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Genetic Analysis of Phosphoprotein and Matrix Protein of Rabies Viruses Isolated in Brazil

Yuki KOBAYASHI[1)], Hiromi OKUDA[1)], Kana NAKAMURA[1)], Go SATO[1)], Takuya ITOU[1)], Adolorata A. B. CARVALHO[2)], Marlon V. SILVA[3)], Carla S. MOTA[4)], Fumio H. ITO[4)] and Takeo SAKAI[1)]*

[1)]*Nihon University Veterinary Research Center, 1866 Kameino, Fujisawa,Kanagawa 252–8510, Japan,*
[2)]*Faculty of Agriculture and Veterinary Science, UNESP, Via de Acesso Prof. Paulo Donato Castellane, Jaboticabal, São Paulo 14884–900,*
[3)]*Jorge Vaitsmann Municipal Institute, Av. Bartolomeu de Gusmão, 1120, São Cristóvão, Rio de Janeiro and*
[4)]*Faculty of Veterinary Medicine and Zootechny, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, Cidade Universtiátria, São Paulo 05508–000, Brazil*

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ABSTRACT. To investigate the genetic characteristics of phosphoprotein (P) and matrix protein (M) genes of variable rabies virus (RV) prevalent in Brazil, the authors genetically characterized the P and M genes from 30 Brazilian RV field isolates. Phylogenetic analysis based on the P and M genes revealed the presence of six RV variants that consisted primarily of three insectivorous bats, the vampire bat, dog and fox in Brazil. Specific amino acid substitutions corresponding to these phylogenetic lineages were observed, with Asp_{42} and Glu_{62} in the P protein found to be characteristic of Brazilian chiroptera- and carnivora-related RVs, respectively. Amino acid sequence motifs predicted to associate with a viral function in the P and M proteins were conserved among Brazilian RV variants.
KEY WORDS: Brazil, genetic analysis, matrix protein, phosphoprotein, rabies virus.

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Rabies virus (RV) is a member of the *Lyssavirus* genus, which belongs to the *Rhabdoviridae* family. Lyssavirus is characterized as having seven genotypes (GTs) comprising, rabies virus (GT 1), Lagos bat virus (GT 2), Mokola virus (GT 3), Duvenhage virus (GT 4), European bat lyssavirus type (EBL) 1 (GT 5), EBL 2 (GT 6) and Australian bat lyssavirus (GT 7). Lyssaviruses have approximately 12-kb of unsegmented negative-stranded genomic RNA for encoding the genes of the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L).

RV has an almost global distribution and infects a wide range of mammalian species in which it causes a lethal form of encephalopathy. In Brazil, the principal RV transmitters to humans and domestic animals - dogs and vampire bats - have caused serious problems in the public health sector and the livestock industry [6, 19]. In addition, RVs have been isolated from other animal species [8]. Previously, phylogenetic analyses targeting the N and G genes revealed that there were several RV variants which varied depending on the host species, which included vampire bats, insectivorous bats, dogs and foxes. Furthermore, RV isolates from cattle and frugivorous bats (*Artibeus* (*A.*) sp.) have frequently been typed as vampire bat-related RVs in Brazil [19, 25, 26, 41, 42, 44, 45]. Variability has also been reported in the G protein, a major contributor to the pathogenicity of the virus, in which several amino acid substitutions at antigenic sites associated with the pathogenicity and immunogenicity of the virus have been identified in Brazilian RV variants [41]. However, differences in the pathogenicity and antigenic characteristics of these Brazilian RV variants are not yet known. Recently, in addition to the G protein, both P and M proteins have also been reported to be associated with the pathogenicity of the virus [43]. The P protein forms a ribonucleoprotein (RNP) with N and L proteins, which then wraps around the viral RNA, and plays an important role in transcription and replication in conjunction with the L protein [3, 5, 11]. In addition, the P protein acts to counteract the host's interferon (IFN) responses in infected cells [1, 2]. The M protein is responsible for recruiting RNPs to the cell membrane, as well as their condensation into tightly coiled 'skeleton'-like structures [36]. In addition, the M protein is involved in viral assembly and budding, and is associated with regulating the balance between viral transcription and replication [9, 10, 20]. Furthermore, the M protein acts as a major inducer of apoptosis in neuronal cells [23].

However, although P and M proteins have been demonstrated to have several important functions in viral infection, these investigations have all been conducted using laboratory-adapted strains. In addition, few studies on P and M proteins in wild-type RV strains have been reported to date [30, 31, 32, 33], and no reports have genetically characterized the P and M genes in Brazilian RV isolates. In this study, we conducted genetic analyses of P and M genes of several RV field isolates prevalent in Brazil.

MATERIALS AND METHODS

Viruses: Thirty RV isolates were collected from brain specimens of frugivorous bats (*A. lituratus, A. planirostris*), insectivorous bats (*Eptesicus (E.) furinalis, Molossus (M.) molossus, Nyctinomops (N.) laticaudatus*), vampire bats (*Desmodus (D.) rotundus*), cattle, cats, dogs, and foxes

* CORRESPONDENCE TO: Prof. SAKAI, T., Nihon University Veterinary Research Center, 1866 Kameino, Fujisawa, Kanagawa 252–8510, Japan.
e-mail: sakai@brs.nihon-u.ac.jp

Table 1. Brazilian rabies virus isolates used in this study

Isolate No.	Species of origin	Geographic origin		Year of isolation	Accession No.	
		City	State		P gene	M gene
BR-AL1	*Artibeus lituratus*	Itapira	São Paulo	1998	AB291108	AB291078
BR-AL2	*Artibeus lituratus*	São José do Rio Preto	São Paulo	1998	AB291109	AB291079
BR-AL3	*Artibeus lituratus*	Novo Horizonte	São Paulo	1998	AB291110	AB291080
BR-AP1	*Artibeus planirostris*	São José do Rio Preto	São Paulo	1998	AB291111	AB291081
BRbv303	Cattle	Casimire de Abrue	Rio de Janeiro	2002	AB291112	AB291082
BRbv306	Cattle	Vassouras	Rio de Janeiro	2004	AB291113	AB291083
BRct112	Cat	Morrinhos	Goiás	1999	AB291114	AB291084
BRct116	Cat	Itaberaí	Goiás	1999	AB291115	AB291085
BRct333	Cat	Gonçarves Dias	Maranhão	2003	AB291116	AB291086
BRdg96	Dog	Itaguaçu	Goiás	2001	AB291120	AB291090
BRdg97	Dog	Itaguaçu	Goiás	2001	AB291121	AB291091
BRdg101	Dog	Sta Tereza	Goiás	2001	AB291117	AB291087
BRdg317	Dog	Rio de Janeiro	Rio de Janeiro	1985	AB291118	AB291088
BRdg335	Dog	Barra do Corda	Maranhão	2003	AB291119	AB291089
BR-DR1	*Desmodus rotundus*	Lindóia	São Paulo	2000	AB291122	AB291092
BR-DR2	*Desmodus rotundus*	Lindóia	São Paulo	2000	AB291123	AB291093
BR-DR3	*Desmodus rotundus*	São José do Barreiro	São Paulo	2001	AB291124	AB291094
BR-EF1	*Eptesicus furinalis*	São José do Rio Preto	São Paulo	1998	AB291125	AB291095
BR-EF2	*Eptesicus furinalis*	Olímpia	São Paulo	2001	AB291126	AB291096
BR-EF3	*Eptesicus furinalis*	São José do Rio Preto	São Paulo	2001	AB291127	AB291097
BR-EF4	*Eptesicus furinalis*	Catanduva	São Paulo	2002	AB291128	AB291098
BR-Pfx1	*Dusicyon* sp.	Patos	Paraíba	2002	AB291133	AB291103
BR-Pfx3	*Dusicyon* sp.	Patos	Paraíba	2001	AB291134	AB291104
BR-Pfx4	*Dusicyon* sp.	Patos	Paraíba	2002	AB291135	AB291105
BR-Pfx5	*Dusicyon* sp.	Patos	Paraíba	2002	AB291136	AB291106
BR-Pfx6	*Dusicyon* sp.	Patos	Paraíba	2002	AB291137	AB291107
BR-MM1	*Molossus molossus*	Jales	São Paulo	1999	AB291129	AB291099
BR-MM2	*Molossus molossus*	Ilha Solteira	São Paulo	2002	AB291130	AB291100
BR-NL1	*Nyctinomops laticaudatus*	São José do Rio Preto	São Paulo	1998	AB291131	AB291101
BR-NL3	*Nyctinomops laticaudatus*	Ipiguá	São Paulo	2001	AB291132	AB291102

Table 2. Primers used for RT-PCR and sequencing of the P and M genes

Primer	Sequence	Position[a)]	Sense
Psense1	CGAATCATGATGAATGGAGG	1292–1311	+
Psense2	CAAATAGTCAGACAAATGA	1820–1838	+
Psense3	CAAATGGTCAGACGAATGA	1820–1838	+
RVP860–879	TGCAAGACGACCTGAACCGT	2273–2292	+
P781–802	TGTGT(A/G)CTGGGATGGGT(C/T)GCTT	2294–2315	+
Panti1	TCATTTTATCAGTGGTGTTG	2499–2480	–
Panti2	AAGTTCCTCATGTTCTTCTTGC	2653–2632	–
Panti3	AAGCTCTCAGCAATCTGGTGAGC	2139–2117	–
Panti4	TTATACAAGAATATCCCTGA	2190–2171	–
DG129	GATGATGTATGTCAATCGGG	3438–3419	–
RVG62–81	TGGTATCGTGTAGACGGGGA	3398–3379	–
GfoM1	CTTGAGTGGAG(A/G)GA(C/T)TTGTCGTA	3827–3805	–

a) Nucleotide positions are numbered according to the PV sequences (M13215).

(*Dusicyon* sp.), that had been diagnosed as rabies positive by the immunofluorescence antibody assay and the mouse inoculation test [7, 27] (Table 1).

RT-PCR and sequencing: RT-PCR and sequencing were performed as previously described [41]. The primers used for RT-PCR and sequencing for complete P and M genes are shown in Table 2. Reference data used in the phylogenetic analysis are shown in Table 3.

Phylogenetic analysis: Phylogenetic trees were generated using the neighbor-joining method of Saitou and Nei [40, 46]. Mokola virus was used as an outgroup. Bootstrap values were calculated using 1,000 replicates, and homologies and multiple alignment between nucleotide and deduced amino acid sequences were identified using BioEdit software [14].

Hydropathic profiles: The hydropathic profiles were characterized using the Kyte and Doolittle parameter (GENETYX Version 6.0.3, Software Development, Tokyo,

Table 3. Rabies virus isolates used in this study

Isolate No.	Species of origin	Country	Accession No.	
			P gene	M gene
058.BBB	*Eptesicus fuscus*	Canada	AF369338	–
WCS1	Skunk	Canada	AF369285	AF360848
WCS2	Skunk	Canada	AF369286	AF360849
3694.MYO	*Myotis* sp.	Canada	AF369349	–
6832.RB	*Lasiurus borealis*	Canada	AF369351	–
FL.RAC	Raccoon	U.S.A.	AF369294	AF360856
FT2891.DG	Dog	U.S.A.	AF369308	–
l15.DG	Dog	India	AF369309	–
l19.DG	Dog	India	AF369310	–
IR5.DG	Dog	Iran	AF369311	–
KY2877.SK	Dog	U.S.A.	AF369292	–
M4.VB	Cattle	Mexico	AF369366	–
M29.DG	Cat	Mexico	AF369313	–
NY.RAC	Raccoon	U.S.A.	AF369293	AF360857
ONT1.RFX	Red fox	Canada	AF369265	AF360850
ONT5.RFX	Red fox	Canada	AF369269	AF360854
P4.VB	Cattle	Paraguay	AF369364	–
V027.DG	Dog	Tunisia	AF369322	–
V037.MG	Mongoose	Botswana	AF369300	–
V077.SHB	*Lasionycteris noctivagans*	Canada	AF369346	–
V078.BBB	*Eptesicus fuscus*	Canada	AF369341	–
V084.BBB	*Eptesicus fuscus*	Canada	AF369340	–
V089.LBB	*Myotis lucifugus*	Canada	AF369343	–
V103.HB	*Lasiurus cinereus*	Canada	AF369347	–
V113.DG	Dog	Sri Lanka	AF369320	–
V118.DG	Dog	Sri Lanka	AF369321	–
V121.DG	Dog	Nepal	AF369317	–
V211.SK	Skunk	U.S.A.	AF369287	–
V213.SK	Skunk	U.S.A.	AF369289	–
V216.SK	Skunk	U.S.A.	AF369291	–
V230.BBB	*Eptesicus fuscus*	U.S.A.	AF369342	–
V235.FTB	*Tadarida brasiliensis*	U.S.A.	AF369359	–
V264.MG	Mongoose	S. Africa	AF369302	–
V265.BFX	Bat-eared fox	Tanzania	AF369296	–
V280.FX	Red fox	France	AF369278	–
V461.DG	Dog	Nigeria	AF369326	–
V464.DG	Dog	Nigeria	AF369328	–
V660.FX	Fox	Israel	AF369280	–
SHBRV-18	*Lasionycteris noctivagans*	U.S.A.	AY705373	AY705373
USASK	Skunk	U.S.A.	–	AY170396
ZAMRAV5100	Dog	Zambia	AB285215	AB285215
PV	Unknown	Unknown	M13215	M13215
ABL	Human	Australia	AF418014	AF418014
Mokola	Unknown	Unknown	Y09762	Y09762

Japan).

RESULTS

The ORF of the P gene contained 894 nucleotides encoding 297 amino acids in the most of the RV isolates studied, while isolates BR-Pfx3 and BR-Pfx4 contained 906 nucleotides encoding 301 amino acids, as well as raccoon RV isolates from the U.S.A. (NY.RAC and FL.RAC), and BR-Pfx5 contained 891 nucleotides encoding 296 amino acids, as well as dog RV isolates from Asia (l15.DG, V113.DG and V118.DG) (Fig. 1). The similarities between the nucleotide and amino acid sequences within the Brazilian RV isolates were greater than 77.5% and 80.6%, respectively.

A phylogenetic tree based on partial P genetic data revealed two large genetic clusters (Fig. 2). One cluster consisted of chiroptera-related RV isolates and could further be divided into several monophyletic lineages according to host bat species. The other cluster consisted mainly of carnivora-related RV isolates, and was further divided into several lineages according to geographic origin and the host species. Some skunk and raccoon RV isolates from North America were placed in outlying lineages of the chiroptera-related RV cluster.

Brazilian RV isolates consisted of six RV variants which were associated with specific host species; the three insec-

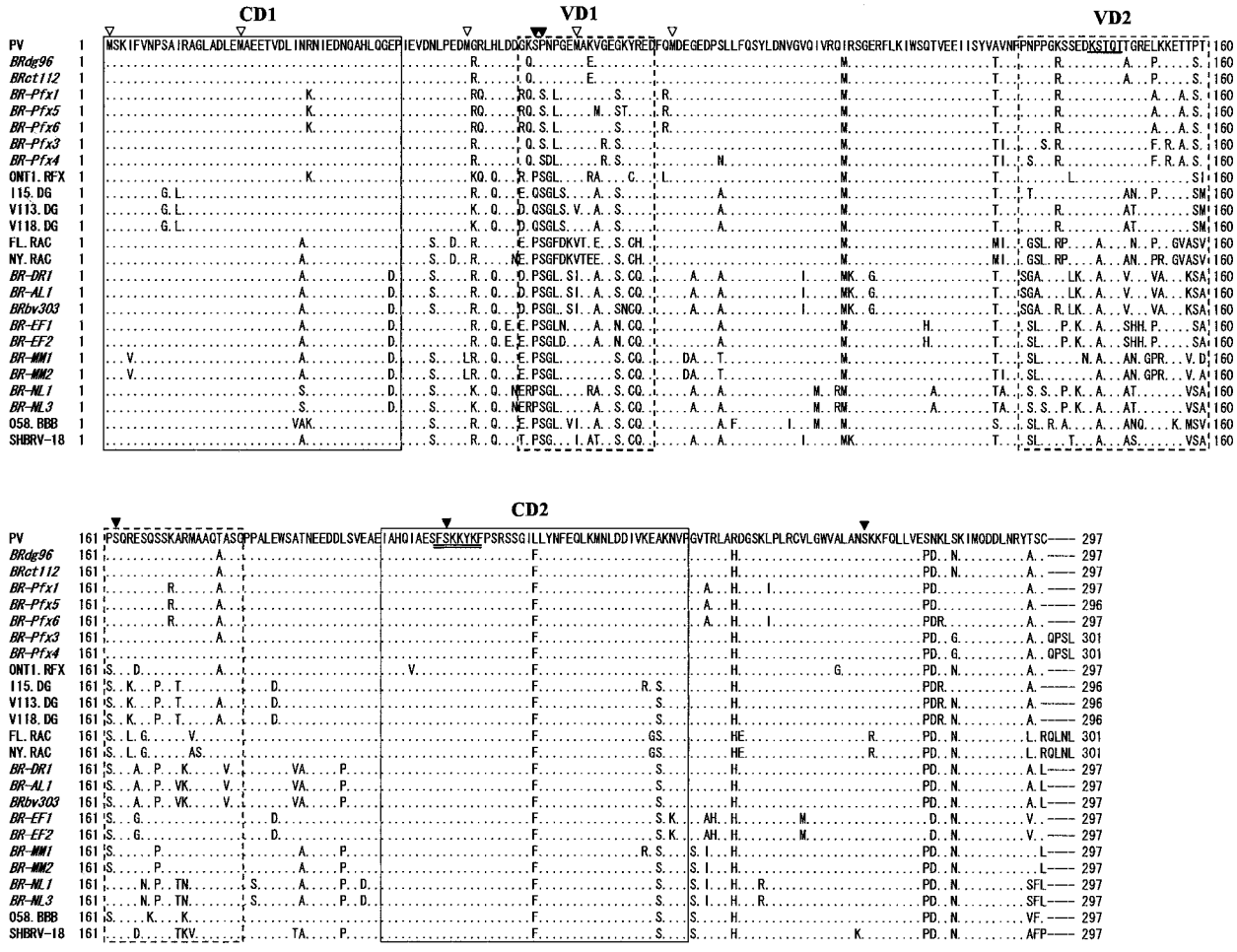

Fig. 1. Multiple alignments of P protein amino acid sequences. Italic font indicates the samples for which nucleotide sequences were determined in this study. Dots indicate amino acids that are in agreement with the sequence in the first line. Boxes with continuous lines delimit conserved domains (CD1 and 2), while those with dashed lines delimit variable domains (VD1 and 2). Black triangles indicate the positions of serine residues identified as phosphoacceptors in the P protein of the CVS strain. White triangles indicate the positions of methionine residues and confirmed translation initiation in the CVS strain. Continuous and double-continuous underlining show the LC8 binding motif and the lysine-rich motif, respectively.

tivorous bat RV lineages consisted of *N. laticaudatus, M. molossus* and *E. furinalis* and the vampire bat-related RV lineage consisted of *D. rotundus, Artibeus* sp. as well as cattle RV isolates (Fig. 2). In addition, Brazilian carnivora-related RVs formed a monophyletic cluster and were divided into dog- and fox-related RV lineages.

Based on multiple alignments of P protein amino acid sequence data, two conserved domains (CD1 and 2) comprising positions 1–50 and 201–245, respectively, and two variable domains (VD1 and 2) comprising positions 61–80 and 134–180, respectively, have been identified [30, 32]. These conserved and variable domains were observed in the Brazilian RV isolates of this study (Fig. 1). Specific amino acid substitutions corresponding to the phylogenetic lineages were observed throughout the P protein, as the result that Asp_{42} and Glu_{62} were found to be retained among Brazilian chiroptera- and carnivora-related RV isolates, respectively. The distribution of amino acid substitutions was particularly concentrated on the VD2, in which the hydropathic profiles associated with specific RV variants were observed in the hydrophilic region (data not shown).

The lysine-rich motif, FSKKYKF, which was identified as an important component of C-terminal N protein-binding [22], was conserved in amino acids 209–214 in all Brazilian RV isolates analyzed (Fig. 1). The binding site for the cytoplasmic light chain of dynein (LC8), which is involved in viral nucleocapsid axoplasmic transport [21, 39], has been reported as the RSSEDKSTQTTGR sequence of amino acids 139–151 in the P protein of the PV strain [38]. However, Lo *et al.* reported that the consensus sequence (K/R)XTQT is the common target-accepter of LC8 [28]. In this study, the consensus sequence of Brazilian RV isolates was

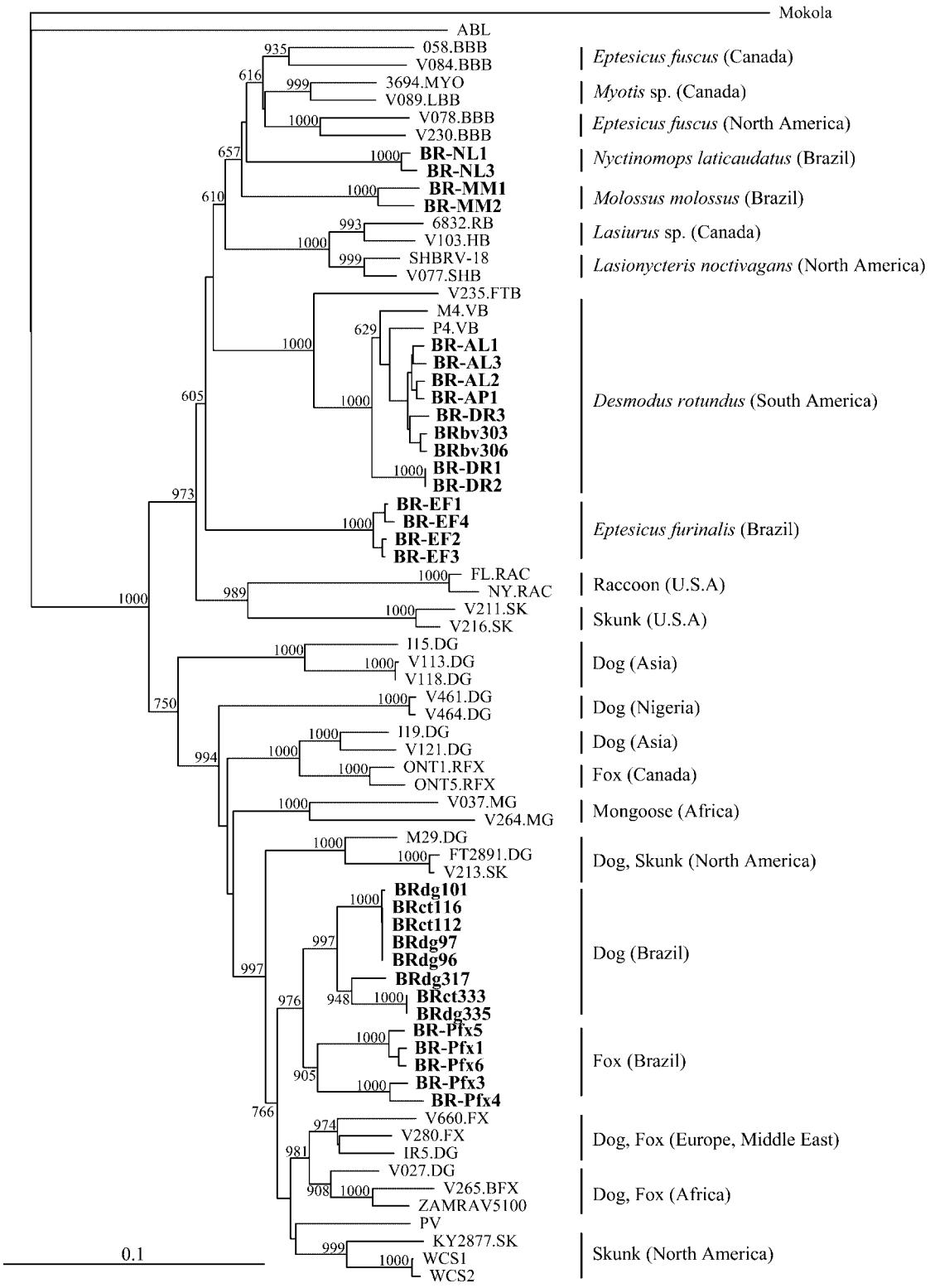

Fig. 2. Phylogenetic tree based on the P gene (1514–2404 compared to PV strain). Bold font indicates the samples for which nucleotide sequences were determined in this study. Bootstrap values are shown at major branches.

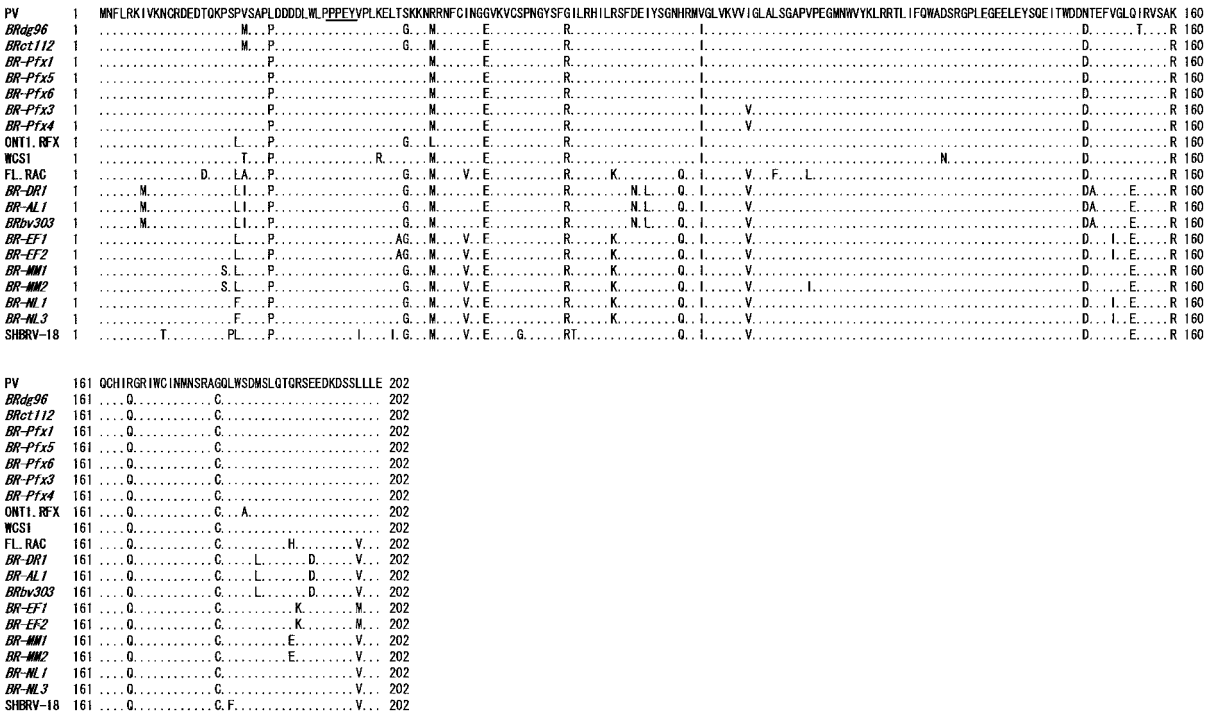

Fig. 3. Multiple alignments of M protein amino acid sequences. Italic font indicates the samples for which nucleotide sequences were determined in this study. Dots indicate amino acids that are in agreement with the sequence in the first line. Continuous underline shows the PPxY motif.

K(S/A)TQT encoded by amino acids 144–148. In addition, Ser_{145} was found to be conserved predominantly carnivora-related RV isolates while Ala_{145} was predominant in chiropteran-related RV isolates.

Phosphoacceptors associated with protein kinase C (PKC) or RV protein kinase (RVPK) were identified as Ser_{63}, Ser_{64}, Ser_{162}, Ser_{210} and Ser_{271} in the P protein of the CVS strain [13]. The phosphoacceptors associated with PKC, Ser_{162}, Ser_{210} and Ser_{271}, were retained in all Brazilian RV isolates, while those of RVPK, Ser_{63} and Ser_{64}, were not. Ser_{63} was retained in Brazilian carnivora-related RVs, and was substituted by Pro_{63} in Brazilian chiroptera-related RVs. Ser_{64} was retained in Brazilian fox- and chiroptera-related RVs, and was substituted by Pro_{64} in Brazilian dog-related RVs. Of the four methionine residues located in-frame of the single P protein sequences, Met_{20}, Met_{53}, Met_{69} and Met_{83}, all have been shown to translate four proteins of various lengths in the CVS strain [4]; Met_{20} and Met_{83} were conserved in all Brazilian RV isolates.

The ORF of the M gene contained 609 nucleotides encoding 202 amino acids in Brazilian RV isolates (Fig. 3). The similarities between the nucleotide and amino acid sequences in the Brazilian RV isolates exceeded 82.4% and 92.5%, respectively. As with the analysis of the P gene, phylogenetic trees based on the ORF of the M gene revealed two large genetic clusters, one chiroptera- and one carnivora-related RV cluster, containing six Brazilian RV lineages associated with the host species and geographic distributions (Fig. 4). Some raccoon RV isolates from the U.S.A. were placed within outlying lineages to the chiroptera- and carnivora-related RV clusters. Specific amino acid substitutions associated with host species were found scattered throughout the sequences of the M protein, for example Met_7, Ile_{22}, Asn_{80}, Leu_{82}, Ala_{148}, Leu_{184} and Asp_{192} were all retained in vampire bat-related RVs (Fig. 3). However, the hydropathic profiles did not differ significantly among the Brazilian RV isolates (data not shown). The proline-rich motif (PPxY motif), which interacts with the WW domain of cellular proteins [15, 16], formed consensus sequence PPEY at amino acids 35–38 in all Brazilian RV isolates.

DISCUSSION

Phylogenetic analyses based on the P and M genes revealed the existence of several Brazilian RV variants corresponding to phylogenetic analyses targeting the viral N and G genes [19, 25, 26, 41, 42, 44, 45]. Phylogenetic analysis showed two clusters consisting of chiroptera- or carnivora-related RV isolates. The chiroptera-related RV cluster was further divided into several lineages depending on host bat species, with four Brazilian chiroptera-related RV lineages observed. While several Brazilian fox RV isolates were characterized as encoding either extended or deleted P proteins, these formed a monophyletic cluster with other Brazilian carnivora-related RVs encoding general P

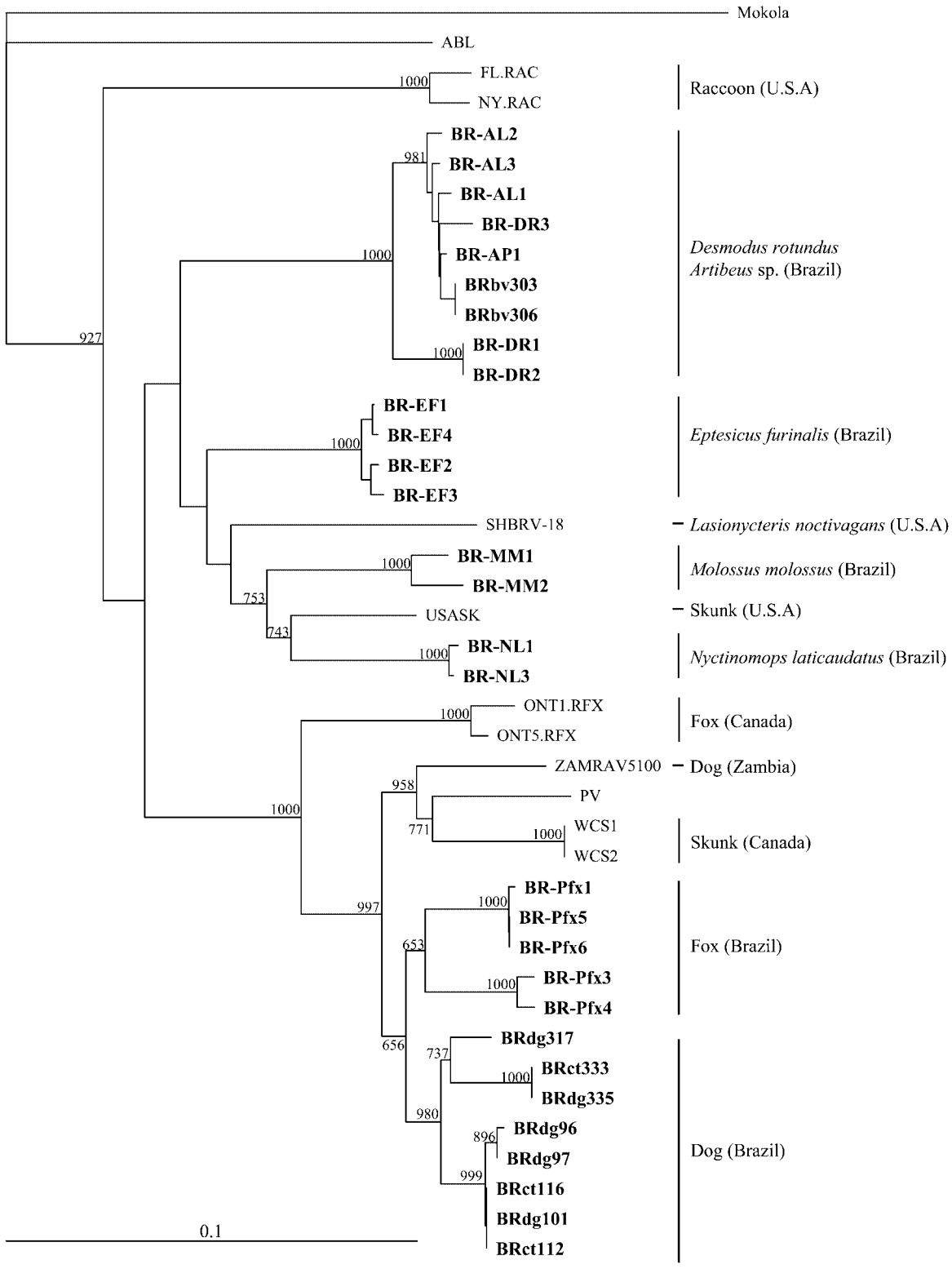

Fig. 4. Phylogenetic tree based on the M gene (2496–3104 compared to PV strain). Bold font indicates the samples for which nucleotide sequences were determined in this study. Bootstrap values are shown at major branches.

protein length, and did not group with carnivora-related RV isolates from other regions that encoded P proteins of different length. In addition, specific amino acid substitutions with respect to host species and geographic origin were found. Taken together, these findings suggest that phylogenetic analyses using these two genes are capable of identifying epidemiological characteristics of Brazilian RVs.

Conservation of the P protein in Brazilian RV isolates was low, with two conserved (CD1 and 2) and two variable domains (VD1 and 2), which corroborates the findings of Nadin-Davis *et al.* [30, 32]. Variable amino acid substitutions characteristic of Brazilian RV variants occurred at high frequencies in the variable domains, and this was reflected in the hydrophilic regions of the observed hydropathic profiles. Additionally, the LC8-binding motif, involved in viral nucleocapsid axoplasmic transport [21, 39], formed consensus sequence K(S/A)TQT in the VD2 region. With the exception of several RV isolates, the Ser and Ala differences were observed in the consensus sequences of isolates from either the carnivora- or chiroptera- related RV isolates, as reported by Nadin-Davis *et al.* [32]. The variable domains thought to be located on the surface structure of the P protein have been suggested to be involved in host/viral interactions and adaptation to the host environment [31, 32]. Our results support this hypothesis and studies of the variable domains are expected to be useful in elucidating the adaptive evolution of the virus.

At least 2 independent site of the P protein that confer N protein-binding activity have been identified; one is located within the N-terminal half of the protein, and another within 50 residues of the C-terminus [3, 11]. The first 19 N-terminal residues of the P protein confer L protein-binding ability, as does the N-terminal region containing the L protein-binding site [5]. Conserved domains contain N and L protein-binding sites and the conserved lysine-rich motif, FSKKYKF, which is an important component in N protein-binding [22, 32]. These two conserved domains containing the lysine-rich motif were observed in all Brazilian RV isolates, supporting the hypothesis that they may be essential to activities such as granule formation and general functioning of the virus.

Phosphorylation of the P protein is essential for transcription to occur in the vesicular stomatitis virus (VSV) [18], which belongs to the *Rhabdoviridae* family. In five serine residues which constitute the phosphoacceptors in the P protein of the CVS strain [13], Ser_{210} and Ser_{271} within the PKC phosphoacceptor target were notably conserved in all lyssavirus genotypes (GT 1 to 7) [32], including the Brazilian RV variants examined in this study. Studies on VSV and paramyxovirus have shown that P gene-encoded multiple proteins are important in the viral replication cycle and pathogenicity of the virus [12, 24, 37]. The four methionine residues located in-frame of single P protein sequences are translated into four proteins of various lengths with no known function in the CVS strain [4]. Nonetheless Met_{20} has been retained in all lyssavirus genotypes [32] and conservation of Met_{20} was observed in all Brazilian RV variants. Notably these conserved amino acid residues in the wild-type RVs are likely to be associated with important viral functions. Further laboratory research of these conserved amino acid residues would facilitate the identification of the biological characteristics of potential functions in wild-type RVs.

The M protein binds to the RNP core, and collaborates with the viral G protein in the infected cells to produce progeny virions by budding at the cell membrane [34, 35]. The M protein is also required for the development of a typical bullet-shaped rhabdovirus [29]. Consequently, the M protein is involved in binding to several viral components. In this study, although the M protein exhibited RV variant-associated amino acid substitutions, the hydropathic profiles conformed closely with each other. In addition, the PPxY motif, which interacts with the WW domains of cellular components [15], was conserved in all Brazilian RV variants. Laboratory-adapted and carnivora RV strains from North America have been characterized as having identical hydropathic M-protein profiles [17, 30], suggesting that the primary structure of the M protein may be important for the retention of viral structure and function.

The present study reveals that Brazilian RV isolates possess sufficient variability with respect to host species and geographic origin, while retaining conserved sequences (motifs) thought to be associated with a viral function in the P and M genes of RV. Studies on wild-type RVs are likely to increase our understanding of the relationship between the virus and the host, as well as the biological characteristics of the virus in the wild. Such genetic data would be helpful in investigating pathogenicity associated with RV.

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