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Preferential host switching and its relation with *Hantavirus* diversification in South America

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In recent years, the notion of co-speciation between *Hantavirus* species and their hosts was discarded in favour of a more likely explanation: preferential host switching. However, the relative importance of this last process in shaping the evolutionary history of hantaviruses remains uncertain, given the present limited knowledge not only of virus–host relationships but also of the pathogen and reservoir phylogenies. In South America, more than 25 hantavirus genotypes were detected; several of them act as aetiological agents of hantavirus pulmonary syndrome (HPS). An understanding of the diversity of hantaviruses and of the processes underlying host switching is critical since human cases of HPS are almost exclusively the result of human–host interactions. In this study, we tested if preferential host switching is the main process driving hantavirus diversification in South America, by performing a co-phylogenetic analysis of the viruses and their primary hosts. We also suggest a new level of amino acid divergence to define virus species in the group. Our results indicate that preferential host switching would not be the main process driving virus diversification. The historical geographical proximity among rodent hosts emerges as an alternative hypothesis to be tested.

INTRODUCTION

Hantaviruses are rodent and insectivore-borne negative-stranded RNA viruses of the *Bunyaviridae* family. It was suggested that, in general, hantaviruses are highly selective toward a single host species (Plyusnin *et al.*, 1996; Plyusnin & Morzunov, 2001). Early phylogenies of the genus showed three consistently well-defined clades, each of them associated with one of the three subfamilies of muroid rodents (Arvicolinae, Murinae and Sigmodontinae), suggesting a history of co-speciation between host and pathogen (Plyusnin *et al.*, 1996; Hughes & Friedman, 2000; Jackson & Charleston, 2004). In recent years, this well accepted notion has been questioned because new evidence has appeared. First, to establish co-speciation, not only should both phylogenies be largely congruent but also sister species of host and parasite should have originated roughly at the same time (Coyne & Orr, 2004). Ramsden *et al.* (2008) posed that hantaviruses would be evolving substantially faster than was predicted on the basis of shared divergence times with their rodent hosts, pointing out the inconsistency between *Hantavirus* biology and the hypothesis of co-speciation. Another line of evidence is the recent discovery of hantaviruses in the order Soricomorpha (Song *et al.*, 2007; Arai *et al.*, 2008), a group of mammals distantly related to rodents. Based on these new data, Ramsden *et al.* (2009) concluded that at a continental level, preferential host switching (i.e. the transmission of the pathogen to a new host closely related to the original one, followed by pathogen speciation) modelled by geographical proximity is driving *Hantavirus* diversification. This process involves a virus cross-species transmission. Then, the new and original virus populations evolve, gaining and losing variants, until all those in the original host population form a monophyletic clade, different from the one in the new host. So, the two viruses will appear as sister clades and will match the host phylogeny as long as the original and the new host species are also sister clades (Charleston & Robertson, 2002). At a regional level, the hypothesis of preferential host switching modelled by geographical proximity posed by Ramsden *et al.* (2009) has not been tested. A study in this direction would be particularly interesting for South American hantaviruses, since the greatest diversity in the western hemisphere lies clearly in this region (Firth *et al.*, 2012). One of the main limitations to understanding the importance of preferential host switching is the disagreement among different studies about the virus–host relationships they assess (see González-Ittig *et al.*, 2014), often as a consequence of poorly resolved taxonomies in some rodent lineages (Almeida *et al.*, 2007; Haag *et al.*, 2007; González-Ittig *et al.*, 2010; Hanson *et al.*, 2011). For example, Maporal and Choclo viruses were detected in specimens originally assigned to *Oligoryzomys fulvescens* but later Hanson *et al.* (2011) demonstrated that the rodent hosts were *Oligoryzomys*

One supplementary table and one supplementary figure are available with the online Supplementary Material.

delicatus and *Oligoryzomys costaricensis*, respectively. Besides, several studies do not differentiate the primary host (i.e. the species that remains infected and maintains the infection in the population by horizontal transmission) from other hosts accidentally infected by a hantavirus, a process known as spill-over.

Hantavirus nomenclature is another source of discrepancy in virus–host relationships since different authors label new lineages as virus, strain, genotype or species in quite an arbitrary way. Most of them do not take into account the rules for species delineation proposed by the International Committee on Taxonomy of Viruses (ICTV) or do not specify which criteria they follow (as in Levis *et al.*, 1998; Rosa *et al.*, 2005). The ICTV states that a *Hantavirus* species should have: (i) been found in a unique ecological niche, i.e. in a specific primary reservoir species or subspecies, (ii) at least 7 % amino acid divergence in the complete nucleocapsid (N) and glycoprotein precursor (GPC) proteins, (iii) at least a fourfold difference in a two-way cross neutralization test, and (iv) no naturally occurring reassortants (Plyusnin *et al.*, 2012). In many cases, these rules contradict each other since many species of hantaviruses found in different rodent hosts present less than 7 % amino acid divergence in the N protein, e.g. Andes and Juquitiba viruses. Besides, if the existence of a unique primary reservoir is considered a proxy to define a *Hantavirus* species, a lineage found in a new rodent host will be described as a different one, regardless of the genetic similarity it may have with other lineages. On the other hand, a fixed level of amino acid divergence may not correspond to every speciation event in *Hantavirus*. Based on the distribution of amino acid distances among hantaviruses worldwide, Maes *et al.* (2009) proposed adjusting the second rule of the ICTV classification guidelines to: 'a 10 % difference in the N protein and a 12 % difference in the GPC protein'. The *Hantavirus* genus may have experienced, in South America, a rapid speciation process given the low amino acid divergence among genotypes (Firth *et al.*, 2012).

Given that hantaviruses are transmitted directly between hosts (Plyusnin & Morzunov, 2001), human cases of infection are almost exclusively the result of human–rodent interactions. The switching of a virus onto a novel host with different ecological patterns and geographical distribution could lead to the emergence of new outbreaks, this being a process of great concern to human health. In South America, several species of *Hantavirus* act as etiological agents of hantavirus pulmonary syndrome (HPS), a disease having high mortality rate, with hundreds of cases recorded each year (Hjelle & Torres-Pérez, 2010). Since there is no specific treatment available for hantavirus infection, prevention measures are essential to decrease HPS cases. Mills *et al.* (1999) recommended: (i) identifying the reservoir host, (ii) delimiting the geographical range of the host and the range of infection by the pathogen within the host range, and (iii) defining the relative risk to humans by determining host and pathogen distribution in distinct habitats. Hence, a more complete understanding of the diversity and distribution of hantaviruses in South America and of the processes influencing host switching is critical in order to develop effective methods to control and prevent this human zoonosis.

In this study, we tested if preferential host switching is the process driving hantavirus diversification in South America, by performing a co-phylogenetic analysis of the viruses and their primary hosts. Previously, we analysed the phylogenetic relationships among *Hantavirus* lineages, using DNA sequences of the N protein gene obtained by reverse transcription PCR (RT-PCR), with the purpose of clarifying virus–host associations. We also compare our results with previously published data on virus taxonomy, geographical distribution and host associations to shed light on contradictory reports.

RESULTS

Phylogenetic reconstruction

A total of 274 strains representing all the *Hantavirus* genotypes detected in South America were analysed in our phylogenetic reconstruction, encompassing 114 localities distributed along the entire region (Fig. 1a, b). GenBank accession number, genotype name, strain name, sequence length, host, locality and geographical coordinates are listed in Table S1 (available in the online Supplementary Material). The number of informative characters was 674. The trees obtained with maximum-parsimony (MP) and Bayesian inference (BI) showed similar topology when estimating clustering among the sequences, but the MP consensus tree presents a polytomy involving major clades that appears solved in the BI tree. Fig. 2 shows the topology of the BI tree with the support values of both phylogenetic reconstructions.

Based on tree topologies, we identified 20 different *Hantavirus* lineages (i.e. a well-supported monophyletic clade or a different sequence that did not group within any clade) for South America (Fig. 2, Table S1). They form a clearly separate group from the Central and North American hantaviruses. The most basal South American clade comprises all the sequences named Caño Delgadito, obtained from *Sigmodon alstoni* specimens from two localities in Venezuela. Then, a separate clade is formed including the Calabazo and Necocli strains recovered from rodents of the genus *Zygodontomys* in Panama and Colombia, respectively. These are the sister lineages of the Maporal sequences recovered from *O. delicatus* from Venezuela. Next, the tree shows two main groups. The first one includes seven well-defined clusters, one of which is the clade of the Choclo strains detected in a specimen of *O. costaricensis* and in a HPS patient from Panama. This is the sister lineage of a clade encompassing mainly strains of the Jabora genotype but also the Ape Aime Itapua and the AC210py strains, obtained from different species of the genus *Akodon*.

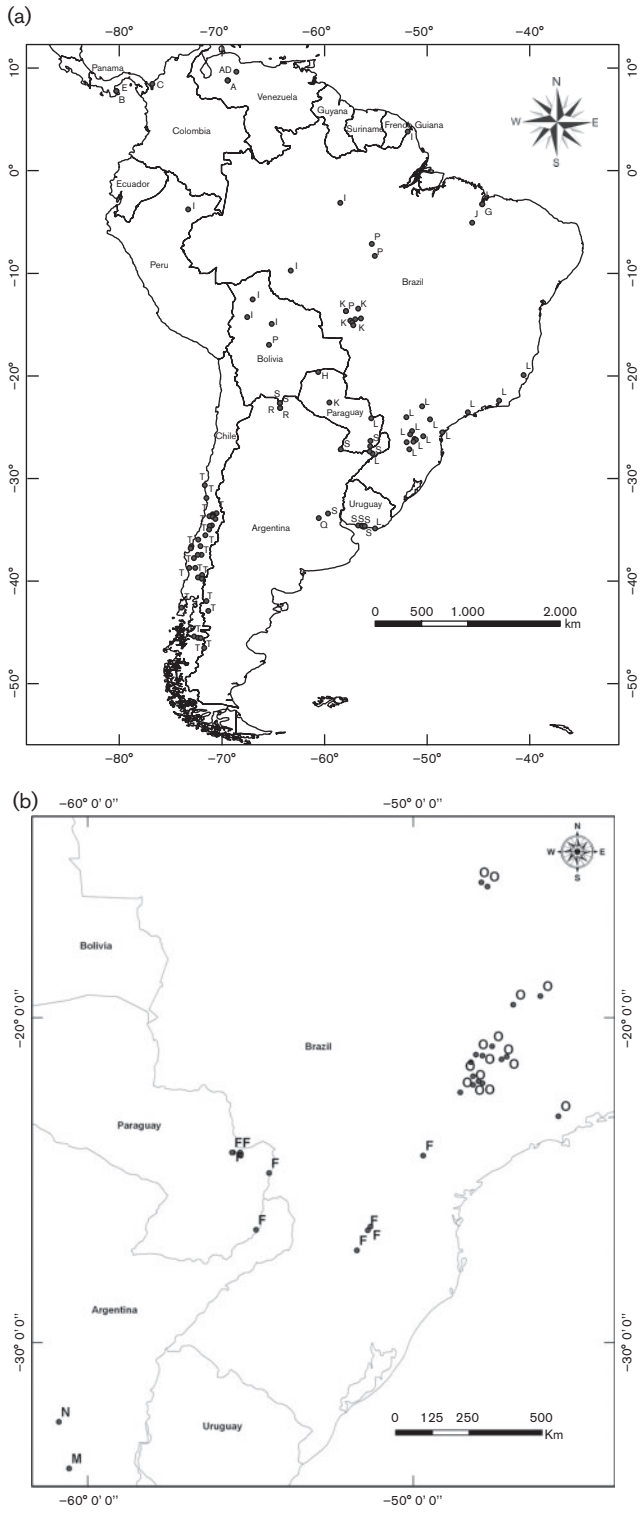


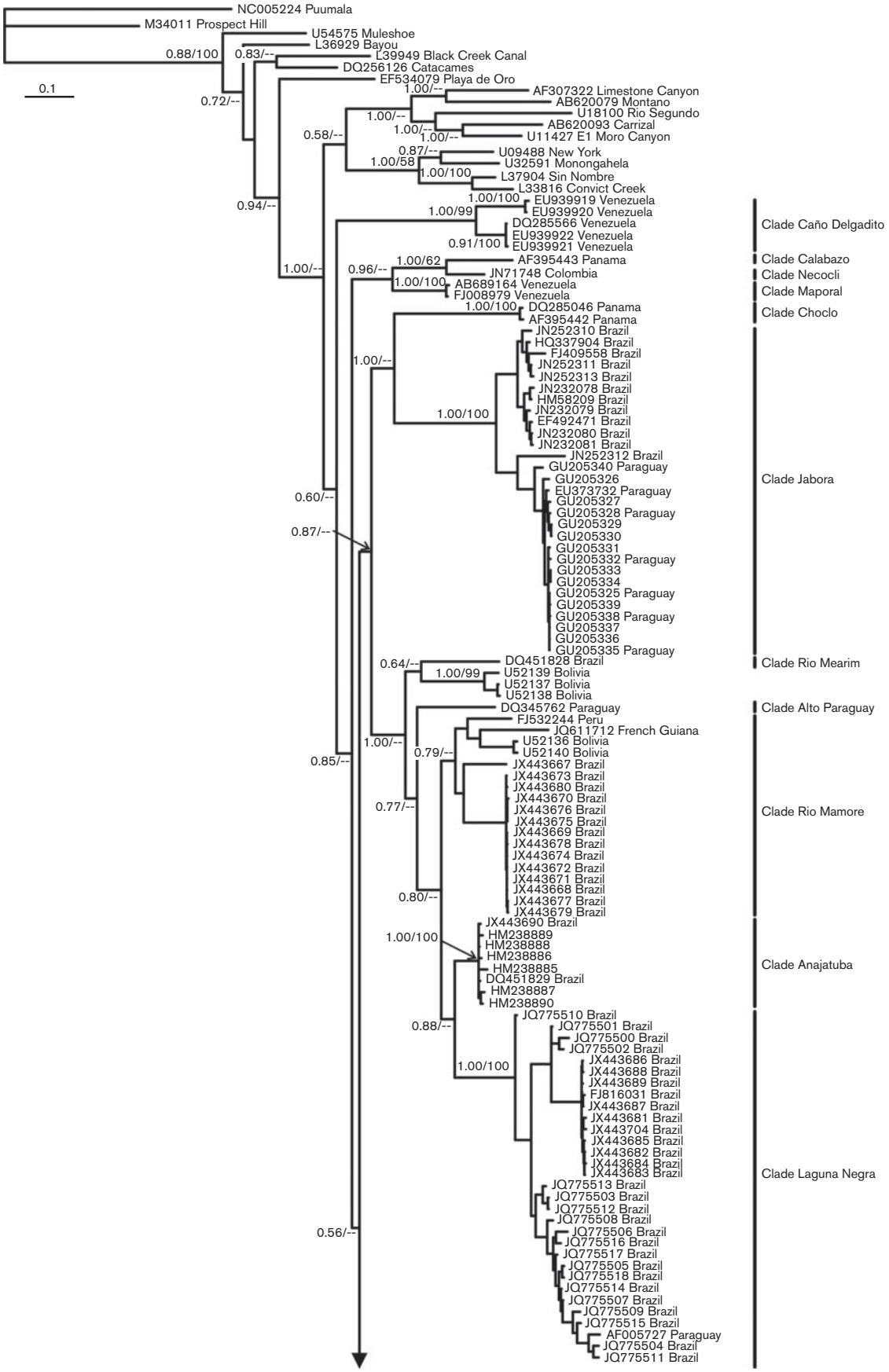
Fig. 1. Sampling sites in South America. Letters indicate *Hantavirus* genotypes. (a) A, Caño Delgadito; B, Calabazo; C, Necocli; D, Maporal; E, Choclo; G, Rio Mearim; H, Alto Paraguay; I, Rio Mamore; J, Anajatuba; K, Laguna Negra; L, Juquitiba; P, Castelo do Sonhos; Q, Hu39694; R, Oran; S, Lechiguanas; T, Andes. (b) F, Jabora; M, Pergamino; N, Maciel; O, Araraquara.

Two well-supported subclades are noticeable within the above-mentioned clade, one of them grouping sequences from eastern Paraguay and the other, sequences from south-eastern Brazil (Fig. 2).

The Choclo–Jabora group appears related to another group including five well-defined clades: Rio Mearim, Alto Paraguay, Rio Mamore, Anajatuba and Laguna Negra. Rio Mearim was found in northern Brazil and Alto Paraguay in western Paraguay. Both of them were detected in rodents of the genus *Holochilus*. In the BI tree, the Rio Mearim genotype is related to three sequences from Bolivia identified as Rio Mamore; however, it is worth noting that these three sequences are quite short (397 bp), which could affect their position in the phylogenetic reconstruction. The remaining sequences associated with the Rio Mamore genotype, i.e. Rio Mamore, RIOMV-3, RIOMV-4 and Maripa, are grouped together. All these sequences are distributed along the Amazonian biogeographic region (see Cabrera & Willink, 1973; Fig. 1a), although no geographical association can be inferred from our phylogenetic reconstruction. The strains were recovered from two rodent lineages, *Oligoryzomys microtis* and a genetic sister species named *Oligoryzomys* sp. RT-2012, and from one HPS patient. All the Anajatuba strains obtained from different rodent hosts and HPS patients in northern Brazil group together and appear as the sister clade of the one formed by all the genotypes associated to the Laguna Negra virus, i.e. Laguna Negra, Laguna Negra-like and LANV-2. The strains belonging to the Laguna Negra clade were mainly recovered from HPS patients but were also detected in two species of the genus *Calomys*, all of them distributed in western Paraguay and western Brazil (Fig. 1a).

In the second main group, the first clade that separated from the rest includes the genotypes Juquitiba, Juquitiba-like and Araucaria and the strains Itapua 37 and Itapua 38, occurring mainly in the Atlantic Rainforest biogeographic region but also found in the Pampas region (Fig. 1a). These strains were obtained from numerous HPS patients and from several rodent species, mainly *Oligoryzomys nigripes* (Table S1). The Juquitiba clade is the sister lineage of a group that includes the Pergamino genotype detected in *Akodon azarae*, the Maciel genotype obtained from *Necromys* sp. from central Argentina and a clade encompassing all the strains of Araraquara, Araraquara-like and Paranoa genotypes. Paranoa was detected only in HPS patients, whereas Araraquara was recovered from HPS patients as well as from specimens of *Necromys lasiurus* and *Akodon* sp.

The Tunari genotype from western Bolivia and Castelo do Sohnos, CASV and CASV-2 genotypes from central Brazil group together with a high support value in the phylogenetic reconstruction (Fig. 2). Only one sequence from this clade was obtained from rodent specimens identified as *Oligoryzomys utiaritensis*. The Castelo do Sohnos clade is the sister lineage of the group that comprises the Hu39694, Oran and Lechiguanas clades. The Hu39694 strain was found in central Argentina. The Oran strains were recovered from HPS patients and from one specimen



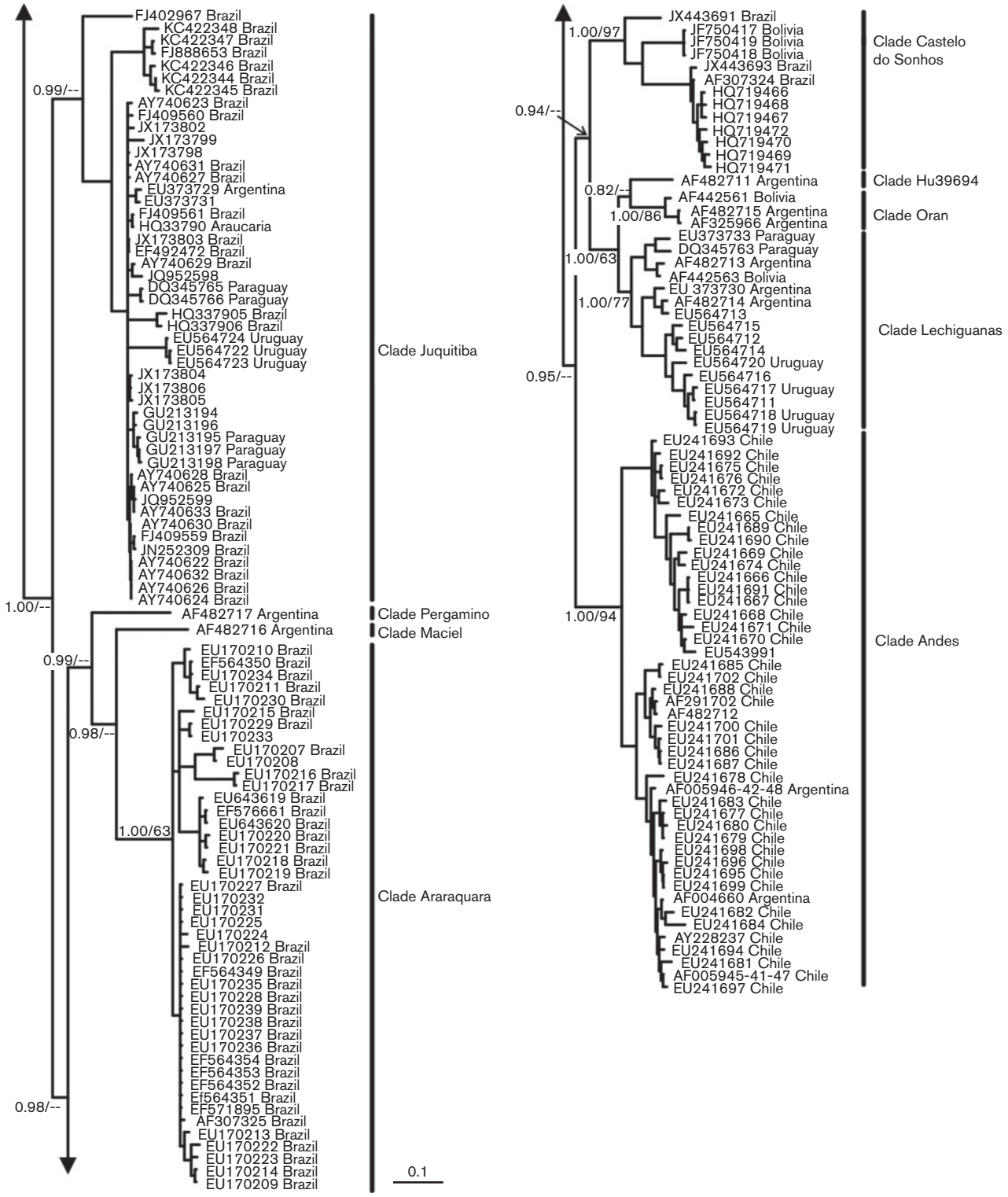


Fig. 2. Phylogenetic relationships among South American *Hantavirus* genotypes based on Bayesian analysis of the N gene dataset after 100 million generations. Bayesian posterior probabilities and MP bootstrap values, respectively, are given only for nodes defining genotypes and their relationships. The tree is rooted relative to the position of Puumala virus and one sequence of each Central and North American *Hantavirus* was added to the analysis. Bar, 0.1 substitutions per nucleotide position. Genotype name, strain name, sequence length, host, locality and geographical coordinates for each GenBank accession number are given in Table S1.

of *Oligoryzomys chacoensis*, in northern Argentina and southern Bolivia. The Lechiguanas clade includes the Bermejo, Ñeembucu, Lechiguanas and Central Plata genotypes from Argentina, Uruguay and western Paraguay, which were recovered from several specimens of *Oligoryzomys flavescens*, one of *O. chacoensis* and several HPS patients. The last clade in the tree encompasses all the Andes strains from the temperate rain forest of southern Argentina and Chile, with *Oligoryzomys longicaudatus* being the primary host. However, this virus was also found in other native rodents like *Loxodontomys micropus* and *Abrothrix longipilis*, and in the introduced species *Rattus norvegicus* and *Rattus rattus*. The group shows two subclades that reflect the strains' different latitudinal origin.

When we graphed, on the phylogenetic tree obtained, the virus lineage distributions according to the main zoogeographic regions (Fig. S1), no biogeographic pattern was observed.

Amino acid divergence

A total of 129 complete or partial sequences were analysed to calculate amino acid divergences within and among South American isolates of the genus *Hantavirus* (Table 1). Only 18 of the 20 different clades were analysed since the Calabazo and Necocli genotypes have available sequences that are too short (376 and 427 bp, respectively) to be included in the measurement of amino acid divergence, according to Maes *et al.* (2009). The upper value of amino acid divergence within clades ranged from 0.2 % in the Oran clade to 3.6 % in the Rio Mamore and Laguna Negra clades. The value of amino acid divergence among clades ranged from 20.1 % between Caño Delgadito and Jabora virus to 0.6 % between Lechiguanas and Hu39694. However, most clades presented amino acid divergence values among them higher than the maximum upper value within a clade (3.6 %).

Co-phylogeny

For the phylogenetic reconciliation analysis 17 virus–host relationships were considered. Those clades whose sequences were detected only in HPS patients (Hu39694) or presented controversies regarding the primary host (Anajatuba and Maciel) were excluded in the analysis. For Rio Mamore, we refer to *O. microtis* as the primary host since the so-named *Oligoryzomys* sp. RT-2012, from which several strains of Rio Mamore were obtained, is still not considered a valid species. The following sequences were used to infer the rodent phylogeny: *Akodon azarae* (AY702963), *Akodon montensis* (EU251018), *Calomys fecundus* (AF385592), *Holochilus sciureus* (EU579497), *Holochilus chacarius* (GU185898), *Necromys lasiurus* (EF622509), *O. chacoensis* (GU185903), *O. costaricensis* (EU192164), *O. delicatus* (DQ227457), *O. flavescens* (GU185913), *O. longicaudatus* (GU185912), *O. microtis* (FJ374766), *O. nigripes* (JQ013778), *O. utiaritensis* (JK443655), *S. alstoni* (AF293397), *Zygodontomys brevicauda* (GU397417), *Zygodontomys cherriei* (EU579520). The result of the phylogenetic reconciliation analysis shows discordances, in most comparisons, between host phylogeny based on cyt-*b* sequences and virus phylogeny based on N gene sequences (Fig. 3).

Table 1. Percentage of amino acid sequence divergence from the N gene within and among clades of South American hantaviruses

Numbers in bold type, values below the criterion recommended by the International Committee on Taxonomy of Viruses to define *Hantavirus* species.

Clade	A	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
A. Caño Delgadito	–																	
D. Maporal	14.8	–																
E. Jabora	20.1	14.0	≤2.1															
F. Choclo	15.9	11.4	14.5	–														
G. Rio Mearim	19.8	12.0	12.3	14.6	–													
H. Alto Paraguay	16.2	10.8	12.1	13.2	**5.1**	–												
I. Anajatuba	18.8	10.2	11.9	12.3	**4.6**	**4.9**	≤1.0											
J. Laguna Negra	16.1	11.1	14.2	12.8	9.8	8.0	8.2	≤3.6										
K. Rio Mamore	16.7	9.3	12.4	11.6	**6.1**	**4.3**	**4.6**	8.3	≤3.6									
L. Juquitiba	16.5	10.6	14.9	12.8	13.5	12.7	10.9	11.1	11.6	≤2.7								
M. Pergamino	17.3	10.1	16.3	11.1	13.5	11.1	11.9	9.6	10.8	7.6	–							
N. Maciel	17.9	10.6	16.0	11.6	15.0	11.4	12.7	11.4	11.4	7.8	**4.1**	–						
O. Araraquara	17.0	10.5	14.9	10.2	13.9	10.8	12.0	11.4	10.9	8.1	**5.9**	**4.7**	≤1.2					
P. Castelo dos Sonhos	16.2	9.9	14.3	11.0	13.9	11.6	12.6	10.3	10.8	**5.2**	**5.8**	**6.6**	**6.6**	≤2.8				
Q. Hu39694	15.6	9.6	14.2	9.8	13.5	11.1	11.7	9.4	10.1	**4.4**	**4.1**	**5.5**	**5.4**	**2.8**	–			
R. Oran	16.1	9.9	14.9	10.7	13.5	11.2	11.8	9.5	10.2	**3.6**	**4.2**	**5.6**	**5.8**	**3.3**	**1.1**	≤0.2		
S. Lechiguanas	17.0	10.3	14.7	10.8	13.9	12.8	12.9	11.6	11.6	**5.2**	**5.2**	**6.8**	**6.5**	**3.0**	**0.6**	**1.8**	≤1.0	
T. Andes	14.7	9.7	14.2	11.1	13.5	12.4	10.5	11.5	10.4	**4.4**	**5.6**	**6.7**	**7.3**	**4.9**	**3.5**	**3.8**	**4.5**	≤1.3

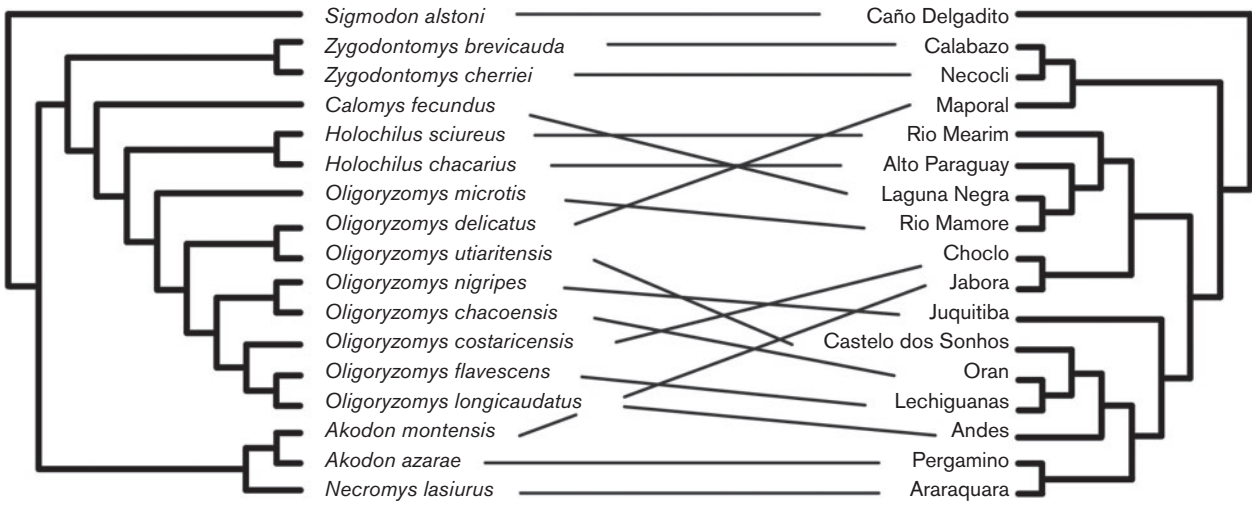


Fig. 3. Tanglegram reconstructed with TreeMap software showing rodent–hantavirus relationships. The host tree on the left was generated from cyt-*b* gene sequences and the virus tree on the right was generated from N gene sequences.

DISCUSSION

Hantavirus diversity in South America

This is the first phylogenetic study, to our knowledge, that includes most of the available sequences of hantaviruses from South America, a total of 274. The inclusion of strains from different geographical origins allowed us to estimate the level of genetic variability that may correspond to a particular genotype in the region. The advantages and disadvantages of including incomplete sequences in a phylogenetic reconstruction has been discussed (see Wiens, 2006 and references therein). In our analysis we included several partial sequences; however, in general they grouped as they were originally named, in clades with very high supporting values (see Fig. 2 and Table S1). We identified a total of 20 *Hantavirus* clades, which represents a lower lineage number than those described up to now (Firth *et al.*, 2012). Only six groups showed consistent results regarding their taxonomic status and virus–host relationships. (1) Caño Delgadito appears as the most basal lineage of the South American hantaviruses; a similar position was recovered in a previous phylogenetic reconstruction of the group (Firth *et al.*, 2012). Fulhorst *et al.* (1997) isolated this virus from *S. alstoni* rodents in Venezuela. (2) Maporal *Hantavirus* was detected in a rodent originally named *O. fulvescens* (Vincent *et al.*, 2000). Later, Hanson *et al.* (2011), in a study of the systematics of the genus *Oligoryzomys*, demonstrated that that specimen corresponded in fact to *O. delicatus*. (3) Choclo genotype has been responsible for hantavirus cardiopulmonary syndrome cases in Panama (Nelson *et al.*, 2010). The only rodent specimen where a Choclo strain was detected corresponds to the species *O. costaricensis* (Hanson *et al.*, 2011). (4) Rio Mearim and (5) Alto Paraguay viruses were detected only once in *H. sciureus* and *H. chacarius*, respectively (Chu *et al.*, 2003; Rosa *et al.*, 2005). (6) Pergamino genotype was isolated from a specimen of *Akodon azarae* in central Argentina (Levis *et al.*, 1998). It is worth noting that these six genotypes were defined either from one or from a few sequences obtained in very restricted geographical areas (Fig. 1a, b); it is probable that new data on these groups could change what we know so far on virus–host relationships in these lineages.

Five clades grouped strains assigned to two or more genotypes. To achieve monophyly we reclassified lineages on the basis of phylogenetic reconstruction, genetic distances and geographical distribution as follows. (1) One clade groups all the Jabora strains together with the Ape Aime Itapua and the AC210py strains. The two subclades observed, one from eastern Paraguay and the other from south-eastern Brazil (Fig. 2), show a low amino acid divergence between them (2.1 %), suggesting that they represent geographical variants of the Jabora genotype and not a lineage split. Hence, we consider that Ape Aime Itapua and AC210py are actually Jabora strains. (2) The clade Juquitiba includes numerous strains most of which were named Juquitiba or Araucaria. This virus was originally detected in a HPS patient (Vasconcelos *et al.*, 1997); later, it was found in other humans and in several rodent species (Delfraro *et al.*, 2008). The Araucaria genotype was also identified as responsible for HPS cases (Raboni *et al.*, 2005). The synonymy of these two viruses has already been reported in previous studies based on the genetic similarity among the sequences (Padula *et al.*, 2007; Delfraro *et al.*, 2008) but, despite these observations, some authors are still considering them different lineages (Raboni *et al.*, 2009; Firth *et al.*, 2012). When all the available sequences without any previous assumption on lineage identity are included in the phylogenetic reconstruction, as in the present analysis, it clearly appears that Araucaria, IP37 and IP38 genotypes are variants of the Juquitiba virus. (3) The Araraquara clade includes all sequences named Araraquara

and Paranoa. The former genotype was detected in HPS patients and in *Necromys lasiurus* specimens from southern and central Brazil (Johnson *et al.*, 1999; Ramsden *et al.*, 2009), whereas the latter was detected only in HPS cases from one locality in central Brazil (Melo-Silva *et al.*, 2009). In our phylogenetic reconstruction, the Paranoa and Araraquara sequences from this locality (Federal District, Brazil) cluster together, suggesting that Paranoa is a synonym of Araraquara. (4) The Castelo do Sonhos clade includes the Tunari strains from western Bolivia (Cruz *et al.*, 2012) and all the variants of Castelo do Sonhos genotype from central Brazil (Travassos da Rosa *et al.*, 2011; Firth *et al.*, 2012). These two groups present a low amino acid divergence between them (2.7 %) suggesting that Tunari sequences are geographical variants of Castelo do Sonhos. (5) The Lechiguanas clade groups all the strains of Bermejo, Ñeembucu, Lechiguanas and Central Plata genotypes from Argentina, Uruguay and western Paraguay (Bohlman *et al.*, 2002; Chu *et al.*, 2006; Delfraro *et al.*, 2008). Since the mean amino acid divergence among these strains is very low ($\leq$1.0 %), a different denomination of genotypes within this clade is not justified.

Regarding Maripa virus, this has already been recognized as a variant of Rio Mamore (Matheus *et al.*, 2012); our analysis confirms the authors' suggestion. That is why we did not include the Rio Mamore clade in the above reclassification.

Three groups deserve special attention concerning taxonomy. One comprises Calabazo and Necocli genotypes. The available sequences of both viruses are too short to be included in the measurement of amino acid divergence, hence the possibility that these two lineages are actually geographical variants of the same genotype cannot be discarded; here we consider them as different genotypes. Another conflictive group comprises Maciel and Araraquara; these two genotypes show an amino acid divergence of 4.7 % (higher than the upper divergence within clades) and their type localities are 1600 km apart, suggesting that the divergence could be the result of their geographical isolation. However, it is worth noting that only Araraquara was isolated from humans (Johnson *et al.*, 1999), and is responsible for many HPS cases in Brazil (Figueiredo et al., 2009). On the basis of the present data, there is not enough evidence to reject the hypothesis that they represent two separate taxonomic entities; here we consider them as different genotypes. The last group comprises Hu39694, Oran and Lechiguanas genotypes, with amino acid divergence values among clades lower than the maximum upper value within a clade (Table 1). However, Oran and Lechiguanas appear as well separated clades in the phylogenetic reconstruction, with high support values. For this reason we considered that Hu39694, Oran and Lechiguanas genotypes are separated lineages.

Two clades, Rio Mamore and Laguna Negra, show an upper value of amino acid divergence within clade higher than the other genotypes (3.6 %; Table 1), suggesting that they include more than one lineage. Further evidence is needed to accurately determine if this divergence includes a phylogenetic break originated by a recent lineage split or if it is the result of a clinal pattern of population differentiation explained by processes such as gene flow, drift and/or local selection. Note that Rio Mamore is the most widely distributed genotype among South American hantaviruses (Fig. 1), and it is probable that this species includes geographical variants.

Despite the fact that reclassifying hantavirus lineages lowered the total number reported up to now for South America, the region remains one of the most speciose for the genus. The overall phylogenetic reconstruction shows that the basal lineages in the South American hantavirus group are found in the north of the region. A north–south expansion could be proposed, following a similar colonization pattern to that postulated for South American rodents (subfamily Sigmodontinae), which have experienced a sudden and explosive diversification since the arrival of their ancestors from North America, about six million years ago (Steppan *et al.*, 2004). The availability of new niches (new rodent species) could have favoured the diversification of the genus *Hantavirus* in the region, explaining the pattern observed here.

Hantavirus–host relationships

When accurate hantavirus–host relationships need to be defined, different problems emerge. Jabora genotype was originally detected in western Paraguay in *Akodon montensis* (Chu *et al.*, 2003) and in *Akodon cursor* (Padula *et al.*, 2007); it was named Ape Aime-Itapua genotype and AC210py strain, respectively. Later, it was also found in *Akodon montensis* and in *Akodon paranaensis* from south-western Brazil (de Oliveira *et al.*, 2011, 2012). No association was observed between the virus sequences and the host species from which they were obtained (de Oliveira *et al.*, 2012). Although *Akodon montensis* appears as the primary host for this genotype based on the frequency of virus–host association, the detection of Jabora in several species of the genus *Akodon* raises the question of whether it might persist in nature through a group of species acting as natural hosts rather than through a unique primary host, which would contradict the hypothesis that hantaviruses are highly selective towards a single host species (Plyusnin *et al.*, 1996; Plyusnin & Morzunov, 2001). However, it has not been tested whether all of these *Akodon* species act as natural reservoirs, maintaining the infection in the population by horizontal transmission, or if it is the result of spill-over infection from the primary rodent host into other species of the same genus. The same situation was observed for Laguna Negra, which was originally isolated from specimens of *Calomys laucha* from Paraguay (Johnson *et al.*, 1997) and identified as the epidemiological agent that causes HPS cases in Bolivia, western Paraguay, western Brazil and northern Argentina (Johnson *et al.*, 1997; Levis *et al.*, 2004; Travassos da Rosa *et al.*, 2012). Laguna Negra was also detected in rodents originally named *Calomys callosus* (Levis *et al.*, 2004), later identified molecularly as *C. fecundus* (R. E. Gonzalez-Ittig, personal communication) and in *Calomys callidus* (Travassos da Rosa *et al.*, 2012). Our phylogenetic analysis does not

show any association between strains and rodent hosts. These results can be explained in at least two ways: the different rodent species involved are the result of misidentification, so Laguna Negra virus has a unique primary host, or, as stated above for Jabora virus, Laguna Negra can be maintained in nature by a group of related species of the genus *Calomys*.

Calabazo was detected in *Zygodontomys brevicauda cherriei* from Panama (Vincent *et al.*, 2000) and Necocli in *Z. cherriei* from Colombia (Londoño *et al.*, 2011). According to Musser & Carleton (2005) *Z. cherriei* is a junior synonym of *Z. brevicauda*, which is distributed from south-eastern Costa Rica through Panama, Colombia, Venezuela, Guyana, Suriname and French Guiana to northern Brazil. Clearly, the taxonomic status of these rodent hosts should be revised.

Anajatuba genotype was originally recovered from rodents identified as *Oligoryzomys fornesi* (Rosa *et al.*, 2005). The taxonomic identification of this host has been questioned (González-Ittig *et al.*, 2014), since the localities where the rodents were captured are very far from the known distribution of *O. fornesi* (north-east Argentina, east of Paraguay and south of Brazil; Musser & Carleton, 2005). Anajatuba genotype was also detected in *Necromys lasiurus* (Travassos da Rosa *et al.*, 2010); the authors attribute this finding to a spill-over transmission. However, up to now, molecular identification of the infected specimens has not been performed. An accurate determination of the Anajatuba host is critical since this genotype is responsible for several HPS cases in northern Brazil (Mendes *et al.*, 2001).

Maciel genotype was isolated from a rodent identified as *Necromys benefactus* (Levis *et al.*, 1998). A systematic study of the genus *Necromys* reported that *Necromys benefactus* is not a valid species and that specimens so named would correspond to *Necromys lasiurus* or to *Necromys obscurus* (D'Elía *et al.*, 2008). Since the rodent host of the Maciel genotype was not identified molecularly, we referred to it as *Necromys* sp.

Castelo do Sonhos genotype has been associated with several cases of HPS (Johnson *et al.*, 1999; Medeiros *et al.*, 2010); it was obtained from specimens of *O. utiaritensis* (Travassos da Rosa *et al.*, 2011; Agrellos et al., 2012), which was originally considered a junior synonym of *Oligoryzomys eliurus* (Musser & Carleton, 2005). However, *O. eliurus* is distributed in central and south-eastern Brazil, whereas the HPS cases associated with Castelo dos Sonhos (including Tunari strains) and the rodent specimens from which this genotype was recovered occur in the Amazonia biogeographic region (Johnson *et al.*, 1999; Travassos da Rosa *et al.*, 2011; Cruz *et al.*, 2012). Further studies delimiting the geographical range of *O. utiaritensis* are needed to infer the potential distribution of its associated virus.

Andes genotype was originally obtained from a HPS patient from southern Argentina (López *et al.*, 1996). Levis *et al.* (1998) identified *O. longicaudatus* as its host; later, Padula *et al.* (2004) demonstrated that this rodent acts as a natural reservoir, maintaining the infection in the population by horizontal transmission. Although less frequently, this viral genotype has also been detected in other native or introduced rodents. It was argued that these cases correspond to spill-over transmission since numerous seropositive rodents did not present viral RNA, suggesting a brief infection, as expected in nonhost species (Toro *et al.*, 1998).

O. nigripes would act as the Juquitiba primary host according to the frequency of virus detection in this rodent. This assumption is supported by the fact that the HPS cases associated with Juquitiba virus (Fig. 1) are restricted to the distribution range of this rodent (Carbajo & Teta, 2009).

Level of divergence among South American hantaviruses

Our analysis suggests that, for South American hantaviruses, amino acid divergence values above 4 % for the N protein clearly define different genotypes. Values below this level, however, deserve further attention, taking into account intra-lineage genetic variability, geographical distribution and host relationships. The 4 % criterion supports the reclassifications suggested in our present study, since the distances between Juquitiba and Araucaria, Paranoa and Araraquara, and Tunari and Castelo dos Sonhos strains fall below this level. Only the taxonomic status of the Hu39694, Oran and Lechiguanas clades remains uncertain. If we adopt the criterion established by the ICTV (7 %) to define the taxonomic status of South American hantaviruses, in several cases the genotype taxonomy is incongruent with the phylogenetic reconstruction. For example, clades Rio Mearim, Alto Paraguay, Rio Mamore and Anajatuba would correspond to the same virus species, separated from Laguna Negra. However, in the present phylogenetic reconstruction, the Anajatuba clade is closer to the latter than to the former ones. We cannot ignore the fact that the level of amino acid divergence within a group is related to the evolutionary history of its lineages and, hence, to the biogeographic history of the region where such a group is distributed. The result of our study clearly shows that the amino acid divergence values observed between South American hantaviruses are lower than those reported for European and Asian ones (Arai *et al.*, 2008), suggesting that a worldwide amino acid divergence level to define the taxonomic status of a virus lineage is not accurate. Therefore, we propose the application of the 4 % amino acid divergence criterion for South American hantaviruses.

Co-phylogeny

The phylogenetic reconciliation analysis clearly indicates that preferential host switching does not explain virus diversification at a regional scale in South America. On the other hand, host switching mediated by geographical host proximity would not be supported, since closely related *Hantavirus* lineages are allopatrically distributed

(e.g. Choclo–Jabora, Castelo do Sonhos-Lechiguanas; Fig. 1a, b). Furthermore, we did not find a biogeographic pattern in the *Hantavirus* phylogeny, since closely related genotypes are associated with different main zoogeographic regions (e.g. the group that comprises Andes, Castelo do Sonhos, Lechiguanas, Hu39694 and Oran is distributed in Patagonia, Amazonia, Pampas and Chaco–Cerrado–Caatinga). However, host switching mediated by geographical host proximity could have occurred previous to rodents' range expansions or shifts, after the last glacial maximum. Further studies taking into account not only present but also past host distribution would help to test if historical geographical host proximity contributes to explain South American *Hantavirus* diversification.

METHODS

Phylogenetic analyses. The phylogenetic analysis included all the available N gene sequences of members of the genus *Hantavirus* from South America. Non-coding regions were excluded from the analysis. The tree was rooted relative to the position of Puumala virus. One sequence of each Central and North American *Hantavirus* was added to test the monophyly of the South American group.

The matrix was analysed using MP and BI. MP was performed with TNT 1.1. (Goloboff *et al.*, 2008). Characters were unordered and equally weighted; no gaps were detected in the dataset. A heuristic search of 1000 iterations of random taxon addition was performed using the tree bisection–reconnection branch swapping algorithm. The most parsimonious trees resulting from the MP analysis were summarized in a strict consensus tree. Non-parametric bootstrap support values were calculated based on 1000 replicate searches.

For the BI, the GTR + I + G was selected as the best-fitting model of sequence evolution using jModeltest 0.1.1 (Posada, 2008). The BI was performed using MrBayes 3.2.2 (Ronquist *et al.*, 2012) in the CIPRES Science Gateway v3.3portal (http://www.phylo.org/portal2/; Miller *et al.*, 2010) with two independent Markov chain Monte Carlo (MCMC) runs, with one cold and three heated chains each. Runs were performed for 100 million generations and trees were sampled every 10 000 generations. Mixing, convergence to stable values and effective sample size (ESS) were checked with Tracer v1.5 (Rambaut & Drummond, 2007). The two runs converged on very similar posterior estimates with an average standard deviation of split frequencies of 0.01. We discarded the first 25 % of the samples as 'burn in' and used ESS and probability distribution values for all parameters higher than 500.

We also analysed if there is a biogeographical pattern in the phylogeny of South American hantaviruses by graphing, on the phylogenetic reconstruction, the virus distribution in accordance with the main zoogeographic regions described for mammals in Central and South America (Upham & Patterson, 2012): (1) Central America, including tropical Mexico, and Choco, (2) Andes (northern and central formations), (3) Amazonia, (4) Chaco–Cerrado–Caatinga, (5) Atlantic Forest, (6) Pampas and (7) Patagonia, including the Monte Desert and Southern Andes. We used this biogeographical classification since South American hantaviruses are rodent-borne.

Amino acid divergence calculation. Pairwise percentage amino acid sequence divergence for the N gene within and among clades was calculated with MEGA v4.0 software (Kumar *et al.*, 2004) using the Poisson correction model. We considered the minimum length of 300 aa which, according to Maes *et al.* (2009), is needed to accurately identify *Hantavirus* species.

Co-phylogeny. Before performing the phylogenetic reconciliation analysis, we condensed all viral sequences from the same clade into a single representative taxon. Then, to infer the primary host for each clade, we considered information from literature and the frequency a virus was detected in a specific rodent host in our database (Table S1). To obtain the rodent phylogeny we used the cyt-*b* gene as molecular marker. Whenever possible, we selected sequences of individuals from which *Hantavirus* genotypes were detected. In the cases where the sequence of an infected specimen was not available, we selected a cyt-*b* sequence of the species analysed in reliable phylogenies (Hanson *et al.*, 2011; González-Ittig *et al.*, 2014). The tree was rooted relative to the position of *Peromyscus maniculatus* (JF489123) and *Neotoma lepida* (AF307833). The matrix was analysed using Bayesian methods. For the BI, the GTR + I + G was selected as the best-fitting model using jModeltest 0.1.1. The BI was performed using MrBayes 3.2.2 in the CIPRES Science Gateway v3.3 portal with two independent MCMC runs, with one cold and three heated chains each. Runs were performed for two million generations and trees were sampled every 1000 generations. Mixing, convergence to stable values and ESS were checked with Tracer v1.5. The two runs converged on very similar posterior estimates with an average standard deviation of split frequencies of 0.01. We discarded the first 25 % of the samples as 'burn in' and used ESS and probability distribution values for all parameters higher than 500.

We used the program TreeMap3b124 (https://sites.google.com/site/cophylogeny/treemap) to perform a host–parasite phylogenetic comparison, which attempts to explain both the observed virus phylogeny and the distribution of viruses in the host lineages in the most parsimonious manner (Charleston, 1998; Jackson & Charleston, 2004). Based on this, the concordances of host and virus cladograms are considered preferential host switching and the discordances, random host switching.

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