Journal of General Virology (1997), **78**, 2319–2328. Printed in Great Britain

Evolution of European bat lyssaviruses

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Forty-seven European bat lyssaviruses (EBL) and two African insectivorous bat lyssaviruses (Duvenhage viruses) were selected for a comparison to be made of their evolutionary relationships. Studies were based on direct sequencing of the PCR-amplified products of the 400 nucleotides coding for the amino terminus of the nucleoprotein. Phylogenetic relationships were analysed after bootstrap resampling using the maximum parsimony and the neighbour-joining methods. Analyses of both the nucleotide and amino acid sequences placed these viruses in three separate clusters, namely genotype 4 (Duvenhage), genotype 5 (EBL1) and genotype 6 (EBL2). Evolutionary analysis of the nucleoprotein gene of EBL1 and EBL2 indicated low intrinsic heterogeneity mainly due to synonymous substitutions. In addition, both EBL1 and EBL2 evolved into at least two genetically distinguishable lineages (a and b) following geographical drifting. We can speculate that subsequently the lineages EBL1a and EBL1b were introduced into parts of northern Europe from two different geographical directions; EBL1b was probably introduced most recently and was from North Africa. *Eptesicus serotinus* appears to be the principal reservoir for EBL1 and *Myotis dasycneme* and *M. daubentonii* the reservoirs for EBL2.

Introduction

Rabies in non-haematophagous bats was first diagnosed in frugivorous and insectivorous species in Trinidad in 1931 (Pawan, 1936). Not until 1953, however, was the disease confirmed in insectivorous bats of the USA (Venters *et al.*, 1954) and it rapidly became clear that the disease had been widespread but undetected in North American bats for many years. The interest aroused by these findings led workers in other countries to examine bats for rabies viruses and it was not long before a bat virus was found in European insectivorous bats (Nikolic & Jelesic, 1956), in West African fruit bats (Boulger & Portefield, 1958) and in a human known to have been bitten by an insectivorous bat in South Africa (Meredith *et al.*, 1971). A striking difference between the bat rabies (lyssa) viruses of the Americas and those of Africa, Europe and the former Soviet Union was found. The American bat lyssaviruses were closely related to classical lyssaviruses (Smith, 1988), but those in bats from Africa and Europe were less closely related to classical lyssaviruses and required separate classification as different serotypes (Koprowski *et al.*, 1985; Schneider *et al.*, 1973; Wiktor *et al.*, 1984) and genotypes (Bourhy *et al.*, 1992, 1993). Bats were also found to be virus-positive in Asia but no strain identifications were reported (Smith *et al.*, 1967). More recently, in May 1996, a lyssavirus which is phylogenetically related to classical rabies viruses (serotype/genotype 1) and to European bat lyssaviruses (EBL) was isolated in Australia from five bats known as flying foxes (*Pteropus alecto* and *P. scapulatus*) and from a human who died of rabies (Fraser *et al.*, 1996).

Between 1954 and 1984, lyssavirus infection in European bats, all of which are insectivorous, was rarely reported. However, in Denmark in 1985, the isolation of lyssaviruses from ten *Eptesicus serotinus* bats and from a *Myotis daubentonii* and a *M. dasycneme* (Mollgaard, 1985), together with the death in Finland of a human from presumed bat rabies (Lumio *et al.*, 1986) increased interest and awareness of lyssavirus infection in European bats (Kappeler, 1989). During 1986 and 1987, a further 262 cases were reported, predominantly from Denmark and the Netherlands, but since that time the number of reports has declined to an average of 12 per year between 1991 and 1995. Nevertheless, the bat lyssavirus 'epidemic' has resulted in the reporting of 483 cases from ten European countries, including the Russian Federation, from 1954–1995 (Anon., 1995; Kappeler, 1989; Müller, 1994). In addition, one infected

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Table 1. Origin of the isolates

Virus	Year	Country of isolation	City	Species isolated from	Strain number	Reference or source*	GenBank accession no.
EBL1a	1987	Denmark	Christiansfeld	*E. serotinus*	R 94110DEN	A. King	U89474
EBL1a	1987	Denmark	Horsens	*E. serotinus*	9479DEN	A. King	U89471
EBL1a	1987	Denmark	Juelsminde	*E. serotinus*	94109DEN	A. King	U89473
EBL1a	1968	Germany	Hamburg	*E. serotinus*	9395GER	A. King	U89466
EBL1a	1970	Germany	Stade, Niedersachsen	*E. serotinus*	9398GER	A. King	U89457
EBL1a	1982	Germany	Bremerhaven	*E. serotinus*	9399GER	A. King	U89468
EBL1a	1985	Germany	Rostock	*E. serotinus*	9396GER	A. King	U89467
EBL1a	1986	Germany	Nienburg, Niedersachsen	*E. serotinus*	9477GER	A. King	U89469
EBL1a	1986	Germany	Stade, Niedersachsen	*E. serotinus*	9436GER	A. King	U89458
EBL1a	1987	Germany	Bremen, Bremen	*E. serotinus*	9437GER	A. King	U89459
EBL1a	1988	Germany	Neumunster, Schleswig-Holstein	*E. serotinus*	9438GER	A. King	U89460
EBL1a	1989	Germany	Bad Iberg, Niedersachsen	*E. serotinus*	9439GER	A. King	U89461
EBL1a	1989	Germany	Braunschweig, Niedersachsen	*E. serotinus*	9440GER	A. King	U89462
EBL1a	1990	Germany	Walsrode, Niedersachsen	*E. serotinus*	9441GER	A. King	U89463
EBL1a	1990	Germany	Ratzeburg, Schleswig-Holstein	*E. serotinus*	9442GER	A. King	U89464
EBL1a	1990	Germany	Lubeck, Schleswig-Holstein	*E. serotinus*	9481GER	A. King	U89475
EBL1a	1987	Holland	Joure	*E. serotinus*	9474HOL	A. King	U89476
EBL1a	1987	Holland	Bellingwolde	*E. serotinus*	9480HOL	A. King	U89472
EBL1a	1989	Holland	Jubbega	*E. serotinus*	94116HOL	A. King	U89450
EBL1a	1989	Holland	Rolde	*E. serotinus*	9478HOL	A. King	U89470
EBL1a	1992	Holland	Goor	*E. serotinus*	9366HOL	J. T. van Oirschot[a]	U89451
EBL1a	1992	Holland	Neede	*E. serotinus*	9368HOL	J. T. van Oirschot	U89452
EBL1a	1992	Holland	Valthermond	*E. serotinus*	9372HOL	J. T. van Oirschot	U89453
EBL1a	1993	Holland	Alblasserdam	*E. serotinus*	9374HOL	J. T. van Oirschot	U89454
EBL1a	1985	Poland	Gdansk	*E. serotinus*	8615POL	D. Seroka[b]	U22844
EBL1a	1990	Poland	Ketrzyn	*E. serotinus*	9394POL	D. Seroka	U89456
EBL1a	1994	Poland	Nietoperz	*E. serotinus*	96031POL	D. Seroka	U89455
EBL1a	1985	Russia	Belgorod	Human	9397RUS	A. King	U89477
EBL1a	1987	Ukraine	Volyn region	*V. murinus*	9443UKR	A. King	U89465
EBL1b	1989	France	Briey	*E. serotinus*	8918FRA	Institut Pasteur	U22845
EBL1b	1989	France	Bainville	*E. serotinus*	8919FRA	J. Barrat[e]	U89440
EBL1b	1995	France	Bourges	*E. serotinus*	9603FRA	J. Barrat	U89441
EBL1b	1992	Holland	Schagen	*E. serotinus*	94113HOL	A. King	U89448
EBL1b	1992	Holland	Spanbroek	*E. serotinus*	94114HOL	A. King	U89449
EBL1b	1992	Holland	Bovenkarspel	*E. serotinus*	94115HOL	A. King	U89444
EBL1b	1992	Holland	Wassenaar	*E. serotinus*	9367HOL	J. T. van Oirschot	U89445
EBL1b	1993	Holland	Moerkapelle	*E. serotinus*	9376HOL	J. T. van Oirschot	U89446
EBL1b	1993	Holland	Apeldoorn	*E. serotinus*	9377HOL	J. T. van Oirschot	U89447
EBL1b	1987	Spain	Granada	?	9483SPA	Institut Pasteur	U89443
EBL1b	1994	Spain	Granada	*E. serotinus*	94285SPA	A. Téllez[d]	U89442
EBL2a	1987	Holland	Wommels	*M. dasycneme*	9018HOL	J. Haagsma[e]	U22847
EBL2a	1987	Holland	Tjerkwerd	*M. dasycneme*	9482HOL	A. King	U89480
EBL2a	1989	Holland	Andijk	*M. dasycneme*	94112HOL	A. King	U89481
EBL2a	1993	Holland	Roden	*M. dasycneme*	9375HOL	J. T. van Oirschot	U89482
EBL2a	1996	United Kingdom	Newhaven	*M. daubentonii*	EBL2GB	J. E. Whitby	U89478
EBL2b	1986	Finland	Helsinki	Human	9007FIN	Lumio *et al.* (1986)	U89846
EBL2b	1993	Switzerland	Versoix	*M. daubentonii*	9337SWI	R. Zanoni[f]	U89479
Duvenhage	1971	Republic of South Africa		Human	86132SA	Meredith *et al.* (1971)	U22848
Duvenhage	1981	Republic of South Africa		*Miniopterus sp.*	94286SA	Van der Merwe (1982)	U89483

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Fig. 1. Geographical distributions of the isolates.

bat was recently found in England (Whitby *et al.*, 1996). No evidence of EBL infection has been observed in terrestrial animals. However, three human cases have been reported, two from the former Soviet Union (Selimov *et al.*, 1989, 1990) and the third from Finland in 1985 (King *et al.*, 1990; Lumio *et al.*, 1986). Only three isolations of lyssaviruses of African insectivorous bats (Duvenhage viruses; DUV) have been reported: one from the index case in South Africa (Meredith *et al.*, 1971), one from an unidentified, but believed to be insectivorous bat in South Africa (Van der Merwe, 1982) and one from an insectivorous *Nycteris thebaica* bat in Zimbabwe in 1986 (King & Crick, 1988).

Preliminary results based on monoclonal antibody studies established that EBL were related to, but different from DUV and that two different antigenic profiles could be identified in EBL (King *et al.*, 1990; Montano Hirose *et al.*, 1990). Further studies based on the sequences of the nucleoprotein gene of four EBL established that these viruses can be further subdivided into two different genotypes of lyssavirus: genotype 5 (or EBL1) and genotype 6 (or EBL2) (Bourhy *et al.*, 1992, 1993). Protection and seroneutralization experiments (Fekadu *et al.*, 1988; Lafon *et al.*, 1986, 1988) and evaluations of the cellular immune response after vaccination (Celis *et al.*, 1988; Herzog *et al.*, 1991, 1992; Joffret *et al.*, 1990; Perrin *et al.*, 1991) have demonstrated the weak efficacy of conventional rabies vaccines of genotype 1 against infection by EBL1, EBL2 and DUV isolates. This has lead to public health concerns and to the possible need to develop more specific vaccines against other genotypes of lyssaviruses (Tordo *et al.*, 1993).

In order to evaluate the intrinsic genetic variability and to determine the spatio-temporal and phylogenetic relationships between the EBL, an extensive molecular analysis of a section of the nucleoprotein-coding region of 47 EBL and 2 DUV isolates was carried out. The putative ancestral sequence of the different lineages of these viruses was estimated and the nucleotide substitution patterns of the four nucleotides were analysed. On the basis of the results, the characteristics of the nucleotide substitutions of the nucleoprotein gene of EBL are discussed and hypotheses concerning the origin of these viruses in European bats are explored.

Methods

■ **Selection criteria for the samples.** All species of European bats are protected under law and as such they may not be taken for surveys or experimentation without a license. This restricts any studies of rabies

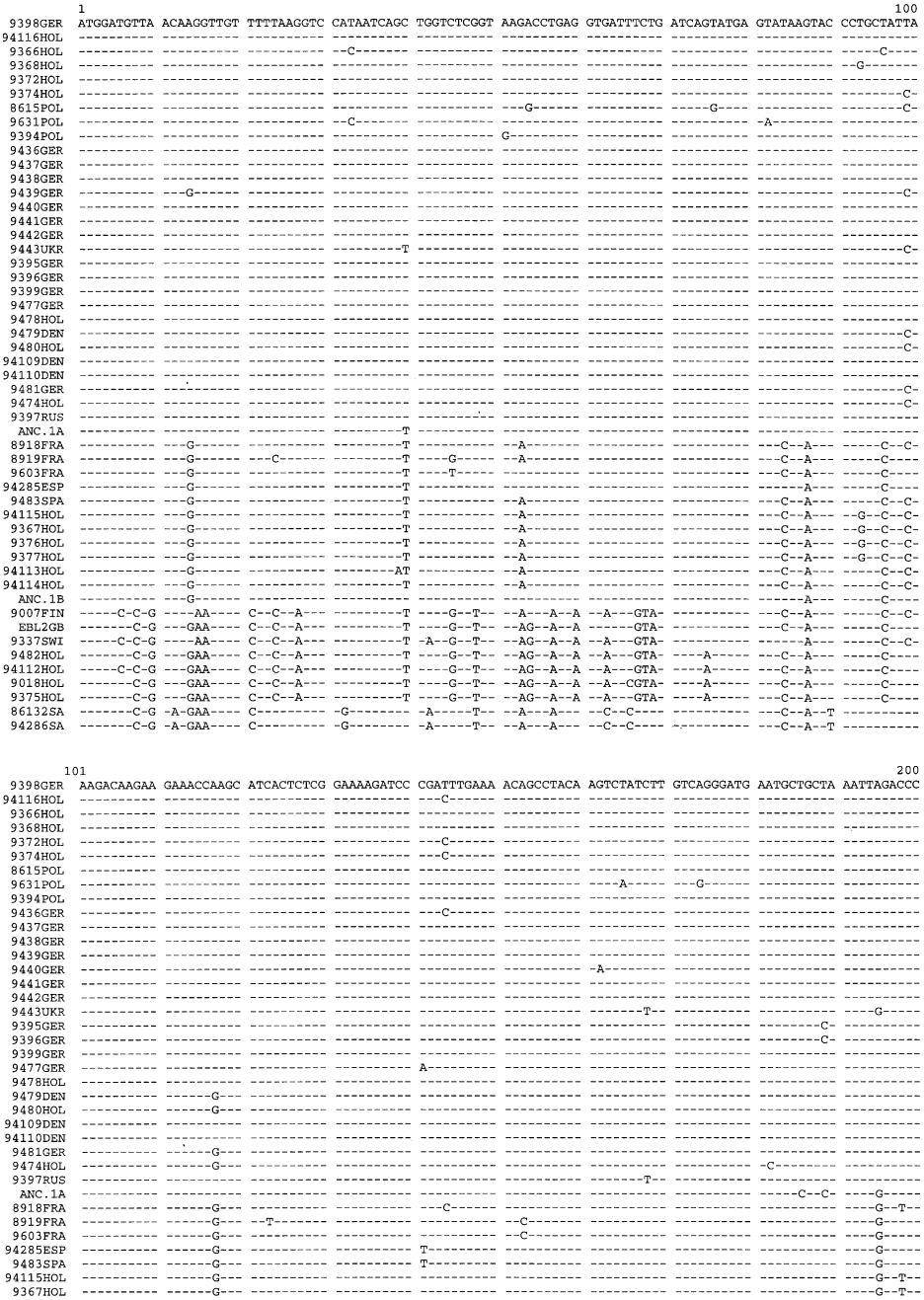

Fig. 2 For legend see facing page.

in bats to those which are dead or dying. The EBL isolates analysed in this study represented about 10% of the reported cases and were selected according to their availability and to best reflect spatio-temporal diversity (Table 1).

■ **Extraction of RNA, PCR and sequencing.** Original or first mouse passage brain material of 43 positive samples of European bats (Fig. 1), together with one African DUV was selected (Table 1). Briefly, original infected brains or suckling mouse brains infected with the

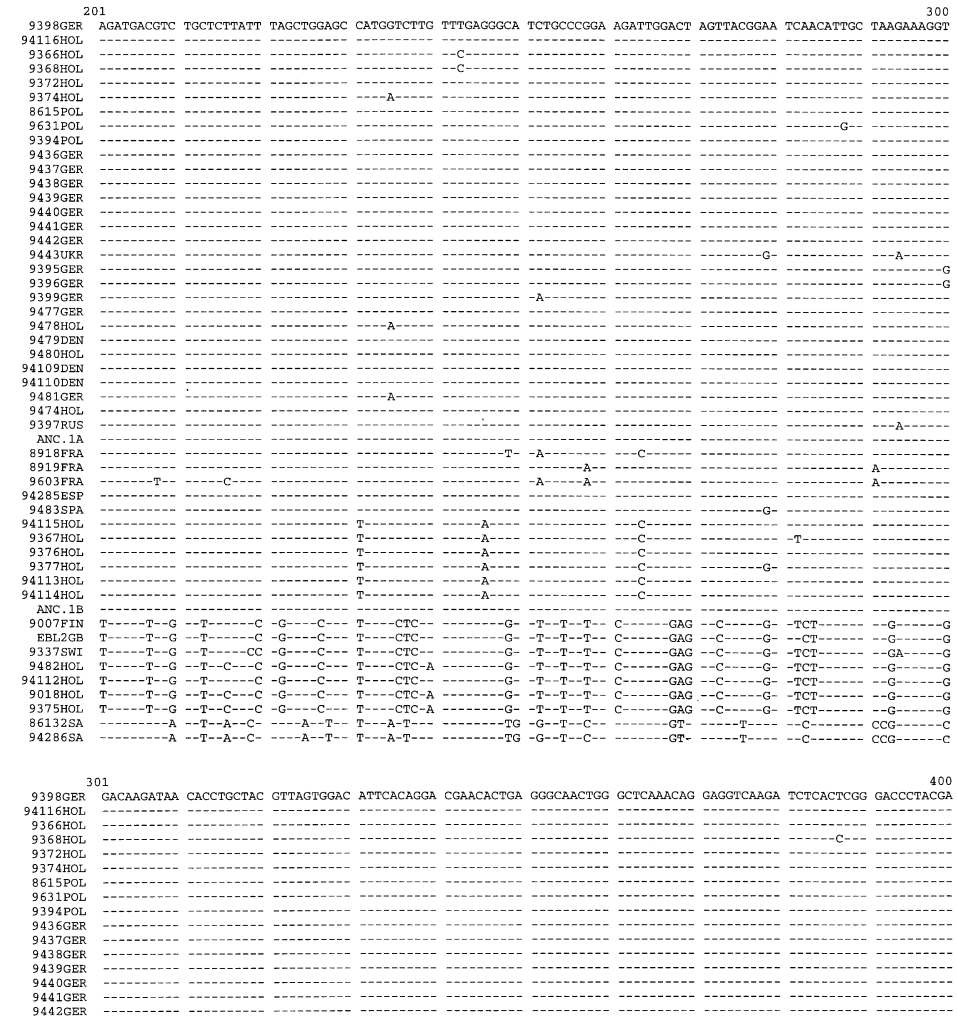

Fig. 2. Alignment of the 400 bases coding for the amino terminus end of the nucleoprotein. Only nucleotide changes relative to the sequence of 9338GER are indicated. ANC.1A, putative ancestral strain of EBL1a; ANC.1B, putative ancestral strain of EBL1b.

original virus were subjected to total RNA extraction, cDNA synthesis and PCR amplification according to the method described by Sacramento *et al.* (1991) and with primers N41 (ACCAGAGATGCATGTTCA, nucleotides 492–475 of the PV sequence; Tordo *et al.*, 1986) and N12, N6, N8 and N19 as previously described (Bourhy *et al.*, 1993). Amplified products were purified by electrophoresis on 0·7% Nu sieve GTG

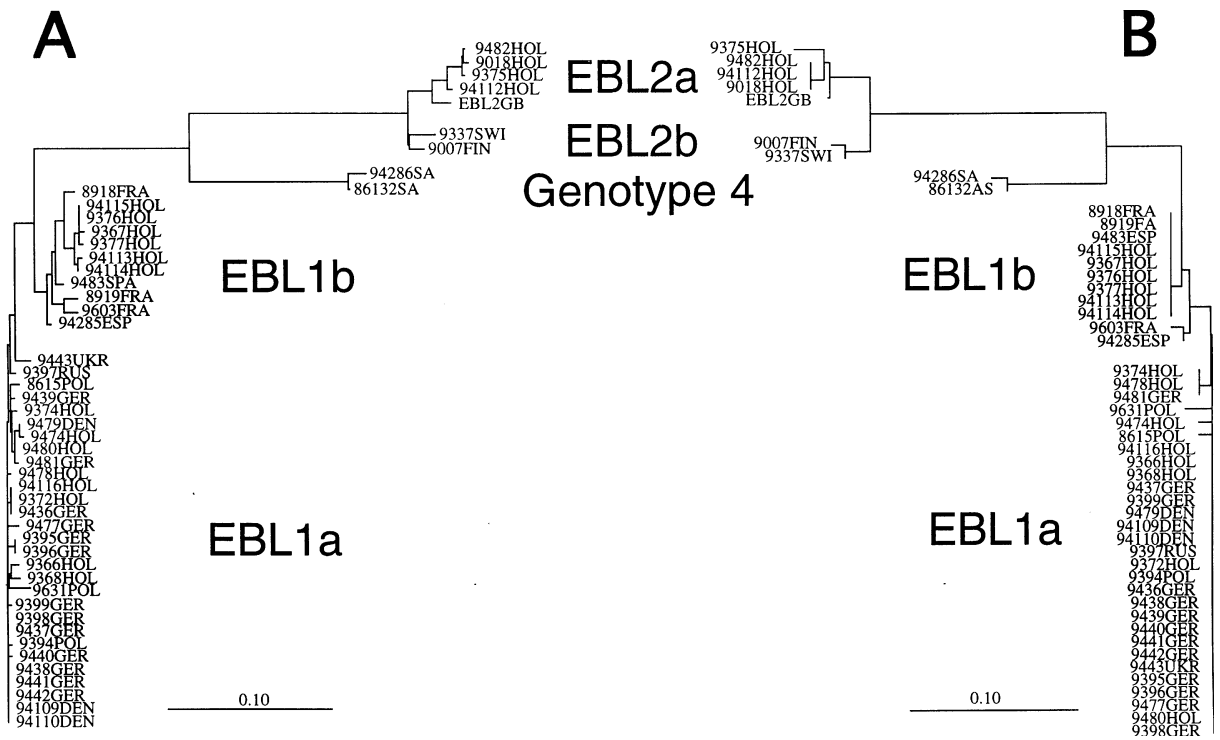

Fig. 3. Phenogram showing the phylogenetic relationships between the nucleotide (400 bases; A) and the deduced polypeptide (133 amino acids; B) sequences of the amino terminus of the nucleoprotein of 46 isolates of EBL1, EBL2 and DUV. Data were obtained using the neighbour-joining method (Saitou & Nei, 1987). Vertical branches are non-informative and set for clarity only. The length of the horizontal branches reflects phylogenetic distance relationships. Numbers and letters at the ends of the branches refer to the isolate code. The name of the lineages and sublineages described in the text are indicated. The scale is shown below each tree.

agarose gel (FMC) and were sequenced on both strands of the 400 nucleotides coding for the amino terminus of the nucleoprotein using the T7 sequencing kit (Pharmacia). The sequenced region corresponds to the most variable part of the nucleoprotein and has previously been shown to be suitable for epidemiological investigations (Kissi *et al.*, 1995).

■ **Phylogenetic analysis.** The nucleotide (400 nt) and deduced amino acid (133 residues) sequences of the 44 isolates, together with those of five isolates previously determined (Bourhy *et al.*, 1993), were aligned by using the CLUSTALW 1.60 program (Higgins & Sharp, 1989). Aligned sequences were analysed using the neighbour-joining method (Saitou & Nei, 1987) applied to a distance matrix of substitution per site and implemented using CLUSTALW. The BOOTSTRAP option of CLUSTALW (1000 replicate data sets) was used to assess the robustness of the method. A phylogenetic analysis was also performed by the maximum parsimony method (Fitch, 1971), implemented using PHYLIP package version 3.52c (Felsenstein, 1993). Firstly, SEQBOOT was run to produce 100 bootstrapped data sets; then the phylogeny estimate for each of these sets was found using DNAPARS or PROTPARS for nucleotide or amino acid sequences, respectively. CONSENSE was used to generate the majority rule consensus tree and the bootstrap confidence limits. Finally, the TREETOOL program was used to produce graphic outputs of phylogenetic trees. The sequences of the internal nodes were determined with the algorithm described by Fitch (1971).

■ **Genetic distances between isolates.** Distances between sequences are the percentage divergences and were calculated according to Saitou & Nei (1987). Synonymous and non-synonymous nucleotide substitutions between the reconstructed sequences of the ancestor nodes and those of the isolates were computed by the *p*-distance method (Nei & Gojobori, 1986) using the DISTANCE ESTIMATION program of the MEGA package version 1.01 (Kumar *et al.*, 1993). Rates of change were estimated by linear regression of the number of differences against either the time (year) of collection of the isolates or the geographical distance (km) separating the place of collection of the isolates.

Results

Comparison of nucleotide and deduced amino acid sequences of EBL and DUV isolates

Alignment of the 47 sequences corresponding to the 400 nucleotides coding for the amino terminus of the nucleoprotein showed no insertions or deletions (Fig. 2). Analysis of both the nucleotide and deduced amino acid sequence grouped these viruses into three genetic clusters, namely genotype 4 (DUV), genotype 5 (EBL1) and genotype 6 (EBL2), confirming previous results (Bourhy *et al.*, 1993). A representative phylogeny using the neighbour-joining method is shown in Fig. 3. The maximum parsimony method showed the same overall arrangements of the sequences (not shown). The percentage of nucleotide and amino acid sequence similarity between the two DUV was high: 98·7 and 99·2%. Both EBL1 and EBL2 were further subdivided into two different phylogenetic lineages (a and b). These relationships between the nucleotide or amino

Table 2. Bootstrap confidence limits (%) of the major phylogenetic clusters obtained by the analysis of 400 bases coding for the amino terminus of the nucleoprotein

NJ values were obtained by the BOOTSTRAP option (1000 replicates) of CLUSTALW and PARS values were obtained by the SEQBOOT option (100 replicates) and the maximum parsimony method implemented using the PHYLIP package version 3.52c. The sequence of isolate 94286SA was selected as the outgroup.

	Nucleotide sequence		Amino acid sequence	
Lineage	NJ	PARS	NJ	PARS
Genotype 4	100	100	100	100
EBL2	100	100	100	100
EBL2a	96	91	90	89
EBL2b	75	73	99	44
EBL1	100	100	100	100
EBL1a	99	96	76	34
EBL1b	68	35	x	58

x, Value not included in the bootstrap confidence limits.

acid sequences of the isolates were supported by bootstrap resampling, which revealed that clusters delineating EBL1a, EBL2a, EBL2b and DUV are found in a high proportion of the trees tested. The major exception is lineage EBL1b (Table 2). However, the two EBL1 groups are unequivocally differentiated by substitutions at ten positions (bp 14, 40, 87, 96, 117, 195, 333, 334, 336 and 337). Five isolates from Holland and England constituted the lineage EBL2a (98·4 and 98·8% average similarity at the nucleotide and amino acid level, respectively) and two isolates represented the lineage EBL2b (97·2 and 99·2% average similarity at the nucleotide and amino acid level, respectively). The recent case of rabies in a Daubenton's bat in the south of the UK was shown to belong to the lineage EBL2a. The majority of the isolates studied belonged to EBL1. The distribution of type EBL1a (99·1 and 99·1% average similarity at the nucleotide and amino acid level, respectively) joins 29 samples of the northern and eastern parts of Europe (Denmark, Holland, Germany, Poland, Ukraine and Russia), whereas EBL1b (98 and 99·1% average similarity at the nucleotide and amino acid level, respectively) joins 11 samples from countries of the western part of Europe (Holland, France and Spain) (Fig. 1).

Nucleotide substitution pattern

When nucleotide substitutions among the different lineages were compared (Table 3), values for transitions (p) and transversions (q) indicated a predominance of the transitions. The ratios p/q, ranging from 1–9, were comparable with those determined in genotype 1 (Kissi *et al.*, 1995). The intrinsic variability of the deduced amino acid sequences within genotype 4 and the four lineages of EBL1 and EBL2 was very low (Fig. 3B), indicating an absence of accumulation of non-synonymous mutations. Synonymous nucleotide substitutions exhibited comparable proportions in all the lineages and tended to occur at low rates compared with those observed in genotype 1 (Kissi *et al.*, 1995) (Table 3). The putative ancestral strains of EBL1a (ANC.1a) and EBL1b (ANC.1b) were determined (Fig. 2). The proportions of synonymous nucleotide substitutions compared to these ancestral sequences were computed for all the EBL1a and EBL1b isolates. These proportions appeared to be stable with time. For example,

Table 3. Comparison of nucleotide substitutions among different lineages

Distance (d), proportions of transitions (p), transversions (q) and synonymous (dsyn) substitutions in the nucleoprotein-coding sequence calculated according to the *p*-distance method (Nei & Gojobori, 1986). N1 indicates the number of values and N2 the number of calculated rates.

	EBL1a	EBL1b	EBL1	EBL2a	EBL2b	EBL2	Genotype 4
N1	406	55	780	10	1	121	1
d	0·0085	0·0203	0·0249	0·0155	0·0275	0·0324	0·0125
SD	0·0051	0·0117	0·0194	0·0099	ND	0·0185	ND
p	0·0066	0·0157	0·0201	0·0135	0·0250	0·0302	0·0100
SD	0·0043	0·0089	0·0161	0·0092	ND	0·0181	ND
q	0·0019	0·0046	0·0048	0·0020	0·0025	0·0021	0·0025
SD	0·0025	0·0041	0·0045	0·0016	ND	0·0016	ND
dsyn	0·0319	0·0846	0·0931	0·0580	0·0968	0·0967	0·0426
SD	0·0075	0·0180	0·0159	0·0352	ND	0·0497	ND
N2	195	43	557	6	1	15	1
p/q	1·02	2·91	2·84	6·25	9	7·81	4
SD	1·49	2·76	2·68	3·37	ND	7·55	ND

ND, Not determined.

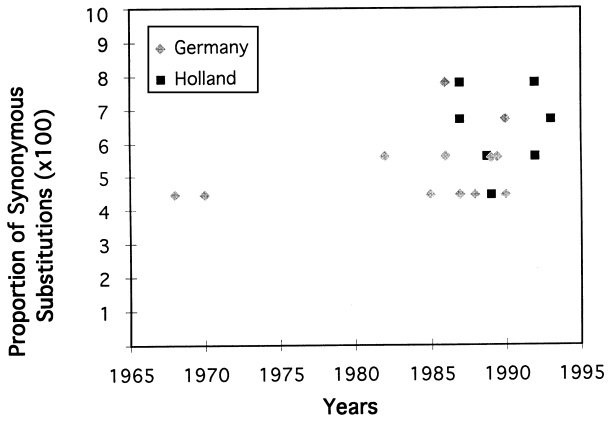

Fig. 4. Evolution of EBL1a in Germany and in Holland from 1968–1990. Synonymous substitutions were computed using the DISTANCE ESTIMATION program of the MEGA package version 1.01 (Kumar *et al.*, 1993) with the common ancestor node of EBL1a in reference. Number of substitutions was plotted against year of isolation.

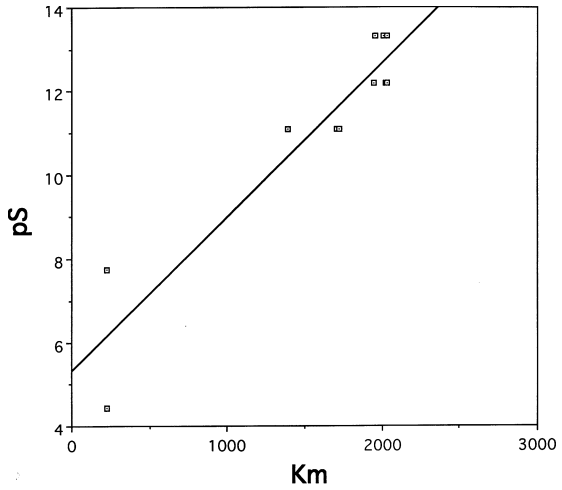

Fig. 5. Evolutionary distance for the nucleoprotein-coding sequence according to the geographical distance. The evolutionary rate is estimated by regression of the proportion of synonymous mutations $\times 100$ (pS) of the nucleotide sequence of the isolates from the common ancestor node of EBL1b against the distance in km (Km) to Gibraltar. Synonymous mutations were computed by the method of Nei & Gojobori (1986) using the DISTANCE ESTIMATION program of the MEGA package version 1.01 (Kumar *et al.*, 1993).

samples of EBL1a collected in Germany and in Holland at intervals of 22 and 9 years, respectively, did not exhibit significant linear variation (Fig. 4). The only correlation that was observed linked the proportion of synonymous mutations and the geographical origin of the isolates of EBL1b. According to our data, the isolates phylogenetically closer to ANC.1b were those from Spain. From south to north, the distance of EBL1b isolates of France and Holland increase proportionally to the distance from Gibraltar ($r = 0{\cdot}89$; $P < 0{\cdot}01$) (Fig. 5). No correlation between the proportions of synonymous nucleotide substitutions and the geographical distance to one hypothetical introduction point was observed with lineage EBL1a.

Discussion

This phylogenetic study confirmed that EBL include two independent lineages of lyssavirus which we named, respectively, genotype 5 (EBL1) and genotype 6 (EBL2) and that they are different from genotype 4 (DUV). It also established the relatively low intrinsic heterogeneity (less than 3·3% divergence at the nucleotide level) of both EBL genotypes compared with that which is known for genotype 1 (Kissi *et al.*, 1995). Within EBL1 and EBL2, two lineages (a and b) can also be differentiated by their nucleotide and amino acid sequences. EBL1a and EBL1b, the most frequently reported, are widely distributed. EBL1a exhibits a west–east distribution whereas that of EBL1b is north–south. Holland is the only country from which both lineages have been isolated. The low intrinsic heterogeneity obtained by comparing EBL1a and EBL1b indicates a genetic stasis of the nucleoprotein gene within lineages, connected with high selective constraints on the non-synonymous sites of the sequence. These data favour the existence of a close adaptation of the viruses to its host. Similar findings concerning the conservation of nucleoprotein were obtained with avian influenza A viruses (Gorman *et al.*, 1990) and enterovirus 70 (Takeda *et al.*, 1994). All the EBL1 viruses studied were isolated from *E. serotinus* bats with one exception, an EBL1a isolated in the Ukraine from a *Vespertilio murinus* bat. Furthermore, all the lyssaviruses characterized up to now in *E. serotinus*, either by monoclonal antibody studies or by genetic analyses were of the EBL1 type. EBL1a and EBL1b could then represent two groups of variants adapted to the same animal species. The serotine bat (*E. serotinus*) is by far the most frequently reported rabid bat in Europe (87·9%; $n = 379$) (Kappeler, 1989). This species is found from western Europe through southern Asiatic Russia to the Himalayas, Thailand and China, north to Korea and also in North Africa and most of the Mediterranean islands (Stebbings & Griffith, 1986). Serotines are relatively sedentary bats and feed predominantly on large insects in open sheltered urban and parkland areas, mostly in lowlands. It has also been hypothesized from the data presented here that the introduction of EBL1b to Europe could have occurred from Africa via the south of Spain. If this is confirmed, the rate of evolution of the virus according to the distance of spread from the introduction point could be explained by a 'bottleneck-like' transmission mechanism (Clarke *et al.*, 1993). A recent study of prevalence of EBL1 antibodies in *E. serotinus* bats in southern Spain has shown evidence of circulation of this virus in bat colonies (Pérez-Jorda *et al.*, 1995). In conclusion, EBL1 isolates have evolved into at least two genetically distinguishable groups, following geographical drifting. We can speculate that subsequently, these two groups were introduced into parts of northern Europe from two different geographical directions, EBL1b from North Africa and probably most recently, according to the lower homology observed within this subtype. If we consider that the insectivorous bat lyssavirus of Africa, DUV, was isolated in

Zimbabwe from a Microchiroptera (*Nycteris thebaica*) (King & Crick, 1988) and that these bats are widely distributed in Africa, including North Africa (A. Brosset, personal communication), it would be interesting to explore whether EBL1-like viruses circulate in *Nycteris*, *Miniopterus* or *Eptesicus spp*. in the northern part of Africa.

The infrequency of identification of EBL2 restricted our ability to draw conclusions concerning the geographical range of EBL2 and therefore a more critical survey of *Myotis spp*. is required. *M. dasycneme* bats ('Pond bats') prefer riparian habitats and usually feed over waterways. They are known to migrate and nursery roosts and hibernacula are often 200–300 km apart (Stebbings & Griffith, 1986). *M. daubentonii* bats are more common than *M. dasycneme* bats and share many of their ecological characteristics. At least three foci of EBL2 are now identified: EBL2b in *M. daubentonii* in Switzerland and EBL2a in *M. dasycneme* in Holland. The recent positive case reported from the south of England in a *M. daubentonii* (Whitby *et al*., 1996) is phylogenetically closest to this last focus. There may exist a fourth focus of EBL2b in Finland, although it is not possible to determine whether or not the Swiss bat biologist contracted the disease in Switzerland or in Finland. According to these limited data, EBL2a and EBL2b are not specifically associated with rabies in either *M. dasycneme* or *M. daubentonii*.

The distribution of EBL observed and the putative hypothesis that an extension of the respective infected areas has occurred would suggest that EBL are more widely distributed than recorded cases indicate and that measures to contain the spread would be difficult to apply. A comparison of genetic and antigenic homogeneity (Bourhy *et al*., 1992) within the lineages of EBL1 and EBL2 and the low number of affected bat species contrast markedly with the situation in America. The 38 species of North American bats, representatives of which have been shown to be rabid (Brass, 1994) show a higher level of antigenic variation, a higher frequency of human death and a spillover into terrestrial animal species (Krebs *et al*., 1996; Smith, 1988).

We are deeply indebted to Dr J. Barrat, Dr J. Haagsma, Professor van Oirschot, Dr D. Seroka, Dr A. Téllez and Dr R. Zanoni for sending us EBL isolates. Blanca Amengual's travel was partly supported by el Ministerio de Sanidad y Consumo de Espana.

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Received 17 February 1997; Accepted 28 April 1997