

A new phylogenetic lineage of *Rabies virus* associated with western pipistrelle bats (*Pipistrellus hesperus*)

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Bats represent the major source of human rabies cases in the New World. In the USA, most cases are associated with species that are not commonly found or reported rabid. To understand better the epidemiology and public health significance of potentially important bat species, a molecular study was performed on samples collected from naturally infected rabid western pipistrelle (*Pipistrellus hesperus*), eastern pipistrelle (*Pipistrellus subflavus*) and silver-haired bats (*Lasiurus noctivagans*) from different regions of their geographical distribution in the USA. A 264 bp fragment at the 5' end of the N gene coding region was sequenced and analysed in comparison with rabies virus variants circulating within other North American mammals. Phylogenetic analysis demonstrated that *P. hesperus* bats maintain a unique rabies virus variant. Preliminary data also suggest that *P. subflavus* and *Lasiurus noctivagans* may harbour two different rabies virus variants (Ps and Ln) that are likely to be maintained independently by each bat species, which recently appear to have emerged as major vectors of human disease.

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INTRODUCTION

Despite the existence of highly effective animal rabies prevention and control programmes, this zoonotic disease remains a significant cause of human mortality throughout the world (WHO, 2005). Currently, most human rabies deaths are typically not related to inadequate biologicals, but rather as a result of a failure to recognize the risk of disease transmission and to seek appropriate prophylaxis following the exposure to an infected animal. A key facet to understanding the epidemiology of rabies involves a better appreciation of viral variants maintained by different mammalian species. High levels of genetic and antigenic diversity of *Rabies virus* (RABV) have been reported in association with distinct species of mammals in the Americas (Rupprecht *et al.*, 1991; Nadin-Davis *et al.*, 2001; Smith, 2002; Velasco-Villa *et al.*, 2005). Specific genetic and antigenic patterns exist among RABV lineages. Recent studies suggest that viral

variants tend to be host-associated and maintained in populations predominantly through intraspecific transmission, as exemplified by viral variants or biotypes found among common North American taxa, such as: domestic dogs (*Canis familiaris*), grey foxes (*Urocyon cinereoargenteus*), eastern spotted skunks (*Spilogale putorius*), striped skunks (*Mephitis mephitis*), raccoons (*Procyon lotor*) and bats of several species.

Worldwide, most human rabies cases are caused by rabies viruses associated with dogs. However, in the Americas, RABVs associated with bats are emerging as a disproportionate source for human infection (Rupprecht *et al.*, 1995; Messenger *et al.*, 2002; Belotto *et al.*, 2005). To date, bat species frequently reported rabid in the USA include the big-brown bat (*Eptesicus fuscus*), the Brazilian (Mexican) free-tailed bat (*Tadarida brasiliensis*) and in the western region, the California myotis (*Myotis californicus*). More limited numbers of cases are identified in the hoary bat (*Lasiurus cinereus*), the red bat (*Lasiurus borealis*) and the little-brown bat (*Myotis lucifugus*). Interpretation of surveillance results

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are DQ445308–DQ445382.

is complicated by the fact that certain bat species may be difficult to identify and therefore results could be biased because of misidentification. Despite the frequency of reported rabies cases in *E. fuscus* and *T. brasiliensis*, 32 of 58 human rabies deaths reported from 1958 to 2000 in the USA were caused by RABV variants harboured by other bat species (16 *Pipistrellus subflavus*, eight *Lasionycteris noctivagans*, five *T. brasiliensis*, two *Myotis californicus* and one *E. fuscus*) (Messenger *et al.*, 2002). Two bat species uncommonly submitted to state public health laboratories for diagnosis and rarely found around human dwellings were most frequently associated with human rabies cases: the silver-haired bat (*Lasionycteris noctivagans*) and the eastern pipistrelle (*P. subflavus*). The prevalence of Ln/Ps (*Lasionycteris noctivagans* and *P. subflavus*) RABV variant in recent human rabies cases is puzzling because *Lasionycteris noctivagans* and *P. subflavus* bats are seldom found or reported to be rabid (CDC, 1994). Since both species prefer habitats far from human dwellings, one explanation may be that many bats die of rabies most frequently during summer in forest habitats where they are seldom observed by people. Two other hypotheses have been proposed to explain this phenomenon: (i) increased virulence of Ln/Ps RABV relative to other virus variants, and (ii) the existence of unique host characteristics, such as the so-called 'small vector' hypothesis (Morimoto *et al.*, 1996; Messenger *et al.*, 2003). The latter hypothesis describes a failure to recognize or appreciate the significance of a bite when a small bat is involved, because of the limited severity of the lesion produced. Support of the former hypothesis has been suggested by results from experimental data, comparing infectivity of Ln/Ps RABV variant with those from domestic canids (Morimoto *et al.*, 1996; Dietzschold *et al.*, 2000).

In North America, RABV has been detected in almost every bat species (Constantine, 1979). However, due to problems of passive surveillance, only limited data are available for RABV variants associated with less abundant species. Several bat species, such as *Pipistrellus hesperus*, are more reclusive or restricted in distribution and rarely come into contact with humans or domesticated animals. Relatively few samples obtained from rabid *P. hesperus* have been available for analysis, even though this species is relatively abundant in desert and grassland habitats of the western USA where they roost in rock crevices (Barbour & Davis, 1969). As the smallest bat in the USA, *P. hesperus* is an excellent example for consideration to continue study of the 'small vector' hypothesis, by using a molecular approach.

In the present study, RABV of *P. hesperus* was characterized based on the last 264 bp of the 5' end of the nucleoprotein (N) gene coding region. In Arizona and California, 30 samples were obtained from *P. hesperus* bats during the years 2000–2005, sequenced and compared with nine historical samples (collected during 1981–1997) from *P. hesperus* from Arizona (Flagstaff), available in GenBank. These sequences were also compared to more than 300 sequences of RABV collected from different mammalian species throughout

North America. Additionally, we compared sequences of Ln/Ps RABV from *Lasionycteris noctivagans* and *P. subflavus*, to inquire if they have species-specific markers or should be considered as a single RABV variant.

METHODS

P. hesperus bats were collected as a part of rabies public health surveillance investigations in California and Arizona during 2000–2005. *P. subflavus* and *Lasionycteris noctivagans* were collected via state public health submissions during the period 1976–2004 from different regions of the USA (Fig. 1). Brains were removed and rabies diagnoses were performed by the direct fluorescent antibody test (Dean *et al.*, 1996). In addition, another 30 samples of *P. hesperus*, 14 samples of *P. subflavus* and 22 samples of *Lasionycteris noctivagans* bats were used for further characterization (Table 1). Total RNA was extracted from infected bat brains with TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. RT-PCR was performed with primer sets designed for the rabies virus nucleoprotein (N): forward primer 113fw (5'-GTAGGATGC-TATATGGG-3', position 1013–1029, according to the PV genome); and reverse primer 304 (Trimarchi & Smith, 2002).

Direct sequencing of the RT-PCR products was performed from purified PCR products. We sequenced fragments of RABV N gene by using forward primers: 1066 (5'-GAGAGAAGATTCTTCAGGGA-3', position 1136–1155) and 113fw, and reverse primer: 304 (Trimarchi & Smith, 2002) (all positions are according to the PV genome, GenBank accession no. M13215). Because of limited length of sequences available in GenBank and the abundance of RABV sequences from the last 300 nt of the N gene coding region, further comparisons were focused on the variable 264 nt corresponding to bases 1157–1420 and aa 363–450. Use of this fragment allowed us to increase the robustness of the analysis and provided better resolution for the detection of possible spillover events between species. Previous analyses showed that this short fragment from the 5' end of the N gene generates the same branching pattern as the whole N gene sequence (Smith *et al.*, 1992).

A phylogenetic analysis was undertaken using more than 300 RABV sequences (only 133 unique representatives were used for the final analysis and are shown in the Table 1), originating from different North American mammals, available from GenBank, including nine historical sequences from *P. hesperus* collected in Arizona (Table 1) and sequences from *P. subflavus* and *Lasionycteris noctivagans* available in the CDC archival database or otherwise sequenced for this study. Sequences were edited using BioEdit software (Hall, 1999) and multiple alignments were built using the CLUSTAL X package (Jeanmougin *et al.*, 1998). *Duvenhage virus* and *European bat lyssavirus 2* (EBLV-2) were used as outgroup taxa. A neighbour-joining (NJ) analysis (p-distance model) with 1000 bootstrap replicates was performed using the MEGA computer program, version 2.1. (Kumar *et al.*, 2001). The NJPLOT program from the CLUSTAL X package and the Tree-Explorer module of MEGA were used to obtain graphical output of phylogenetic estimations. Bootstrap values of more than 70% were considered as providing support for phylogenetic grouping. Representatives of particular lineages were selected for the final phylogenetic analysis.

In addition, both maximum-likelihood (ML) and Bayesian analyses were performed on the dataset to support or clarify results of NJ analysis. A total of 133 RABV sequences were analysed, including seven sequences from raccoon, skunk and dog RABVs that were used as outgroup taxa. MODELTEST (Posada & Crandall, 1998) analysed 56 models of evolution to determine the most appropriate selection for the dataset. The HKY85 model (Hasegawa *et al.*, 1985) with a gamma

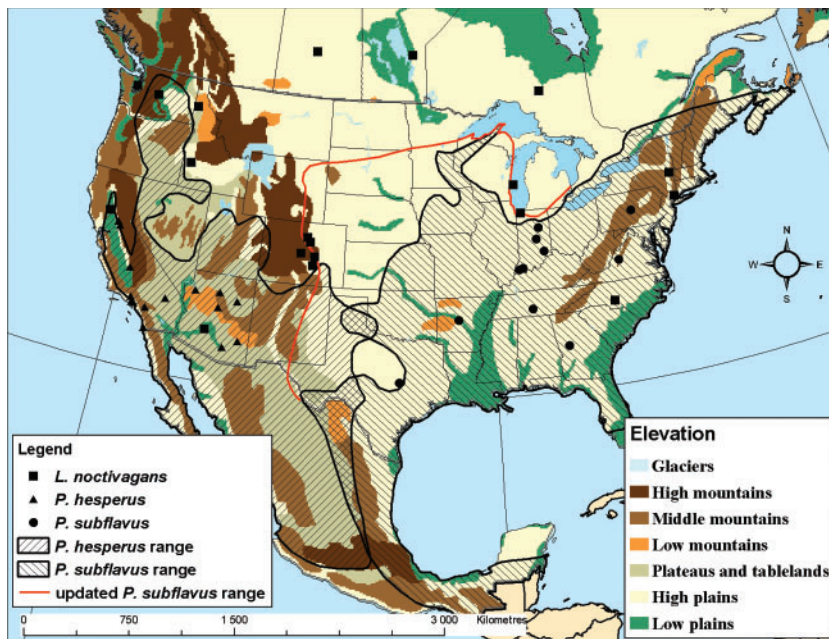


Fig. 1. Locations where *P. hesperus* (California, Arizona), *P. subflavus* (Pennsylvania, Georgia, Tennessee, Texas, Arkansas, Indiana and Virginia) and *Lasionycteris noctivagans* (Ontario, British Columbia, New York, Colorado, Wisconsin, Washington, Idaho, North Carolina and California) were collected (sequences from GenBank were collected in Flagstaff, AZ). *Lasionycteris noctivagans* is distributed throughout the USA and southern Canada (Wilson & Ruff, 1999). Geographical distribution of *P. hesperus* and *P. subflavus* according to Wilson & Ruff (1999). The red line represents recently reported westward expansion of *P. subflavus* (Geluso *et al.*, 2005).

distribution (HKY85 + G) was selected and subsequently implemented for the ML analysis in PAUP*4.0b10 (Swofford, 2002). Nucleotide frequencies were A = 0.34190, C = 0.24190, G = 0.20470, T = 0.21150, the transition-to-transversion ratio = 4.3131 and the gamma shape parameter = 0.3395.

MrBayes 3.1.1 (Ronquist & Huelsenbeck, 2003) was used for a Bayesian analysis with the general time-reversible model incorporating both invariant sites and a gamma distribution (GTR + I + G) to examine the data with a more complex model than used in the ML analysis. Two simultaneous analyses, each with four Markov chains, were run for 2,000,000 generations and sampled every 100 generations. Trees generated prior to the stabilization of likelihood scores were discarded, (burnin = 450). The remaining trees were used to build a 50% majority rule consensus tree. Posterior probability values were used to assess support at each node (≥ 95 = statistical support).

The deduced amino acid sequences from the consensus of particular monophyletic clades were aligned for comparison as well. All new RABV sequences reported in this study were submitted to GenBank (Table 1).

RESULTS

The overall branching pattern of the trees constructed in our study by using the NJ, ML and Bayesian methods was the same regardless of the method used. RABV variants harboured by North American bats formed two major clades. One was formed by the Ph1, Ph2 and *Myotis* clades and another one by *Lasionycteris noctivagans*, *Lasiurus cinereus*, *Lasiurus borealis* and *P. subflavus*. *E. fuscus* 1–3 clades represented groups that branched differently if we used different methods.

In the NJ analysis, 79% (31 samples) of RABV samples originating from *P. hesperus* bats in California and Arizona (a large part of their geographical range in the USA) formed a monophyletic clade (Ph1) with high bootstrap support,

separated from other phylogenetic lineages of RABV (Fig. 2). A high level of nucleotide sequence similarity (98.7%) within this clade originating from different locations was observed over the period of sample collection (1981–2004). Five samples (13%) collected from *P. hesperus* in California in 2002–2005, formed a separate clade (Ph2), which was a sister to a clade consisting of *Myotis* species and *P. hesperus* with inconsistent bootstrap support (Fig. 2). Nucleotide sequence similarity within Ph2 clade was 98.8% and within the *Myotis* species clade was 95.5%. Nucleotide sequence similarity between Ph1 and Ph2 was 94.3%, between Ph2 and the *Myotis* species clade was 94.3% and between Ph1 and *Myotis* species clade was 91.7%. Only three (8%) samples collected from *P. hesperus* belonged to other RABV lineages (*Myotis* species), suggesting occasional infectious spillover events from *Myotis* species. Only four of more than 50 sequences (< 8%) of RABV originating from *Myotis* species were found in the Ph1 clade. The *P. hesperus* (Ph) variants Ph1 and Ph2 shared a most recent common ancestor with a RABV variant associated with *Myotis* species (a RABV variant associated with different bat species from the genus *Myotis*). The separation between the Ph1 and Ph2 clades was based on 11 synonymous nucleotide substitutions within the N gene fragment, G/A¹¹⁶⁵, C/T¹²³⁷, C/T¹²⁴⁰, G/A¹²⁶⁴, C/A¹²⁶⁸, T/A¹²⁸², T/C¹²⁹¹, T/C¹²⁹⁷, G/A¹³¹⁵, A/G¹³²⁴, A/C¹³⁴⁸ and one non-synonymous substitution T/G¹²⁰⁵ (aa L/V³⁷⁹).

While closely related to each other, the viruses originating from *P. subflavus* and *Lasionycteris noctivagans* separated into two clusters with no overlap between clusters. The monophyletic clade of *P. subflavus* (Ps) included 13 sequences of this species originating from different geographical locations including Pennsylvania, Georgia, Tennessee, Arkansas, Indiana, Virginia and Texas, collected

Table 1. List of samples used

Identification no.	Mammalian species*	State	County or city	Year of collection	GenBank accession no.
<i>Pipistrellus hesperus</i>					
AY170251Azph81	<i>P. hesperus</i>	Arizona	Coconino	1981	AY170251
AF394870Azph93	<i>P. hesperus</i>	Arizona	Mohave	1993	AF394870
AY170263Azph93	<i>P. hesperus</i>	Arizona	Maricopa	1993	AY170263
AY170261Azph95	<i>P. hesperus</i>	Arizona	Coconino	1995	AY170261
AY170252Azph96	<i>P. hesperus</i>	Arizona	Navajo	1996	AY170252
AY170254Azph96	<i>P. hesperus</i>	Arizona	Maricopa	1996	AY170254
AY170250Azph97	<i>P. hesperus</i>	Arizona	Pima	1997	AY170250
AY170256Azph97	<i>P. hesperus</i>	Arizona	Pima	1997	AY170256
AY170257Azph97	<i>P. hesperus</i>	Arizona	Pima	1997	AY170257
Cab1306ph00	<i>P. hesperus</i>	California	San Bernardino	2000	DQ445308†
Cab1308ph00	<i>P. hesperus</i>	California	San Bernardino	2000	DQ445309†
Cab1319ph00	<i>P. hesperus</i>	California	San Bernardino	2000	DQ445310†
Cab1324ph00	<i>P. hesperus</i>	California	San Bernardino	2000	DQ445311†
Cab1325ph00	<i>P. hesperus</i>	California	San Bernardino	2000	DQ445312†
Cab1326ph00	<i>P. hesperus</i>	California	San Bernardino	2000	DQ445313†
Cab1328ph00	<i>P. hesperus</i>	California	San Bernardino	2000	DQ445314†
Cab1329ph00	<i>P. hesperus</i>	California	San Bernardino	2000	DQ445315†
Cab1330ph00	<i>P. hesperus</i>	California	San Bernardino	2000	DQ445316†
Cab1332ph00	<i>P. hesperus</i>	California	San Bernardino	2000	DQ445317†
Cab1347ph01	<i>P. hesperus</i>	California	San Bernardino	2001	DQ445318†
Cab1349ph01	<i>P. hesperus</i>	California	San Bernardino	2001	DQ445319†
Cab1354ph01	<i>P. hesperus</i>	California	San Bernardino	2001	DQ445320†
Cab1355ph01	<i>P. hesperus</i>	California	San Bernardino	2001	DQ445321†
Cab1382ph01	<i>P. hesperus</i>	California	San Bernardino	2001	DQ445322†
Cab1385ph01	<i>P. hesperus</i>	California	San Bernardino	2001	DQ445323†
Cab1401ph01	<i>P. hesperus</i>	California	San Bernardino	2001	DQ445324†
Cab1402ph01	<i>P. hesperus</i>	California	San Bernardino	2001	DQ445325†
Car1406ph01	<i>P. hesperus</i>	California	Riverside	2001	DQ445326†
Car1415ph01	<i>P. hesperus</i>	California	Riverside	2001	DQ445327†
Car1416ph01	<i>P. hesperus</i>	California	Riverside	2001	DQ445328†
Car1419ph01	<i>P. hesperus</i>	California	Riverside	2001	DQ445329†
Casdl1420ph01	<i>P. hesperus</i>	California	San Diego	2001	DQ445330†
Car1421ph01	<i>P. hesperus</i>	California	Riverside	2001	DQ445331†
Caaub1424ph02	<i>P. hesperus</i>	California	El Dorado	2002	DQ445332†
Ca2167ph03	<i>P. hesperus</i>	California	Los Angeles	2003	DQ445333†
Ca2149ph03	<i>P. hesperus</i>	California	Castaic	2003	DQ445334†
Ca2022ph04	<i>P. hesperus</i>	California	Encino	2004	DQ445335†
Az01ph04	<i>P. hesperus</i>	Arizona	Maricopa	2004	DQ445336†
Ca164ph05	<i>P. hesperus</i>	California	Tulare	2005	DQ445337†
<i>Myotis</i> species‡					
AF351837ml7alta79	<i>M. lucifugus</i>	Alberta	NA	1979	AF351837
Ny112ml83	<i>M. lucifugus</i>	New York	NA	1983	DQ445338†
Ca1563mc86	<i>M. californicus</i>	California	Shasta	1986	DQ445339†
AF394871Camc87	<i>M. californicus</i>	California	Plumas	1987	AF394871
AF351835me1bc92	<i>M. evotis</i>	British Columbia	NA	1992	AF351835
AF351836mc2bc92	<i>M. californicus</i>	British Columbia	NA	1992	AF351836
AF351839ml4bc92	<i>M. lucifugus</i>	British Columbia	NA	1992	AF351839
AF351834ml5bc92	<i>M. lucifugus</i>	British Columbia	NA	1992	AF351834
Ca2375me93	<i>M. evotis</i>	California	NA	1993	DQ445340†
Ca2373ml93	<i>M. lucifugus</i>	California	NA	1993	DQ445341†
AF351838ml6ns94	<i>M. lucifugus</i>	Nova Scotia	NA	1994	AF351838
Az3850mv96	<i>M. velifer</i>	Arizona	Show Low	1996	DQ445342†

Table 1. cont.

Identification no.	Mammalian species*	State	County or city	Year of collection	GenBank accession no.
AY170249Azmc96	<i>M. californicus</i>	Arizona	Pima	1996	AY170249
AY170260Azmc96	<i>M. ciliolabrum</i>	Arizona	Holbrook	1996	AY170260
AY170253Azmsp97	<i>Myotis</i> species	Arizona	Pima	1997	AY170253
AY170255Azmsp99	<i>Myotis</i> species	Arizona	Pima	1999	AY170255
AY170258Azmsp99	<i>Myotis</i> species	Arizona	Bullhead City	1999	AY170258
<i>Eptesicus fuscus</i>					
AF351832ef34bc72	<i>E. fuscus</i>	British Columbia	NA	1972	AF351832
AF351830ef22bc88	<i>E. fuscus</i>	British Columbia	NA	1988	AF351830
AF351827ef1ont93	<i>E. fuscus</i>	Ontario	NA	1993	AF351827
AF351828ef32ont93	<i>E. fuscus</i>	Ontario	NA	1993	AF351828
Tn3307ef96	<i>E. fuscus</i>	Tennessee	Knox	1996	DQ445343†
AF351854ef72usa98	<i>E. fuscus</i>	Connecticut	NA	1998	AF351854
<i>Lasiurus cinereus</i>					
AF351846lc2ont93	<i>L. cinereus</i>	Ontario	NA	1993	AF351846
<i>Lasiurus borealis</i>					
Al2007lb78	<i>L. borealis</i>	Alabama	Mobile	1978	DQ445344†
Ny71lb81	<i>L. borealis</i>	New York	NA	1981	DQ445345†
AF351844lb1ont94	<i>L. borealis</i>	Ontario	NA	1994	AF351844
AF351857lb7ctusa98	<i>L. borealis</i>	Connecticut	NA	1998	AF351857
In4646lb99	<i>L. borealis</i>	Indiana	Vanderburg	1999	DQ445346†
<i>Pipistrellus subflavus</i>					
In1071ps89	<i>P. subflavus</i>	Indiana	Vanderburg	1989	DQ445347†
AF394881ps1435ar91	<i>P. subflavus</i>	Arkansas	Houston	1991	AF394881
In3173ps92	<i>P. subflavus</i>	Indiana	Vanderburg	1992	DQ445348†
In3189ps94	<i>P. subflavus</i>	Indiana	Warrick	1994	DQ445349†
In3210ps95	<i>P. subflavus</i>	Indiana	Marion	1995	DQ445350†
Tn3315ps96	<i>P. subflavus</i>	Tennessee	Marshall	1996	DQ445351†
In3665ps97	<i>P. subflavus</i>	Indiana	Jackson	1997	DQ445352†
Pa3708ps98	<i>P. subflavus</i>	Pennsylvania	NA	1998	DQ445353†
Pa3709ps98	<i>P. subflavus</i>	Pennsylvania	NA	1998	DQ445354†
Ga4025ps98	<i>P. subflavus</i>	Georgia	Upson	1998	DQ445355†
Va4500ps99	<i>P. subflavus</i>	Virginia	NA	1999	DQ445356†
Va4502ps99	<i>P. subflavus</i>	Virginia	NA	1999	DQ445357†
Tx3775ps02	<i>P. subflavus</i>	Texas	Smith	2002	DQ445358†
Tx5512ps03	<i>P. subflavus</i>	Texas	Smith	2003	DQ445359†
Tx5168ps04	<i>P. subflavus</i>	Texas	Smith	2004	DQ445360†
<i>Lasionycteris noctivagans</i>					
Ca806ln76	<i>L. noctivagans</i>	California	Butte	1976	DQ445361†
AF351841lan13ont80	<i>L. noctivagans</i>	Ontario	NA	1980	AF351841
Co352ln84	<i>L. noctivagans</i>	Colorado	El Paso	1984	DQ445362†
Co353ln84	<i>L. noctivagans</i>	Colorado	Boulder	1984	DQ445363†
AF394880ln2152ny84	<i>L. noctivagans</i>	New York	Westcheste	1984	AF394880
Ny2153ln85	<i>L. noctivagans</i>	New York	Albany	1985	DQ445364†
Co443ln86	<i>L. noctivagans</i>	Colorado	Pueblo	1986	DQ445365†
Co911ln87	<i>L. noctivagans</i>	Colorado	Larimer	1987	DQ445366†
AF351840lan12sask88	<i>L. noctivagans</i>	Saskatchewan	NA	1988	AF351840
Wa898ln89	<i>L. noctivagans</i>	Washington	NA	1989	DQ445367†
Co1105ln89	<i>L. noctivagans</i>	Colorado	Denver	1989	DQ445368†
In3162ln91	<i>L. noctivagans</i>	Indiana	Porter	1991	DQ445369†
AF351842lan8man92	<i>L. noctivagans</i>	Manitoba	NA	1992	AF351842
Wi2284ln93	<i>L. noctivagans</i>	Wisconsin	Ozaukee	1993	DQ445370†
AY170248ln3057az95	<i>L. noctivagans</i>	Arizona	Maricopa	1995	AY170248
Id3275ln96	<i>L. noctivagans</i>	Idaho	Boise	1996	DQ445371†

Table 1. cont.

Identification no.	Mammalian species*	State	County or city	Year of collection	GenBank accession no.
Id3277ln96	<i>L. noctivagans</i>	Idaho	Benewah	1996	DQ445372†
Co5153ln96	<i>L. noctivagans</i>	Colorado	NA	1996	DQ445373†
Wa0323ln97	<i>L. noctivagans</i>	Washington	NA	1997	DQ445374†
Wa341ln97	<i>L. noctivagans</i>	Washington	NA	1997	DQ445375†
Wa410ln97	<i>L. noctivagans</i>	Washington	NA	1997	DQ445376†
Wa580ln98	<i>L. noctivagans</i>	Washington	NA	1998	DQ445377†
Nc4249ln99	<i>L. noctivagans</i>	North Carolina	Stanly	1999	DQ445378†
Wa161ln00	<i>L. noctivagans</i>	Washington	NA	2000	DQ445379†
Co5290ln00	<i>L. noctivagans</i>	Colorado	NA	2000	DQ445380†
Wa1185ln03	<i>L. noctivagans</i>	Washington	NA	2003	DQ445381†
Wa01ln04	<i>L. noctivagans</i>	Washington	Kitsap	2004	DQ445382†
<i>Homo sapiens</i>					
AF394882Tnhm94	<i>Homo sapiens</i>	Tennessee	Roane	1994	AF394882
<i>Canis familiaris</i>					
AF351848cf1alta98	<i>Canis familiaris</i>	Alberta	NA	1998	AF351848
<i>Vulpes vulpes</i>					
AF351851vv2pei93	<i>Vulpes vulpes</i>	Prince Edward IIs.	NA	1993	AF351851

*Species from which virus was collected.

†Sequences generated in our study.

‡*Myotis* species represents bats identified as *Myotis* species; NA, location unknown.

during the time period of 1989–2004 (Table 1, Fig. 1). The cluster of *Lasionycteris noctivagans* (Ln) included 24 sequences of *Lasionycteris noctivagans* originating from Ontario, British Columbia, New York, Colorado, Wisconsin, Washington, Idaho, North Carolina and California, collected during the period of 1976–2004, and two sequences from *Myotis lucifugus* originating from Alberta and British Columbia, collected in 1979 and 1992, respectively (Table 1, Fig. 1). The separation between the Ps and Ln clades was based on three conservative synonymous nucleotide substitutions within the studied N gene fragment, G/A¹¹⁷², A/T¹¹⁸⁴ and G/A¹²⁸³ (according to the PV genome). Spillovers of the *Lasiurus borealis* RABV variant to *P. subflavus*, and spillover of the *E. fuscus* RABV variant to *Lasionycteris noctivagans* were detected (Fig. 2).

Comparison of amino acid consensus sequences from clades of *P. hesperus*, *P. subflavus*, *Lasionycteris noctivagans* and *Myotis* species revealed that most of the nucleotide mutations from the last 264 nt of the N coding region in these particular bat variants were synonymous. Moreover, 100% amino acid similarity was found between the Ln/Ps, *Myotis* species, Ph2 and LBNA (*Lasiurus borealis* North America) variants. The amino acid consensus sequence of *P. hesperus* RABV variant (Ph1) contained a substitution of valine to leucine at position 379 aa (according to PV strain) in comparison with *Myotis* species, Ln/Ps, Ph2 and LBNA variants (Fig. 3). Interestingly, amino acid consensus sequences of five samples collected from *P. hesperus* (Ph2), branching separately from the main *P. hesperus*

clade (Ph1) and separately from the *Myotis* species clade (Fig. 2), while sharing a common ancestor, have a valine at position 379 aa. (Fig. 3).

The trees generated by the ML (Fig. 4) and Bayesian (Fig. 5) analyses resulted in similar topologies. In terms of the overall structure of the trees, two large clades could be identified: one consisting of Ph1, Ph2 and *Myotis* species (Bayesian posterior probability = 94) and another containing *Lasionycteris noctivagans*, *Lasiurus cinereus*, *Lasiurus borealis*, *P. subflavus*, *E. fuscus* 3, *D. rotundus* and *T. brasiliensis* (Bayesian posterior probability = 99). Following the two large clades, a cluster of *E. fuscus* samples (1 and 2) joined as the basal group of bat RABVs (Bayesian posterior probability = 99). Results from the NJ analysis (Fig. 2), however, indicated a different structure. In addition to Ph1, Ph2 and *Myotis* species found in the first large clade, samples from *E. fuscus* (1, 2 and 3), *D. rotundus* and *T. brasiliensis* were also included here, albeit with low bootstrap support. The second largest clade then consisted of *Lasiurus cinereus*, *Lasiurus borealis*, *P. subflavus* and *Lasionycteris noctivagans* with bootstrap support at 98.

Minor differences were noted in the placement of the Ph2 clade, which was basal to a clade containing both Ph1 and *Myotis* species in the ML analysis (Fig. 4). In the Bayesian analysis (Fig. 5), as was also the case in the NJ analysis (Fig. 2), Ph2 was sister to the clade of *Myotis* species, and that clade was then sister to Ph1. High support was found for the Ph1 clade (bootstrap = 93, posterior probability = 100) in all analyses.

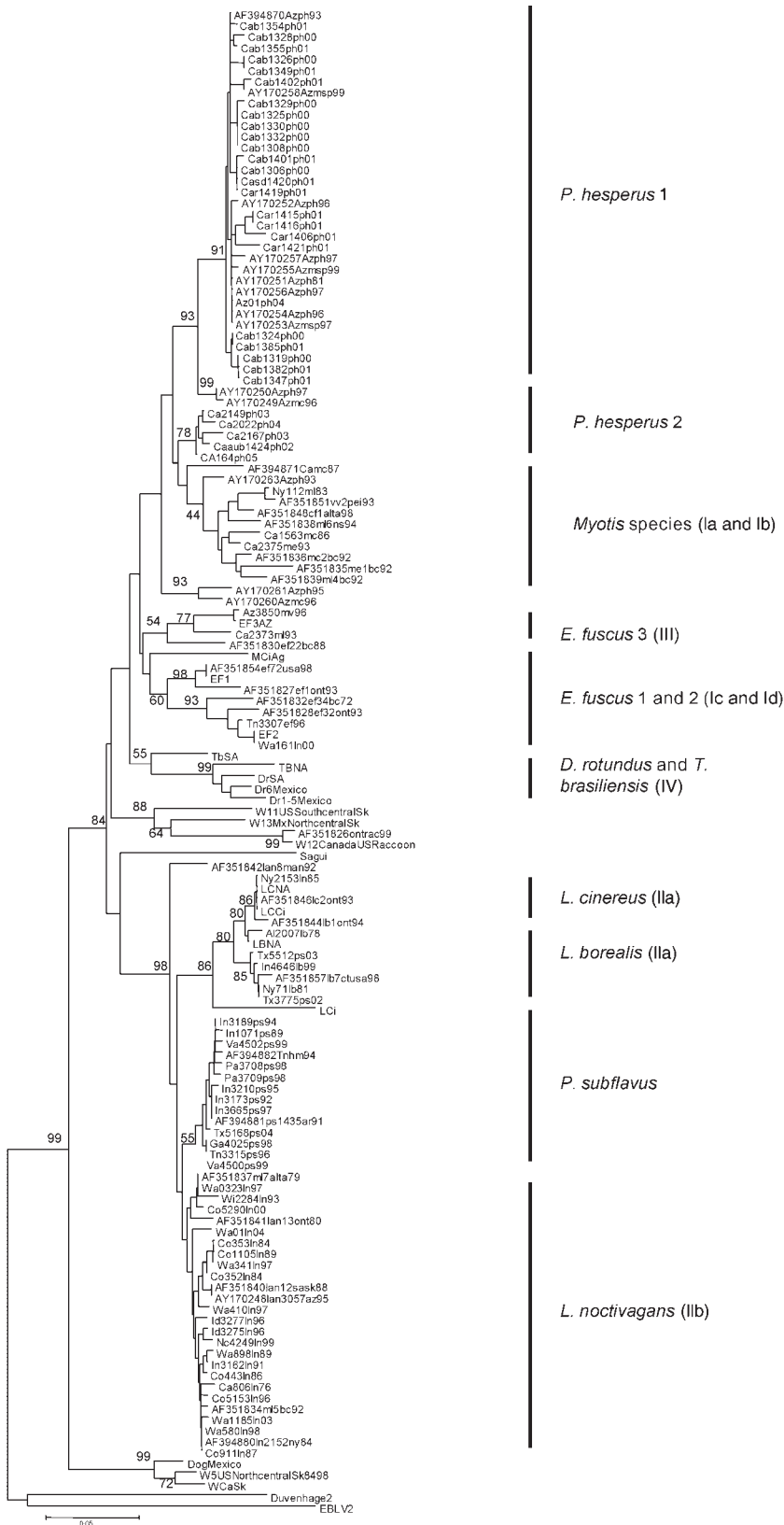


Fig. 2. Phylogenetic tree of bat and terrestrial lyssaviruses using NJ analysis (p-distance model) of a portion of the N gene coding sequence (264 nt) with 1000 bootstrap replicates. Groups I-IV as previously published (Nadin-Davis *et al.*, 2001). Other consensus sequences: EF1 and EF2, *E. fuscus* North America; EF3AZ, *Eptesicus fuscus* Arizona; MCIaAg, *Myotis* species Chile and Argentina; TbSA, *Tadarida brasiliensis* South America; TBNA, *Tadarida brasiliensis* North America; Dr6Mexico and Dr1-5Mexico, *Desmodus rotundus* Mexico; LCI, *Lasiurus* species Chile; LBNA, *Lasiurus borealis* North America; LCNA, *Lasiurus cinereus* North America; LCCi, *Lasiurus cinereus* Chile; W13MxNorthcentralSk, North Central skunk Mexico; W11USSouthcentralSk, South Central skunk USA; W12CanadaUSRaccoon, raccoon Canada and USA; W5USNorthcentralSk8498, North Central skunk USA; EBLV2, European Bat Lyssavirus 2; Sagui, rabies virus from common marmoset (*Callithrix jacchus jacchus*) from Brazil. Consensus sequences as in Velasco-Villa *et al.* (2005, 2006).

	363	370	380	390	400	410	420	430	440	450
Consensus	:	EKELQEYEAALTKTEVALADDGTVNSDDEDFYSSETRSP	EAIVYTRIMMNGGRLKRSHIRRYVSVSSNHQARPN	SAEFLNKTYSSDS						
Myotis	:
Ph1	:L.....
Ph2	:
Ln Ps	:
EF1	:S.....I.....G.....
EF2	:S.....D.....G.....I.....N.....
EF3AZ	:S.....
LCNA	:S.....
LCCi	:S.....
LCi	:D.....NA.P.....I.....Q.....S.....
LBNA	:
TBNA	:ADT.....T.....
TbSA	:M.....N.....
DrMexico	:A.T.....
DrSA	:A.T.....
PV	:D.....G.....I.....

Fig. 3. Consensus amino acid sequences of major bat rabies viruses (5' end of the N gene). Myotis, *Myotis* species; Ph1 and Ph2, *P. hesperus*; Ln Ps, *Lasionycteris noctivagans* and *P. subflavus*; EF1 and EF2, *Eptesicus fuscus* North America; EF3AZ, *Eptesicus fuscus* Arizona; LCNA, *Lasiurus cinereus* North America; LCCi, *Lasiurus cinereus* Chile; LCi, *Lasiurus* species Chile; LBNA, *Lasiurus borealis* North America; TBNA, *Tadarida brasiliensis* North America; TbSA, *Tadarida brasiliensis* South America; DrMexico, *Desmodus rotundus* Mexico; DrSA, *Desmodus rotundus* South America; PV, Pasteur virus.

The placement of taxa within the large clade containing *Lasionycteris noctivagans*, *Lasiurus cinereus*, *Lasiurus borealis* and *P. subflavus* also differed in the three analyses. According to the NJ tree (Fig. 2), a sister relationship was found between *P. subflavus* and *Lasionycteris noctivagans*, but bootstrap support was low. This clade was then sister to a clade comprised of *Lasiurus cinereus* and *Lasiurus borealis*, with a bootstrap value of 98 for the association of the four groups. In the ML analysis (Fig. 4), it appeared that the *Lasionycteris noctivagans*, *Lasiurus cinereus/borealis* and *P. subflavus* clades were unresolved in relation to each other. Finally, a clade of *Lasiurus cinereus* and *Lasiurus borealis* (posterior probability = 100) was sister to *Lasionycteris noctivagans*, which then grouped with the *P. subflavus* clade in the Bayesian tree (Fig. 5). Support for the *Lasionycteris noctivagans*, *Lasiurus cinereus*, *Lasiurus borealis* and *P. subflavus* clade was indicated by a posterior probability value of 100.

DISCUSSION

Previous phylogenetic analyses of nucleotide sequence data from representative RABV variants of North American bat species, at both the N and G (glycoprotein) gene loci, identified two major clades and four principal phylogenetic groups (I–IV), which were associated with particular bat species (Nadin-Davis *et al.*, 2001). Among North American bat RABV variants, a notable division was shown between group (clade) I specimens associated with colonial, non-migratory bats (*Myotis* species and *E. fuscus*) and those of group (clade) II harboured mainly by solitary, migratory species (*Lasiurus* species and *Lasionycteris noctivagans*). These conclusions, however, will need to be re-evaluated, because many publications have suggested the gregarious behaviour of *Lasionycteris noctivagans* (Parsons *et al.*, 1986; Campbell *et al.*, 1996; Mattson *et al.*, 1996; Vonhof & Barclay, 1996; Betts, 1998). Certain *Myotis* species were suggested as reservoirs, an observation often obscured previously by their frequent infection with viral variants

from other Chiroptera. An additional group (III) apparently circulates in *E. fuscus*, while viruses harboured by both *Molossidae* and *Desmodontinae* bats of Latin America form a phylogenetically distinct clade (group IV) (Nadin-Davis *et al.*, 2001). Similar branching patterns were presented by Hughes *et al.* (2005).

In our study RABV variants harboured by North American bats also formed two major clades. However, in contrast with Nadin-Davis *et al.* (2001), the first clade was formed by the Ph1, Ph2 and *Myotis* clades (and not by *Myotis* species and *E. fuscus* 1 and 2 clades) and a second one by *Lasionycteris noctivagans*, *Lasiurus cinereus*, *Lasiurus borealis* and *P. subflavus*. Following the two large clades, a cluster of *E. fuscus* (1 and 2) joined as the basal group of bat RABVs. Interestingly, the overall branching pattern of the trees constructed in our study by using the NJ, ML and Bayesian methods was the same without regards to the method used. The *E. fuscus* 3 clade represents a group that branches differently if we use different methods. The differences in branching of *Myotis* species and *E. fuscus* 1–3 clades between trees constructed in Nadin-Davis *et al.* (2001), Hughes *et al.* (2005), and in our study, could be explained by the different methods and evolution models used, and most importantly by different outgroups and datasets used. We used sequences of RABV collected from *P. hesperus* (Ph), which were not included in the previous publications. These new clades (Ph1 and Ph2) brought novel insights into the relationship between clades, and the different branching of *E. fuscus* is the result of these changes. However, the position of the *E. fuscus* 3 clade in our NJ tree was not supported, similarly as it wasn't supported in the NJ tree published by Nadin-Davis *et al.* (2001).

Our study revealed that 79% of RABV samples originating from *P. hesperus* bats from California and Arizona (a large part of their natural geographical range in the USA) formed a monophyletic clade Ph1 with high bootstrap

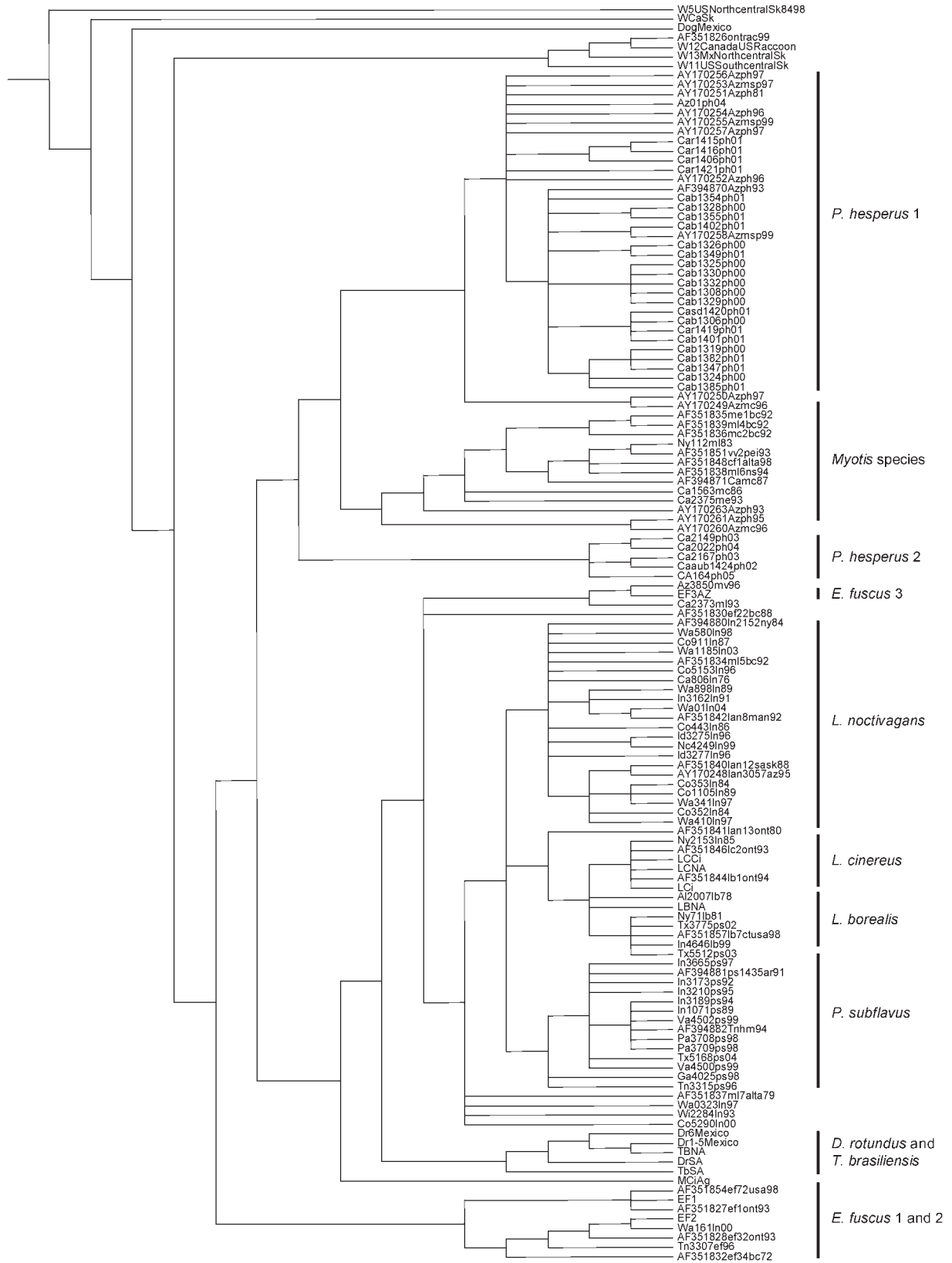


Fig. 4. Phylogenetic tree generated via ML analysis using the HKY85 + G model for a portion of the N gene coding sequence (264 nt). Abbreviations as in Fig. 2.

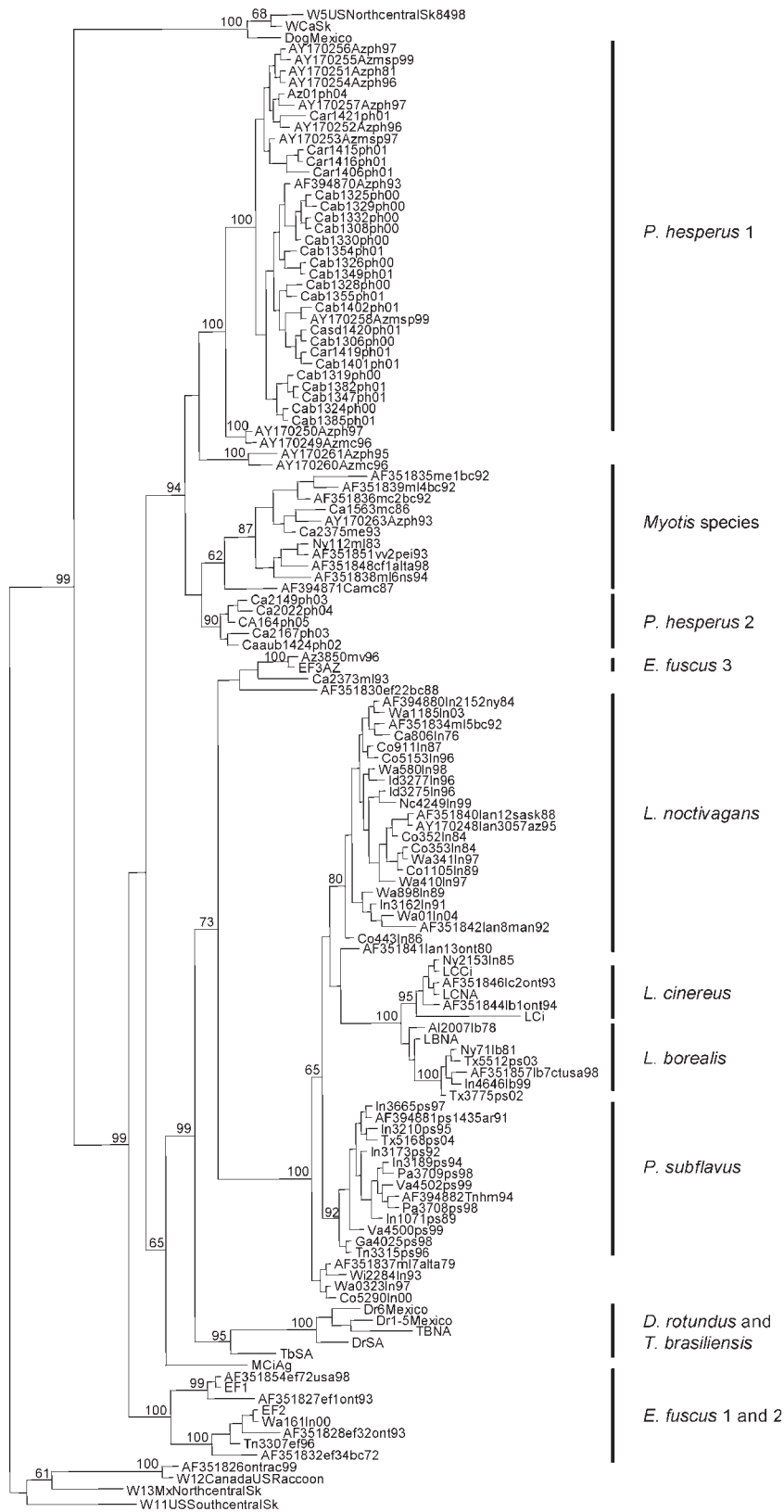


Fig. 5. Phylogenetic tree generated via Bayesian analysis using the GTR+I+G model for a portion of the N gene coding sequence (264 nt). Posterior probability values above 50 were shown for all major nodes (≥ 95 = statistical support). Abbreviations as in Fig. 2.

support and high posterior probabilities, separated from other phylogenetic lineages of RABV (Fig. 2, 4 and 5).

Genetic consistency over space and time, along with the phylogenetic independence of the Ph1 clade, suggests that

this RABV variant has been maintained in the *P. hesperus* population independently. These findings raise a question of adequacy of current passive surveillance methods and are indicative of the public health risk of the *P. hesperus* (Ph) RABV variant. Approximately 13% of *P. hesperus* samples collected in California (Los Angeles, El Dorado; distance between two sites approximately 550 km) during 2002–2005 formed a separate clade (Ph2). This clade was also supported by a moderate bootstrap value of 78%. With only five samples (three from the same county from 2003 to 2004) representing this group, it is difficult to interpret conclusively the relationship of this clade to the *Myotis* species and *P. hesperus* 1 clades. However, from all three trees (Fig. 2, 4 and 5), it is obvious that both *P. hesperus* RABV lineages (Ph1 and Ph2) shared a more recent common ancestor with a RABV variant associated with a *Myotis* species and their clade is separated from the clade formed by *Lasiurus noctivagans*, *Lasiurus* species and *P. subflavus*. Further analyses are needed to explain the relationship between the Ph1, Ph2 and *Myotis* species clades. Likely relevant to the differentiation of *P. hesperus* clades Ph1 and Ph2 through evolution are differences in the geographical and ecological distributions of the *P. hesperus* host populations that maintain the virus of each clade. The hosts of Ph1 were collected in desert areas east of the mountains (Sierra Nevada and Coastal Mt Transverse Ranges: Tehachapi Mts, San Gabriel Mts and San Bernardino Mts) that separate this *P. hesperus* population from a population distributed in more humid valley and coastal areas to the west. The pelage of these bats is pale grey in colour, contributing to their early subspecific distinction as *P. h. hesperus*. Contrastingly, the hosts of Ph2 were collected in more western areas, where the pelage of these bats is more brownish in colour, contributing to their early subspecific distinction as *P. h. merriami* (Hatfield, 1936; Hall & Dalquest, 1950; Findley & Traut, 1970).

Only two samples collected from *P. hesperus* belonged to other RABV lineages (*Myotis* species clade), suggesting relatively rare spillover events from *Myotis* species. As well, only four of more than 50 sequences of RABV collected from *Myotis* species were included in the Ph1 clade, which could be explained by spillover events or by misidentification of bat species.

No spillover between *Lasiurus noctivagans*, *P. subflavus* and *P. hesperus* was detected in the present study. In addition, spillover of the *P. hesperus* RABV variants into terrestrial mammals has not been detected. In contrast, Messenger *et al.* (2003) reported a high prevalence of *Lasiurus noctivagans* and *P. subflavus* variants among terrestrial mammals in the same regions where human cases have occurred. They suggested that increased infectivity of these RABV variants is responsible for relatively frequent spillover events. Our analyses showed that *P. hesperus* harbours RABV variants genetically distinct from the Ln and Ps variants and from other RABV variants. This finding corresponds interestingly to the taxonomic revisions of the

relationship between *P. subflavus* and *P. hesperus*, recently published by Hooper & Van Den Bussche (2003) and Hooper *et al.* (2006). Two bat species previously classified as members of one genus and now, on the basis of genetic classification, assigned to two different genera harbour two distinct RABV variants. New pathogenesis studies focused on the infectivity of all bat RABV variants, together with improved epidemiological analysis of both bat RABV prevalence and accurate bat species identification, are necessary to evaluate the increased infectivity hypothesis.

Data generated by phylogenetic analyses provided us with basic information about relationships between RABV variants harboured by North American bats. Thoughtful interpretation of these data, coupled with relevant epidemiological findings can lead to insights of new hypotheses that seek to explain the frequency of human rabies cases associated with bat RABV variants. As the smallest North American bat *P. hesperus* weighs only 2–6 g, compared with the larger *P. subflavus* (6–10 g) and *Lasiurus noctivagans* (9–12 g). *P. hesperus* becomes aggressive as it develops rabies, and it engages in seemingly unprovoked attacks. This behaviour may be necessary for the Ph RABV variants survival via transmission to other *P. hesperus* bats, which are strictly solitary and may not be readily approached. Such attacks on larger bats by this diminutive butterfly sized bat could end in its demise through retaliatory bites. Given its occasional tendency to apparently attack people when rabid, no human rabies cases have been linked to the Ph RABV variants. However, such overt exposures may signal the need for post-exposure prophylaxis (Constantine, 1970). Of relevant interest, a significantly greater proportion of smaller bats bit people than larger bats: 39 of 279 rabid bats bit people at rates ranging from 67% of *P. hesperus* to only 15% of *E. fuscus* (Constantine, 1967). These results might suggest greater aggressiveness of the smaller bats or greater care by people to avoid bites of the larger bats.

The lack of human cases linked to the Ph variants may be related to the biology and ecology of *P. hesperus*. These bats have few contacts with humans and apparent difficulty inflicting deep wounds by their small teeth. Additionally, specific viral properties such as a higher level of adaptation to a principal host and a relatively lower pathogenicity for other species or limitations in virus excretion in terms of intermittent shedding and viral dose may be operative. The lack of spillover events to terrestrial carnivores and infrequency of spillover to other bat species strongly support the hypothesis that addresses specific viral properties. The thickness of mammalian fur could form a barrier protecting skin from penetration by the small teeth of *P. hesperus*, but this argument fails to explain the absence of human cases associated with Ph RABV variants. Moreover, terrestrial mammals contact bats primarily by the paws and mouth, where fur coverage is absent (lips, mucosae of mouth cavity). One of the most frequently reported rabid bats in California is *Myotis californicus*, which is also a small bat species (weight 3.3–5.4 g). However, no human cases

caused by this RABV variant have been reported in California, suggesting that human exposures are recognized and treated, or these viruses fail to achieve successful infection. Two human cases caused by *Myotis* species RABV were reported in 1984 (Pennsylvania, no history of contact with a bat was reported) and in 1995 (Washington, rabid *Myotis* found in the bedroom, no known animal bite) (Messenger *et al.*, 2002). Interestingly, most of the human rabies cases, which had an unknown history of bite exposure, were caused by different RABV variants, harboured by medium-sized bat species (14 of 16 *P. subflavus* variant, 6–10 g; five of eight *Lasionycteris noctivagans* variant, 9–12 g; four of five *T. brasiliensis* variant, 10–15 g; one of one *E. fuscus* variant, 11–23 g and two of two *Myotis* species variant, weight range according to species 3–13 g). Among 15 human rabies cases reported in California since 1958, seven were acquired indigenously and all were characterized as bat RABV variants [four Ln/Ps variant and three *T. brasiliensis* (Tb) variant] (Messenger *et al.*, 2002).

Thus, given the rather diminutive *P. hesperus*, small body size alone (small vector hypothesis) may be inadequate to explain the association between human rabies cases and certain RABV isolates from bats. More importantly in this regard seem to be viral properties, such as viral infectivity and relative virulence, in association with particular host characteristics (e.g. receptors, body temperature, immune system response, etc.) coupled with host lifestyle (e.g. more solitary versus colonial bat species).

Hughes *et al.* (2005) inferred from the estimated substitution rate of the N gene that the initial branching of parental rabies virus in bats gave rise to the current variants associated with infection of *T. brasiliensis* and *D. rotundus*. Compartmentalization of RABV into lineages associated with infection of solitary bat species (or small group-forming species, such as *P. subflavus* and *Lasiurus* species) and more colonial bat species (*E. fuscus*, *Myotis* species) occurred later. Questions remain, as to the degree of sociality and the dynamics of RABV transmission in different bat species. Can we consider bats as solitary only if individuals are solitary all year or also those which form small (5–50 individuals) maternity colonies? There is an obvious difference between large colonies of *T. brasiliensis* consisting of millions of individuals, seasonal colonies of *E. fuscus* and *Myotis* species with hundreds of individuals, and maternity colonies of *P. subflavus* and *Lasionycteris noctivagans* with only tens of individuals. The results of Hughes *et al.* (2005) showed that adaptation of RABV to colonial bat species occurred more quickly than adaptations for the more solitary species, suggesting dependence of this process on host ecology.

In this regard, one interesting result of our analysis is that the Ln and Ps RABV variants have circulated in two different bat species populations (*P. subflavus* and *Lasionycteris noctivagans*) probably separately from each other and maintain reasonably high genetic stability over relatively long periods (Ps, 1989–1999; Ln, 1976–2004), especially given the error of replication fidelity in RNA viral genomes.

The separation between Ps and Ln clades was based on three conservative nucleotide substitutions within the studied N gene fragment, G/A¹¹⁷², A/T¹¹⁸⁴ and G/A¹²⁸³ (according to the PV genome). Although all three substitutions were synonymous, they suggest that these RABV variants may have circulated independently in these host species. Since bootstrap support for separation of *P. subflavus* and *Lasionycteris noctivagans* clades in the NJ tree and similarly posterior probabilities in the Bayesian tree were low, further examination of the RABV variants collected from *Lasionycteris noctivagans* and *P. subflavus* will be necessary to understand the evolutionary relationships of these two clades. Taking into account the fact that 16 of 24 human rabies cases in the USA over the last 50 years were associated with *P. subflavus*, and our recent finding of possible independent circulation of Ln and Ps RABVs in Ln and Ps populations, the finding of Geluso *et al.* (2005) that *P. subflavus* has expanded westward in the USA to New Mexico, South Dakota and Texas in recent years (Fig. 1) should highlight a need for enhanced surveillance.

All nucleotide mutations in the highly variable 264 nt fragment of the N gene from *P. hesperus* (Ph2), *P. subflavus*, *Lasionycteris noctivagans*, *Myotis* species and LBNA were synonymous with identical amino acid consensus sequences. The only exception was a substitution of the valine for leucine at position 379 aa (according to the PV strain), in Ph1 RABV (Fig. 3). This substitution is, however, structurally conservative. Such findings raise a question of potential constraints of RABV evolution, despite differences in the estimated substitution rate between more solitary or colonial bat species. Comparison of other genomic regions would facilitate a better understanding of differences between these RABV lineages.

Further phylogenetic analysis of additional samples from *P. hesperus*, *P. subflavus* and *Lasionycteris noctivagans* from North America are needed to corroborate the results revealed by our limited dataset. In particular, considering the extent of their distributions and their obvious role in public health in Canada and the USA, greater attention to these bats should occur in Mexico and Central America. Moreover, *in vitro* and *in vivo* pathogenesis studies should be conducted for a better understanding of the features of these particular RABV variants.

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