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Phylogeography, Population Dynamics, and Molecular Evolution of European Bat Lyssaviruses

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European bat lyssaviruses types 1 and 2 (EBLV-1 and EBLV-2) are widespread in Europe, although little is known of their evolutionary history. We undertook a comprehensive sequence analysis to infer the selection pressures, rates of nucleotide substitution, age of genetic diversity, geographical origin, and population growth rates of EBLV-1. Our study encompassed data from 12 countries collected over a time span of 35 years and focused on the glycoprotein (G) and nucleoprotein (N) genes. We show that although the two subtypes of EBLV-1—EBLV-1a and EBLV-1b—have both grown at a low exponential rate since their introduction into Europe, they have differing population structures and dispersal patterns. Furthermore, there were strong constraints against amino acid change in both EBLV-1 and EBLV-2, as reflected in a low ratio of nonsynonymous to synonymous substitutions per site, particularly in EBLV-1b. Our inferred rate of nucleotide substitution in EBLV-1, approximately 5×10^{-5} substitutions per site per year, was also one of the lowest recorded for RNA viruses and implied that the current genetic diversity in the virus arose 500 to 750 years ago. We propose that the slow evolution of EBLVs reflects their distinctive epidemiology in bats, where they occupy a relatively stable fitness peak.

Lyssaviruses (genus *Lyssavirus*, family *Rhabdoviridae*) are RNA viruses with a single-stranded negative-sense genome that infect a variety of mammals, causing rabies-like diseases. Although classical rabies is generally associated with carnivorous mammals, there is also evidence that bats are a major viral reservoir, a phenomenon particularly well studied in North America and Europe (2, 29, 33).

Currently, there are seven recognized genotypes of lyssavirus defined on the basis of their genetic similarity (6, 17): rabies virus (genotype 1), Lagos bat virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (DUVV; genotype 4), European bat lyssavirus type 1 (EBLV-1; genotype 5), European bat lyssavirus type 2 (EBLV-2; genotype 6), and Australian bat lyssavirus (genotype 7). All of the genotypes except Mokola virus have bat reservoirs, hinting that lyssaviruses originated in these mammals (4). Additionally, four new lyssavirus genotypes that infect bats in central and southeast Asia have been proposed: Aravan virus (24), Khujand virus (24), Irkut virus (5), and West Caucasian bat virus (5). Genotype 1 is responsible for classical rabies in terrestrial mammals throughout the world and in bats in North and South America, as well as for most rabies-related human deaths worldwide (39). In Europe, lyssaviruses isolated from bats belong to EBLV-1 and EBLV-2 (2).

More than 600 cases of EBLV infection have been reported throughout Europe, most recently in Scotland (7). EBLV-1 infection has also been reported in a small number of terrestrial mammals, including five sheep in Denmark (32) and a stone marten in Germany (28). These animals exhibited clinical signs consistent with rabies and later died or were euthanized. In addition, four human deaths due to EBLV have been reported (15). The overall rarity of EBLV-associated mortality in terrestrial animals may be because the cross-species transmission of EBLV from bats to other mammals is infrequent, because cases go undetected, or because the virulence of EBLV is low compared to that of genotype 1 lyssavirus. Furthermore, it is possible that bats develop persistent EBLV infection, manifested as the long-term carriage of viral RNA without overt disease. In particular, some wild-caught bats have antibody to EBLV for up to 3 years, and viral RNA has been detected in the tissues of apparently healthy bats (14, 31, 36, 40), although it is also possible that bats experience an unapparent acute infection and are subject to continual reinfection.

Like other lyssaviruses, the negative-sense EBLV genome encodes five proteins: the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA polymerase (L) in the order 3′-N-P-M-G-L-5′. Evolutionary studies of lyssaviruses have tended to focus on the N protein, a well-conserved structural protein, and the G protein, which contains domains responsible for host cell receptor recognition and membrane fusion and which is the major target for the host neutralizing-antibody response (3).

Although several molecular epidemiological studies of genotype 1 lyssaviruses have been undertaken, to date there has been only a single such analysis of EBLV (2). This study, which

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utilized the first 400 bp of the N gene, showed that EBLV-1 can be subdivided into two distinct lineages or subtypes, designated EBLV-1a and EBLV-1b. However, nothing is known about the forces shaping EBLV evolution, particularly compared to genotype 1 lyssaviruses, even though this may provide crucial insights into the propagation of EBLV in bat populations and the evolutionary interaction between the virus and its mammalian hosts. Previous studies of genotype 1 and 7 lyssaviruses have shown that these viruses are subject to stronger selective constraints than many other RNA viruses (18, 21). Whether these constraints reflect a lack of immune-driven selection pressure or are due to the adaptation of lyssaviruses to replicate in a broad range of cell types, producing complex fitness trade-offs, is unknown (21).

Here, we present the first comprehensive evolutionary analysis of EBLV using a variety of analytical methods employed on sequence data encompassing the G and N genes, as well as noncoding gene regions of the viral genome. In particular, we explored whether the selection pressures and rates of evolutionary change observed in these viruses might reflect the peculiarities of their epidemiology in bats, including the possibility that they establish persistent infections and have low virulence.

MATERIALS AND METHODS

Sequence data. Gene sequence data were either newly generated or collected from GenBank. A total of 65 EBLV isolates were obtained, the details of which are given in Table 1.[2] In addition, two complete N and G sequences from DUVV were also generated in this study. Three different data sets were then compiled: (i) 54 G gene sequences (1,575 bp), (ii) 63 N gene sequences (1,350 bp), and (iii) 38 sequences of a concatenated 527-nucleotide (nt) noncoding region combining regions 234 nt and 294 nt 5′ and 3′ to the G gene, respectively.

Brains were obtained from naturally infected animals or from suckling mice after a limited number of passages. RNA extraction and cDNA synthesis were performed as previously described (34). PCR amplification and sequencing were performed according to previous protocols (2) with primer sets for the N gene of N127 (5′-ATGTAACACCTCTACAATGG-3′; nt 55 to 74), N66CS (3′-GTGC TCCARTTTGCRCACA-5′; nt 578 to 596), N512 (5′-GTGGCWGAYAGAAT GGAACA-3′; nt 493 to 512), and N8m (3′-CAGTCTCYTCNGCCATCTC-5′; nt 1567 to 1586). For the G gene, the primer sets were M220 (5′-TGGTGTAT CAACATGRAYTC-3′; nt 2999 to 3028) and GG14 (3′-GGACTCAGTATGA TCCCATTG-5′; nt 4383 to 4403) for EBLV-1 and GG14M (3′-GGACTCAAT ATTATACCGTTR-5′; nt 4383 to 4403) for EBLV-2; G1530 (5′-TGTAAGAC TCCCAAGAWGG-3′; nt 4777 to 6372) and G980 (5′-GGGTTCGGAAAGGC ATACA-3′; nt 4231 to 4250) for EBLV-1 and G980M (5′-GGGTTCGGAAAG GCATACA-3′; nt 4197 to 4215) for EBLV-2; and G1400 (5′-TTGCCCTTCTT GCCTTAGC-3′; nt 4646 to 4664), G1400m (5′-GCCTTGATGATATTTATTG CC-3′; nt 4657 to 4676), L2 (3′-CAAAGGGGAGTTGAGRTTRTA-5′; nt 5520 to 5540), or L4 (3′-TGATTGTCTTGTTATTGTTTCT-5′; nt 5524 to 5543). Sequence positions are numbered relative to the rabies virus PV genome. After the removal of identical sequences, multiple sequence alignments of these data were generated manually using the SE-AL program (http://evolve.zoo.ox.ac.uk/).

Phylogenetic analysis. We estimated phylogenetic trees for a variety of data sets, including (i) 63 N gene sequences combining EBLV-1, EBLV-2, and DUVV; (ii) 54 G gene sequences combining EBLV-1, EBLV-2, and DUVV; (iii) 55 N gene sequences from EBLV-1; (iv) 47 G gene sequences from EBLV-1; (v) 38 concatenated noncoding region sequences from EBLV-1; (vi) 38 concatenated G, N, and noncoding sequences from EBLV-1; and (vii) 49 concatenated G and N gene sequences from EBLV-1. All phylogenetic trees were estimated using a maximum-likelihood (ML) method under the general time-reversible (GTR) model of nucleotide substitution, with the rate of each substitution type estimated from the data and using successive rounds of tree bisection reconnection branch swapping. The ML base frequencies were also estimated from the data, as were the proportion of invariable sites (I) and a gamma distribution of rate variation among sites (Γ) with four rate categories. Parameter values are available from the authors on request. The robustness of each tree was assessed using a bootstrap resampling method, with all 1,000 replicates estimated using the neighbor-joining procedure but with the input genetic distances produced under the ML substitution model. All trees were estimated using the PAUP* package (38).

Recombination tests. Two approaches were employed to determine whether recombination had occurred in the evolutionary history of the EBLVs. First, the N and G gene sequence alignments were split into nonoverlapping fragments of 250 bp. Neighbor-joining trees (incorporating the HKY85-Γ substitution model) were then estimated for each region. Large-scale phylogenetic movements, indicative of recombination, were assessed using bootstrap resampling. Second, Sawyer's run test (GeneConv program) (35) was used to search for gene conversion events between pairs of sequences (that is, runs of sequence similarity that conflicted with correct phylogenetic relationships).

Analysis of selection pressures. To determine the selection pressures acting on the EBLVs, we estimated the numbers of nonsynonymous (d_N) and synonymous (d_S) substitutions per site for individual codons and lineages using a maximum-likelihood method that compares various models of sequence evolution (43). To assess the selection pressures at individual codons, we compared the M7 and M8 models. M7 assumes that codons take 1 of 10 d_N/d_S values, each estimated from the data, although no d_N/d_S category is allowed to exceed 1.0, so that the model specifies neutral evolution. In contrast, M8 adds an 11th category of codons at which d_N/d_S can exceed 1, thereby allowing positive selection. Consequently, positive selection is supported if M8 rejects M7 under a likelihood ratio test and contains a category of codons with d_N/d_S values of >1. The selected codons can then be identified using a Bayesian approach. To assess the selection pressures acting on individual lineages of the EBLV tree, we used a likelihood ratio test to compare the M0 model, which estimates a single d_N/d_S value for all branches and codons, with the "free-ratio" model, which allows a different d_N/d_S ratio for each lineage of the EBLV trees. Finally, M0 was also compared with a "two-ratio" model in which separate d_N/d_S values are estimated for the internal and external branches of the EBLV trees. All of these methods were implemented using the CODEML program of the PAML package (42).

Estimating substitution and population dynamics. Rates of nucleotide substitution per site were estimated using the Bayesian Markov Chain Monte Carlo method available in the BEAST package (http://evolve.zoo.ox.ac.uk/). This method utilizes the differences in branch lengths between viruses sampled at different time points and explores various models of sequence evolution whose parameters include tree topology, substitution model, and substitution rate. Uncertainties in the data are reflected in the 95% high-probability density (HPD) values and hence account for variation in the molecular clocks among lineages (13). As before, this analysis utilized the GTR-I-Γ model of nucleotide substitution with parameters optimized during multiple runs. Three population dynamics models were compared: constant population size, exponential population growth, and logistic population growth. The models were compared with Akaike's Information Criterion, with the constant and exponential and the exponential and logistic models differing by a single parameter and the constant and logistic models differing by two parameters. Finally, for the best-fit model, we estimated the rate of population growth (r), expressed as the number of new infections per individual per year, and also the time taken for the epidemic to double in size (λ) in years using $\lambda = \ln(2)/r$.

Analyses of viral dispersion. The longitude and latitude for each EBLV strain were determined using the Alexandria Digital Library Gazetteer (http://middleware.alexandria.ucsb.edu/client/gaz/adl/index.jsp). The degree of linear association between pairwise geographic and genetic distances was evaluated using the standard Pearson correlation coefficient, with significance determined using Mantel permutation tests with 10,000 row-column permutations (37) and genetic distances estimated under the ML model described previously. To determine the geographical location of the putative common ancestor (PCA) of EBLV-1a and EBLV-1b in the N and G gene data sets, we first inferred the ancestral sequence for each data set using a parsimony reconstruction procedure (in PAUP*) on the ML trees estimated previously. This PCA sequence was then added to the original data set, and a new ML tree was inferred so that the pairwise distance of each viral isolate (of known geographical location) from the PCA could be determined.

Nucleotide sequence accession numbers. The new sequences determined in this study have been submitted to GenBank and assigned accession numbers AY863220 to AY863408 (EBLV) and AY996321 to AY996324 (DUVV).

RESULTS

Phylogenetic relationships of EBLV isolates. We obtained EBLV gene sequences from viruses sampled from 12 European countries over a 35-year period. Using both phylogenetic

TABLE 1. Isolates of EBLV analyzed in this study

Virus	Yr of isolation	City of isolation[a]	Country of isolation	Bat species	Source	GenBank accession no.[b]
EBLV-1a						
9395 GER	1968	Hamburg	Germany	*E. serotinus*	A. King	AY863348 (N) AY863296 (G) AY863273 (G5) AY863235 (G3)
9396 GER	1985	Rostock	Germany	*E. serotinus*	A. King	AY863349 (N) AY863297 (G) AY863269 (G5) AY863231 (G3)
9398 GER	1970	Stade, Niedersachsen	Germany	*E. serotinus*	A. King	AY863350 (N) AY863298 (G) AY863247 (G3)
9399 GER	1982	Bremerhaven	Germany	*E. serotinus*	A. King	AY863351 (N) AY863299 (G) AY863287 (G5) AY863249 (G3)
9436 GER	1986	Stade, Niedersachsen	Germany	*E. serotinus*	A. King	AY863352 (N)
9437 GER	1987	Bremen, Bremen	Germany	*E. serotinus*	A. King	AY863353 (N) AY863300 (G) AY863280 (G5) AY863242 (G3)
9438 GER	1988	Neumunster, Schleswig-Holstein	Germany	*E. serotinus*	A. King	AY863354 (N)
9439 GER	1989	Iberg, Niedersachsen	Germany	*E. serotinus*	A. King	AY863301 (G) AY863279 (G5) AY863241 (G3)
9440 GER	1989	Braunschweig, Niedersachsen	Germany	*E. serotinus*	A. King	AY863355 (N) AY863302 (G) AY863282 (G5) AY863244 (G3)
9441 GER	1990	Walsrode, Niedersachsen	Germany	*E. serotinus*	A. King	AY863356 (N) AY863303 (G) AY863288 (G5) AY863250 (G3)
9477 GER	1986	Nienburg, Niedersachsen	Germany	*E. serotinus*	A. King	AY863357 (N)
9481 GER	1990	Lubeck Schleswig-Holstein	Germany	*E. serotinus*	A. King	AY863358 (N) AY863304 (G) AY863286 (G5) AY863248 (G3)
9366 HOL	1992	Goor	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863359 (N) AY863305 (G) AY863276 (G5) AY863238 (G3)
9372 HOL	1992	Valthermond	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863360 (N) AY863306 (G) AY863274 (G5) AY863236 (G3)
9478 HOL	1989	Rolde	Netherlands	*E. serotinus*	A. King	AY863361 (N) AY863307 (G)
9480 HOL	1987	Bellingwolde	Netherlands	*E. serotinus*	A. King	AY863362 (N) AY863308 (G) AY863283 (G5) AY863245 (G3)
94116 HOL	1989	Jubbega	Netherlands	*E. serotinus*	A. King	AY863363 (N) AY863309 (G) AY863284 (G5) AY863246 (G3)
02017 HOL	2000	Soest	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863364 (N)
02018 HOL	2000	Leiden	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863365 (N)
02020 HOL	1999	Woerden	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863366 (N)
02021 HOL	1998	Leens	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863367 (N)
02022 HOL	1998	Oldeberkoop	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863368 (N)
8615 POL	1985	Gdansk	Poland	*E. serotinus*	D. Seroka	AY863369 (N) AY863310 (G)
9394 POL	1990	Ketrzyn	Poland	*E. serotinus*	D. Seroka	AY863370 (N) AY863311 (G) AY863271 (G5) AY863233 (G3)

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TABLE 1—*Continued*

Virus	Yr of isolation	City of isolation[a]	Country of isolation	Bat species	Source	GenBank accession no.[b]
96031 POL	1994	Dziekanow	Poland	*E. serotinus*	D. Seroka	AY863312 (G)
9397 RUS	1985	Belgorod	Russia	Human	A. King	AY863371 (N)
						AY863313 (G)
						AY863272 (G5)
						AY863234 (G3)
01018 SLO	2001	Presov	Slovakia	*E. serotinus*	R. Franka	AY863382 (N)
						AY863323 (G)
						AY863295 (G5)
						AY863257 (G3)
9443 UKR	1987	Volyn region	Ukraine	*V. murinus*	A. King	AY863372 (N)
						AY863314 (G)
						AY863263 (G5)
						AY863225 (G3)
03002 FRA	2003	Angers, Maine et Loire	France	*E. serotinus*	Institut Pasteur, Paris	AY863381 (N)
						AY863322 (G)
						AY863277 (G5)
						AY863239 (G3)
9479 DEN	1987	Horsens	Denmark	*E. serotinus*	A. King	AY863373 (N)
						AY863315 (G)
						AY863281 (G5)
						AY863243 (G3)
94109 DEN	1987	Juelsminde	Denmark	*E. serotinus*	A. King	AY863316 (G)
						AY863261 (G5)
						AY863223 (G3)
94110 DEN	1987	Christiansfeld	Denmark	*E. serotinus*	A. King	AY863374 (N)
02007 DEN	1993	Århus, East Jutland	Denmark	*E. serotinus*	L. Rønsholt	AY863317 (G)
						AY863262 (G5)
						AY863224 (G3)
02010 DEN	1995	Arden, Northeast Jutland	Denmark	*E. serotinus*	L. Rønsholt	AY863375 (N)
						AY863318 (G)
						AY863259 (G5)
						AY863221 (G3)
02011 DEN	1997	Jerslev, Zealand	Denmark	*E. serotinus*	L. Rønsholt	AY863376 (N)
						AY863319 (G)
						AY863260 (G5)
						AY863222 (G3)
02012 DEN	1999	Holsted, Southwest Jutland	Denmark	*E. serotinus*	L. Rønsholt	AY863377 (N)
02013 DEN	1999	Nr.Nebel, Southwest Jutland	Denmark	*E. serotinus*	L. Rønsholt	AY863378 (N)
						AY863320 (G)
						AY863267 (G5)
						AY863229 (G3)
02015 DEN	2000	Rødding, South Jutland	Denmark	*E. serotinus*	L. Rønsholt	AY863379 (N)
02016 DEN	2002	Janderup, Southwest Jutland	Denmark	Sheep	L. Rønsholt	AY863380 (N)
						AY863321 (G)
						AY863266 (G5)
						AY863228 (G3)
EBLV-1b						
9367HOL	1992	Wassenaar	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863383 (N)
						AY863335 (G)
						AY863275 (G5)
						AY863237 (G3)
9376 HOL	1993	Moerkapelle	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863384 (N)
						AY863339 (G)
						AY863270 (G5)
						AY863232 (G3)
9377 HOL	1993	Apeldoorn	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863385 (N)
						AY863334 (G)
94113 HOL	1992	Schagen	Netherlands	*E. serotinus*	A. King	AY863386 (N)
						AY863336 (G)
						AY863289 (G5)
						AY863251 (G3)
94115 HOL	1992	Bovenkarspel	Netherlands	*E. serotinus*	A. King	AY863387 (N)
						AY863327 (G)
						AY863293 (G5)
						AY863255 (G3)

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TABLE 1—*Continued*

Virus	Yr of isolation	City of isolation[a]	Country of isolation	Bat species	Source	GenBank accession no.[b]
02019 HOL	1999	Limmen	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863388 (N) AY863324 (G)
02024 HOL	1997	Bergen	Netherlands	*E. serotinus*	CIDC-Lelystad	AY863389 (N) AY863338 (G) AY863258 (G5) AY863220 (G3)
9483 SPA	1987	Granada	Spain	*E. serotinus*	Institut Pasteur, Paris	AY863390 (N) AY863340 (G) AY863290 (G5) AY863252 (G3)
94285 SPA	1994	Granada	Spain	*E. serotinus*	A. Tellez	AY863391 (N) AY863337 (G) AY863264 (G5) AY863226 (G3)
8918 FRA	1989	Briey, Meurthe-et-Moselle	France	*E. serotinus*	Institut Pasteur, Paris	AY863392 (N) AY863341 (G)
8919 FRA	1989	Bainville-sur-Madon, Meurthe et-Moselle	France	*E. serotinus*	J. Barrat	AY863393 (N) AY863342 (G) AY863278 (G5)
9603 FRA	1995	Bourges	France	*E. serotinus*	J. Barrat	AY863394 (N) AY863328 (G) AY863294 (G5) AY863256 (G3)
9906 FRA	1995	Morlaix Finistère	France	*E. serotinus*	J. Barrat	AY863395 (N) AY863332 (G)
0001 FRA	2000	Premilhat, Allier	France	*E. serotinus*	J. Barrat	AY863396 (N) AY863326 (G) AY863292 (G5) AY863254 (G3)
0002 FRA	2000	Fouesnant, Finistère	France	*E. serotinus*	J. Barrat	AY863397 (N) AY863330 (G)
0003 FRA	2000	Plounéour-Menez, Finistère	France	*E. serotinus*	J. Barrat	AY863398 (N) AY863331 (G)
0102 FRA	2000	Joinville, Haute-Marne	France	*E. serotinus*	J. Barrat	AY863399 (N) AY863291 (G5) AY863253 (G3)
02031 FRA	2001	Vallon en Sully, Allier	France	*E. serotinus*	J. Barrat	AY863400 (N) AY863329 (G)
02032 FRA	2001	Plonquin Finistère	France	*E. serotinus*	E. Picard	AY863401 (N) AY863325 (G) AY863268 (G5) AY863230 (G3)
02033 FRA	2001	Waville, Meurthe-et-Moselle	France	*E. serotinus*	E. Picard	AY863402 (N) AY863333 (G) AY863265 (G5) AY863227 (G3)
EBLV-2a						
94112 HOL	1989	Andijk	Netherlands	*M. dasycneme*	A. King	AY863405 (N) AY863346 (G)
9018 HOL	1987	Wommels	Netherlands	*M. dasycneme*	CIDC-Lelystad	AY863403 (N) AY863347 (G)
9375 HOL	1993	Roden	Netherlands	*M. dasycneme*	CIDC-Lelystad	AY863404 (N) AY863344 (G)
EBLV-2b						
9007 FIN	1986	Helsinki	Finland	Human	Lumio et al. (1986)	AY863406 (N) AY863345 (G)
9337 SWI	1993	Versoix	Switzerland	*M. daubentonii*	R. Zanoni	AY863407 (N) AY863343 (G)
02053 SWI	2002	NA[c]	Switzerland	NA	R. Zanoni	AY863408 (N)
Duvenhage						
94286 SA	1981	NA	South Africa	*Minopterus* spp.	Van der Merwe	AY996324 (N) AY996322 (G)
86132 SA	1971	NA	South Africa	Human	Meredith	AY996323 (N) AY996321 (G)

[a] The city closest to where the viral isolate was collected.
[b] Letters in parentheses indicate the region of the genome from which the sequence came. N, nucleoprotein gene; G, glycoprotein gene; G5, noncoding sequence 5′ to the glycoprotein gene; G3, noncoding sequence 3′ to the glycoprotein gene.
[c] NA, information not available.

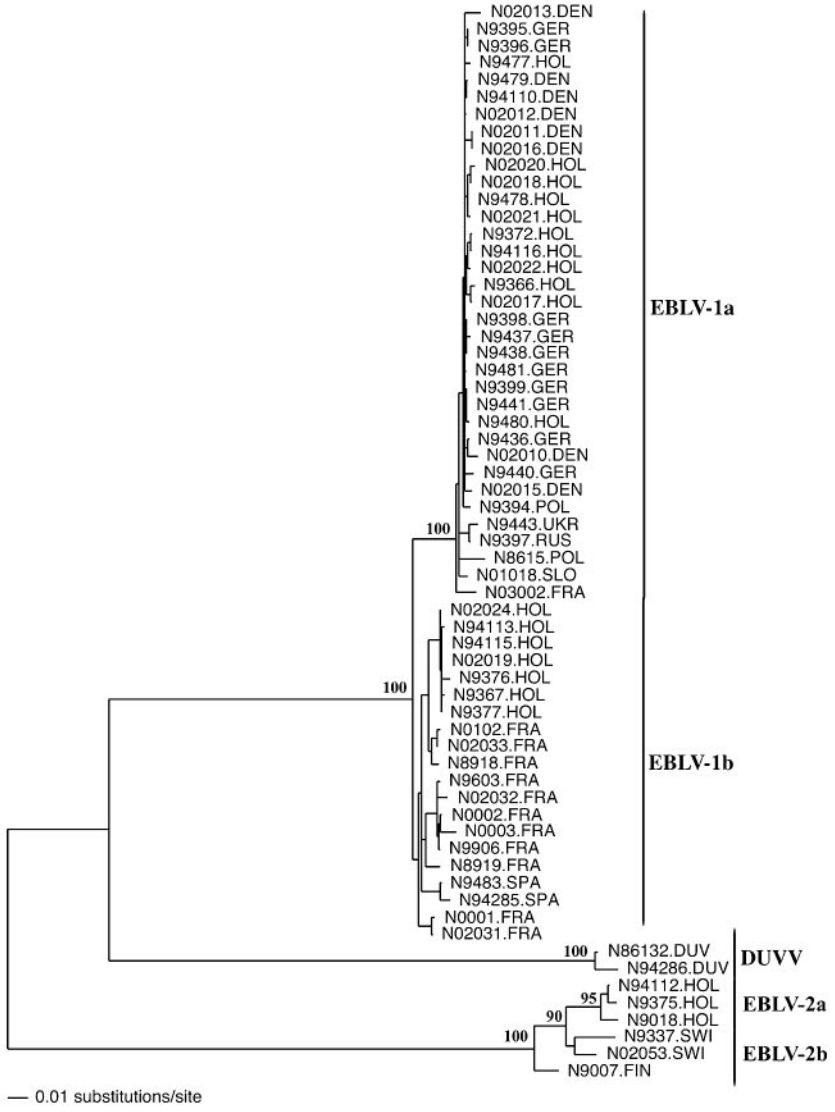

FIG. 1. Maximum-likelihood phylogenetic tree depicting the evolutionary relationships among 63 complete N gene sequences from EBLV-1 and EBLV-2 and two isolates of DUVV. Horizontal branches are drawn to scale, and the numbers at specific nodes indicate the degree of bootstrap support where it is >90%. The tree is midpoint rooted for purposes of clarity only. The "N" before each isolate name signifies the N gene.

and pairwise tests, we found no evidence for recombination in these data, thereby supporting previous studies that concluded that recombination was rare in lyssaviruses (11).

A maximum-likelihood phylogenetic tree of 63 complete N gene sequences is shown in Fig. 1 and has a number of significant features. In particular, the Duvenhage, EBLV-1, and EBLV-2 genotypes were each identifiable with 100% bootstrap values, and there was clear evidence for the existence of two subtypes of EBLV-1 (EBLV-1a and EBLV-1b), although in this tree there was only weak bootstrap support for the EBLV-1b clade. Interestingly, although an EBLV-2a clade was clearly visible, the EBLV-2b group was not monophyletic in this tree, with the EBLV-2b strains from Switzerland clearly related to the EBLV-2a strains from Holland. Unfortunately, the small sample size of the EBLV-2 data set precludes further analysis of these viruses. Also notable was the fact that EBLV-1 and EBLV-2 did not form a monophyletic group, as EBLV-1 and DUVV were more closely related to each other than EBLV-1 was to EBLV-2, although this assumes midpoint rooting. As expected given the lack of recombination, similar tree topologies were inferred for the G gene and the noncoding sequences and when various data sets were combined (all trees are available from the authors on request).

Given the composition of our data, we paid most attention to the phylogenetic relationships within EBLV-1. Trees of the N and G genes for EBLV-1 are shown in Fig. 2 and 3, respectively. Although these trees were similar in topology, we observed an important difference in branching structure between EBLV-1a and EBLV-1b in both genes. Specifically, the EBLV-1a isolates fell into a cluster characterized by little geographical structure, with the lineages defining the German, Dutch, and Danish strains mixed on the tree, suggesting a free movement of strains among these localities. Isolates from eastern Europe occupied more basal positions (with the exception

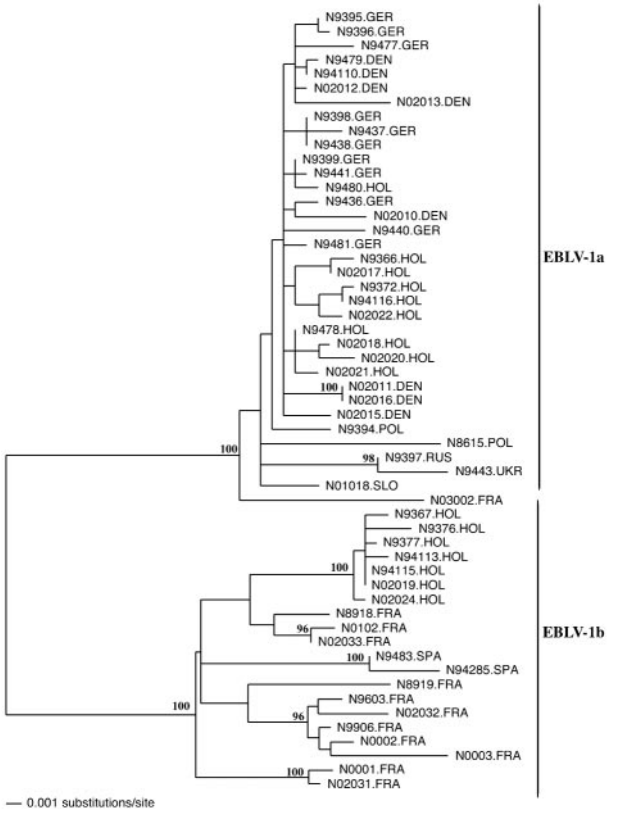

FIG. 2. Maximum-likelihood phylogenetic tree of 55 N gene sequences of EBLV-1. Horizontal branches are drawn to scale, and nodes with >95% bootstrap support are indicated. The tree is rooted between EBLV-1a and EBLV-1b. The "N" before each isolate name signifies the N gene.

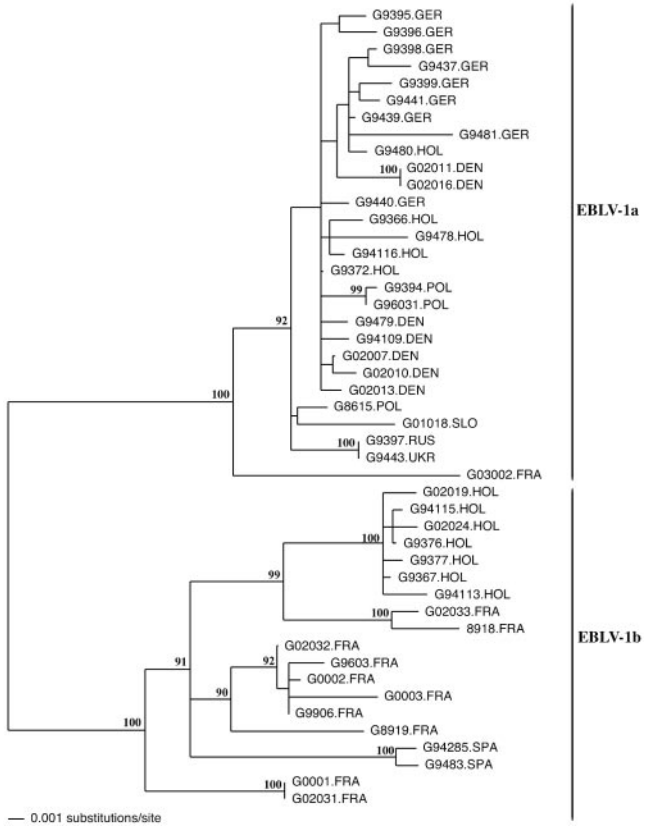

FIG. 3. Maximum-likelihood phylogenetic tree of 47 G gene sequences of EBLV-1. Horizontal branches are drawn to scale, and nodes with >95% bootstrap support are indicated. The tree is rooted between EBLV-1a and EBLV-1b. The "G" before each isolate name signifies the G gene.

of two Polish strains on the G gene tree), while the French isolate 03002FRA was the most divergent lineage of all in both the N and G genes. In contrast, there was more phylogenetic and geographic structure in the EBLV-1b clade. In particular, the Dutch strains form a separate group from the French and the Spanish strains, and the French strains form a basal group. Furthermore, internal branches within the EBLV-1b group were, on average, four times longer than those in EBLV-1a, indicating that this subtype harbors more genetic diversity. For example, in the G gene tree, the mean length of internal branches was 0.004 for EBLV-1a and 0.017 for EBLV-1b.

In a previous phylogenetic analysis, some Spanish isolates of EBLV-1b formed the most divergent group within the subtype, which led to the proposal that EBLV-1b originated in Africa and spread to Europe via Spain (2). To test this hypothesis, we compared the difference in likelihood when the Spanish strains, instead of the French strains, were placed as the most basal group in the ML trees. Using the Shimodaira-Hasegawa test in PAUP*, we found that these trees, which depict different phylogeographic histories for EBLV-1b, were not significantly different ($P = 0.356$ for N; $P = 0.142$ for G). Consequently, the possibility of a Spanish origin for EBLV-1b cannot be refuted based on these data.

Geographic spread of EBLVs. To investigate the pattern of EBLV dispersal in Europe, we examined the association between geographic and genetic distances for the combined N, G, and noncoding data sets. We found a significant positive correlation between geographic and genetic distances for both EBLV-1a (Mantel test; $r = 0.69$; $P < 0.0009$; $n = 23$) and EBLV-1b ($r = 0.71$; $P < 0.0001$; $n = 13$). This association is particularly strong for EBLV-1b; based on the current sample of sequences, the virus appears to have spread along a north-south axis in the west of Europe (Fig. 4). Although the point of origin is difficult to identify, particularly given the uncertainty in the phylogenetic analysis, it is notable that the French isolates tend to fall deep in the tree and have the shortest distances to the putative common ancestor.

A more complex phylogeographic picture was seen in EBLV-1a, although this virus has clearly spread along an east-west axis in northern Europe (Fig. 4). First, for the Danish, Dutch, and German strains, the relationship between geographic and genetic distances was far less pronounced ($r = 0.3$; $P < 0.0016$; $n = 18$), suggesting that there has been some spatial mixing, as was also apparent in the phylogenetic analysis. Second, the probable geographic location of the PCA for EBLV-1a varied depending on which data set was used in the analysis. For the three genes combined and for the N gene only, the PCA was located in Germany. However, for G and N combined, the PCA was located in Denmark, while for G it was located on the Dutch-German border.

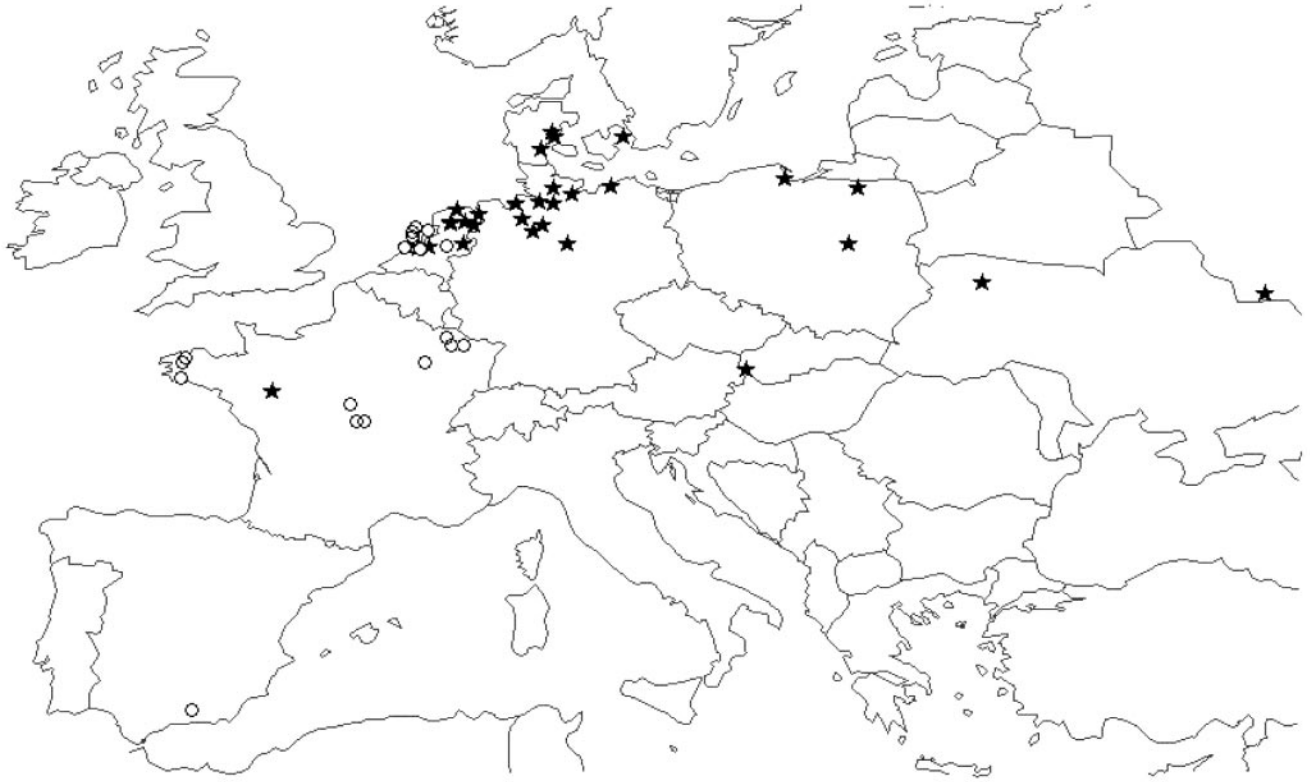

FIG. 4. Sampling locations of all EBLV-1 strains (G, N, and noncoding region) used in this study. EBLV-1a strains are indicated by closed stars, while EBLV-1b strains are indicated by open circles.

Rates of population growth in EBLV-1. The best-fit population dynamic model for EBLV-1a and EBLV-1b was that of exponential population growth (Table 2). Moreover, the estimated rates of population growth were both similar and low for the G and N genes, and we found no clear difference in growth rates between EBLV-1a and EBLV-1b. For the G gene, the mean growth rate in EBLV-1a was 0.049 new infections per individual per year, which gives an average epidemic doubling time of ~14 years, which was faster than that observed in EBLV-1b at 0.010/year (mean doubling time, ~69 years). However, for the N gene the situation was reversed and the growth rate was higher in EBLV-1b (0.053/year; mean doubling time, ~13 years) than in EBLV-1a (0.038/year; mean doubling time, ~18 years). Given this mixed pattern and the fact that the HPD values overlap between EBLV-1b and EBLV-1a, we conclude that these two virus populations have grown at similar and relatively low rates. Because EBLV-1a and EBLV-1b are phylogenetically distinct and do not present a single panmictic population, it is inappropriate to estimate rates of population growth for a combined EBLV-1a and EBLV-1b data set.

TABLE 2. ML estimates of evolutionary parameters in EBLV

ML estimate	Value					
	G Gene			N Gene		
	EBLV-1	EBLV-1a	EBLV-1b	EBLV-1	EBLV-1a	EBLV-1b
Best-fit population model	Exponential growth	Exponential growth	Exponential growth	Exponential growth	Exponential growth	Exponential growth
Mean k[a]	5.10×10^{-5}	5.77×10^{-5}	7.40×10^{-5}	6.11×10^{-5}	5.57×10^{-5}	2.39×10^{-4}
HPD k	3.00×10^{-6}, 9.16×10^{-5}	2.89×10^{-6}, 1.07×10^{-4}	1.33×10^{-6}, 1.70×10^{-4}	1.14×10^{-5}, 1.09×10^{-4}	9.48×10^{-6}, 1.00×10^{-4}	4.67×10^{-5}, 4.28×10^{-4}
Mean age (yr)	739	239	422	494	204	72
HPD age (yr)	159, 1,589	47, 555	52, 1,168	154, 1,029	64, 506	26, 152
Mean growth rate[b]	NA[c]	0.049	0.010	NA	0.038	0.053
HPD growth rate	NA	0.0008, 0.115	−0.005, 0.031	NA	0.004, 0.081	0.002, 0.109
Mean epidemic doubling time (λ) (yr)	NA	14	69	NA	18	13

[a] Rate of nucleotide substitution per site per year.
[b] Number of new infections per individual per year.
[c] NA, not applicable.

Rates and dates of EBLV-1 evolution. The mean rates of nucleotide substitution for EBLV-1 estimated using a Bayesian Markov Chain Monte Carlo approach were very similar for the G and N genes at 5.10×10^{-5} and 6.11×10^{-5} substitutions per site per year, respectively, and with overlapping HPD values (Table 2). Even accounting for the range of HPD values, these are among the lowest measurable nucleotide substitution rates reported for RNA viruses (23). There was no consistent difference between the substitution rates for EBLV-1a and EBLV-1b, and in all cases, the HPD values overlapped. The mean rates for the G gene were 5.77×10^{-5} and 7.40×10^{-5} substitutions/site/year for EBLV-1a and EBLV-1b, while the equivalent rates for the N gene were 5.57×10^{-5} and 2.39×10^{-4} substitutions/site/year for EBLV-1a and EBLV-1b, respectively.

Given these rates of nucleotide substitution, the most recent common ancestor of the genetic diversity sampled in EBLV-1 was estimated to have existed approximately 500 to 750 years ago (Table 2). Estimates of the time of the most recent common ancestor for EBLV-1a were similar for the N and G genes; for G, the mean age was 239 years, and for N, the mean value was 204 years. The values for EBLV-1b were more variable. For the G gene, the value was high compared to that of EBLV-1a (mean age = 422 years), although the HPD was extremely wide (52 to 1,168 years), making it difficult to infer a date. For the N gene, the mean estimate was extremely low at 72 years, but the HPD was narrower than for G (26 to 152 years). The genetic diversities observed within EBLV-1a and EBLV-1b are therefore of roughly the same age.

Selection pressures in EBLV. For both the N and G genes of EBLV-1 and EBLV-2, we found no evidence for positive selection ($d_N/d_S > 1$) acting at any codon or lineage (the full results are available from the authors on request). The mean d_N/d_S values (estimated using the M0 model) were low in all cases, revealing the action of relatively strong selective constraints. In EBLV-1, the mean d_N/d_S value for the G gene, 0.088, was nearly twice that observed in the N gene, 0.049. Mean d_N/d_S values for EBLV-2 were slightly higher than for EBLV-1 at 0.097 and 0.068 for G and N, respectively, but followed the same pattern. Interestingly, the d_N/d_S value was consistently higher in EBLV-1a than in EBLV-1b for both the N and G genes: 0.065 and 0.034 for N and 0.096 and 0.079 for G. Finally, a comparison of the ratio of d_N/d_S in the internal and external branches of the tree (using the "two-ratio" model) revealed more nonsynonymous substitutions on the external branches (tips) of the trees than on the interior branches; the ratios of d_N/d_S on the external branches to that on the internal branches were 4.95 for G and 5.75 for N in EBLV-1.

DISCUSSION

Evolutionary history of EBLV-1. By combining a variety of data, including taxonomic relationships, virus population dynamics, and phylogeography, we are able to reveal some important aspects of the evolutionary history of EBLV-1 in Europe. First, our molecular-clock estimates suggest that the current lineages of EBLV-1 arose some 500 to 750 years ago. Hence, the current genetic diversity in EBLV-1 has a relatively recent origin, as observed in some other lyssaviruses (18, 21). Second, we found that EBLV-1a and EBLV-1b have different patterns of geographical spread and possibly have different points of introduction into Europe, although both viruses exhibit relatively low rates of population growth.

Of particular importance was the observation that there was more population mixing in EBLV-1a than in EBLV-1b. In EBLV-1a, the phylogenetic homogeneity of isolates across geographic regions suggests that there is an established viral traffic among bat populations in northern Europe. This process cannot be explained by the behavior of *Eptesicus serotinus* bats, which are not a migratory species (12) and suffer mortality from EBLV infection. It is therefore possible that long-distance transmission is facilitated by migratory bat species that roost with *E. serotinus*. The large roost sizes and high densities of many bat species make them well suited to the sustained transmission and exchange of RNA viruses (26), as well as a variety of other pathogens (16). Under these conditions, the transmission of EBLV is most likely maintained through the transfer of infectious saliva during licking and biting (16). Although 95% of all EBLV-positive bats submitted for diagnostic tests are *E. serotinus*, this could result from ascertainment bias toward anthropophilic species, those that are not experiencing a decline in population size, or those that suffer fatal infections. However, the bat species involved in this presumed viral traffic in northern Europe are unknown. One migratory bat species that is widely distributed in northern Europe and which has been proposed to play a role in EBLV transmission is *Pipistrellus nathusii*, although there is currently no evidence for its involvement (8, 36).

In contrast, the greater spatial structure in the EBLV-1b tree indicates that there has been less contact between bat populations from diverse regions in Europe. In particular, the Dutch strains form a discrete phylogenetic group, implying that contact networks between northern and southern Europe are not well established. This may, in part, be due to a decline in bat populations, as reported in countries like Germany (1, 27), for reasons that include human intervention. If roosts in intermediary locations disappear, thereby reducing contact between bat communities, then viral population subdivision will also be enhanced. Similarly, there may be an absence of migratory species able to connect the foci of EBLV-1b in southern Europe (Spain and southern France) with those in more western (Brittany) and northern (northern France and The Netherlands) locations. As a case in point, *Tadarida teniotis* and *Miniopterus schreibersii*, which are present in Mediterranean regions, can fly long distances, and are known to carry EBLV-1 (36), are not encountered in northern and western France.

Our analysis of the place of origin for EBLV-1a and EBLV-1b was more complex, particularly given the small number of samples available from southern Europe. In the case of EBLV-1b, a combination of methods suggested that the sampled lineages most likely arose in France and then spread northward and southward, although an initial entry into Europe via Spain and southern France cannot be excluded. The spread of EBLV-1a was even harder to infer, and we were unable to locate a consistent place of origin. However, the observation that the virus occupies an east-west axis across northern Europe implies that the virus spread in a directed manner across the region.

Evolutionary processes in EBLV. A key aspect of our work was the observation that EBLV is subject to strong constraints

against amino acid change. In particular, the d_N/d_S values estimated for EBLV are relatively low for RNA viruses (41), even in comparison to the G gene of genotype 1 lyssaviruses in canids (21). Similarly strong selective constraints have previously been documented in arthropod-borne RNA viruses, presumably because of the fitness trade-offs that are inherent when replicating in cell types as different as those from mammals and insects (41). However, the stronger purifying selection against the N gene relative to the G gene was expected, as envelope glycoproteins, which often interact with host cell receptors and are the main targets of the immune response, show greater amino acid diversity than nucleocapsid proteins in most RNA viruses (41).

The constrained evolution of EBLV-1 was even more apparent in our analysis of rates of nucleotide substitution. Specifically, the substitution rates we estimated for EBLV-1 are not only among the lowest seen in RNA viruses (19, 23), but lower than those previously reported for genotype 1 lyssaviruses. These estimates range from relatively high rates of $\sim 1 \times 10^{-3}$ substitutions/site/year (19, 22) to lower rates of 3.1×10^{-4} to 5.5×10^{-4} substitutions/site/year (4, 6). The lowest evolutionary rate thus far recorded for genotype 1 lyssaviruses was 5.06×10^{-5} substitutions/site/year at nonsynonymous sites (21). However, as the synonymous rate was an order of magnitude greater, at 4.10×10^{-4} substitutions/site/year, the overall substitution rate will be higher than that reported here for EBLV-1.

There are a variety of plausible explanations for the low rates of evolutionary change in EBLV-1. First, it is possible that EBLV-1 replicates slowly in its bat hosts so that few mutational errors are produced per unit time. Very low levels of EBLV RNAs have been detected in several tissues (brain, blood, lung, heart, tongue, and esophagus-larynx-pharynx) and saliva of apparently healthy bats, suggesting either that there is a nonproductive infection in these species or that these RNAs are remnants of an earlier lyssavirus infection (36, 40). Nevertheless, some hosts do develop symptoms and succumb to disease (40). Why this is so is not known. As such, it seems premature to conclude that EBLV is characterized by especially low rates of replication.

A second possibility is that peculiarities of the bat immune system have altered the selection pressures faced by the EBLVs, in turn putting a brake on rates of evolutionary change. Although studies of fruit bats have revealed well-developed immune systems (30), lower levels of agglutinating, hemagglutinating, and complement-fixing antibodies are produced in response to various antigens than in conventional laboratory animals (20), and the peak of primary antibody response after antigenic challenge is delayed (9). Additionally, the activation of T lymphocytes is significantly delayed in bats compared to mice (10, 30). Despite these differences, a previous study of genotype 1 lyssaviruses reported similar d_N/d_S values in bats and terrestrial vertebrates (21), and as yet there is no evidence for reduced rates of nucleotide substitution in rabies viruses isolated from bats (4). This suggests that immune selection is not especially weak in bats compared to other mammals, although this clearly needs to be explored in more detail.

Whatever the explanation for the low rate of evolutionary change in EBLV-1, our study suggests that the virus has reached an adaptive peak, so that most amino acid changes reduce fitness and are therefore removed by purifying selection. This interpretation is supported by a number of observations. First, as discussed above, rates of nucleotide substitution, especially at nonsynonymous sites, were low in EBLV-1. More striking was that the d_N/d_S ratios on the internal branches of the EBLV-1 trees were far lower than the equivalent values seen on external branches. This means that very few nonsynonymous changes have reached fixation during the evolutionary history of EBLV-1, as expected if fitness benefits are difficult to generate. Hence, the nonsynonymous mutations we see in EBLV-1 most likely represent transient deleterious and slightly deleterious amino acid changes that are eventually removed from the population by purifying selection; this explains why they fall at the tips of phylogenetic trees. Intriguingly, unusually low rates of nucleotide substitution have also been recorded in another rhabdovirus, vesicular stomatitis virus (19). The fact that vesicular stomatitis virus induces a persistent infection in its mammalian hosts (25) hints that this form of virus-host relationship might often be associated with reduced rates of nucleotide substitution. Finally, although the population sizes of EBLV in European bats are increasing, the measured growth rates are very low, suggesting that the virus exists in a near-equilibrium state in many bat species.

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