiple endemic cycles of rabies. The fox RV lineage from Paraiba was found to belong to the group of Brazilian carnivore RV isolates but it apparently differed from carnivore RV lineage from southern and central Brazil. The insectivorous bat (*Molossus* sp.)-RV lineage of Paraiba clustered with an isolate from another species of insectivorous bat (*Nictinomops laticaudatus*; BRNL-1) and could be differentiated from other Brazilian RV isolates. Furthermore, the livestock RV lineage from Paraiba was found to belong to Brazilian vampire bat-associated RV lineages, suggesting that the main reservoir of livestock rabies in Paraiba is vampire bats though we could not isolate RV from vampire bats captured in Paraiba. The high bootstrap values of the node of each lineage in the phylogenetic tree indicate that these three RV lineages have been circulating independently of each other in this region.

Considering the population of the fox *Dusysion* sp. in Paraiba and the fact that some residents keep foxes as pets, this species has a potential for transmission of RV to humans.

Vampire bats are responsible for most cases of livestock rabies and some outbreaks of human rabies. Fortunately, the human rabies cases transmitted by insectivorous bats have not been reported recently in Brazil, but the growing incidence of humans bitten by bats including insectivorous bats in Brazil (Mayen *et al.*, 2003) and the growing incidence of human rabies transmitted by insectivorous bats in North America (Messenger *et al.*, 2002; Pape *et al.*, 1999) suggest the importance of insectivorous bats in the epidemiology of rabies in Brazil.

In accord with problem of grouping of RV isolates worldwide, Brazilian RV isolates have been divided in two major phylogenetical groups, the carnivore RV group and the Chiropteran RV group (Tordo *et al.*, 1986). The Brazialian RV isolates from foxes, a dog and a cat belong to the carnivore RV group, while the bat and livestock isolates belong to the Chiropteran RV group. In our phylogenetic analysis, the fox, dog and cat, and insect bat RV lineages formed separate clusters without any isolates from other countries, suggesting that each lineage is circulating in Brazil only. On the other hand, the vampire bat RV lineage clustered with vampire bat isolates from other South American countries.

Brazil is so much diverse in geomorphology and in mammalian fauna including bats (Findley, 1993; Walker, 2001) that it is difficult to completely comprehend or elucidate the

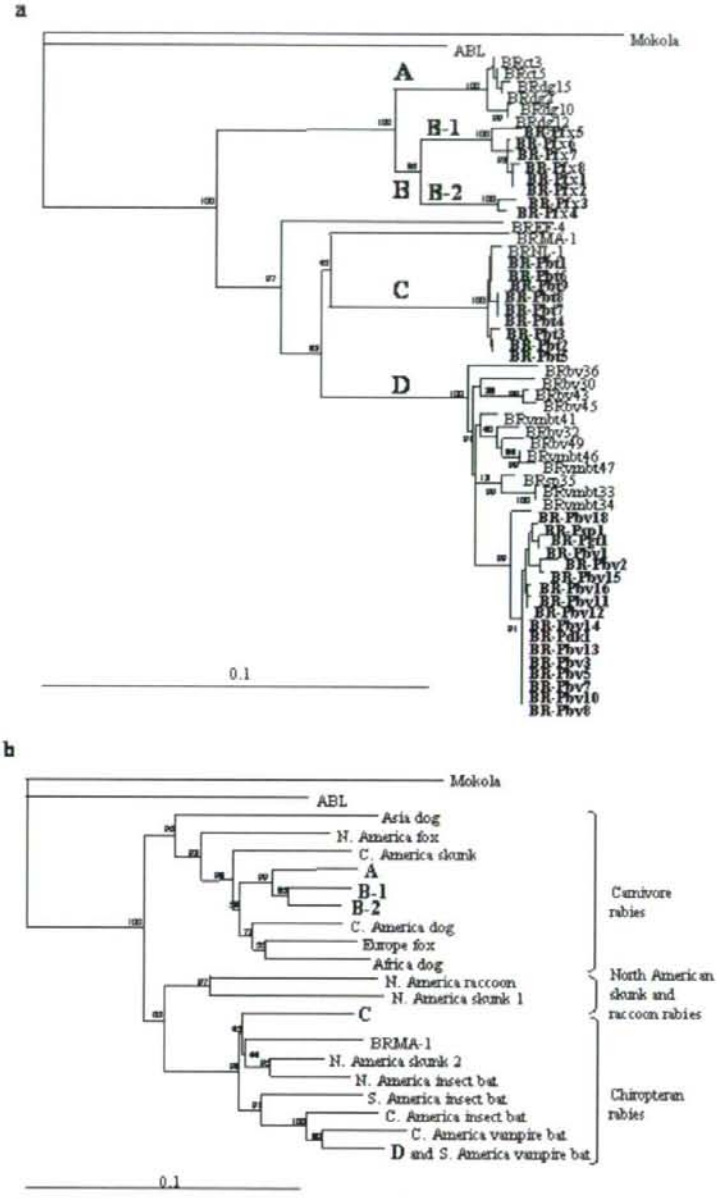

Fig. 2

Phylogenetic analyses of RV isolates based on 890 nt-long sequences of N gene

(a) Phylogenetic tree of Brazilian RV isolates. (b) Phylogenetic tree of representative RV isolates from Brazil and other countries. MOKV (Mokola) and ABLV (ABL) (Acc. Nos. Y09762 and AF418014, respectively) were used as outgroups. Isolates from Paraiba are in bold. A, B (B-1 and B-2), C and D represent individual lineages (sublineages). Percentage bootstrap values out of 1,000 replicates are indicated at each respective node.