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SARS-CoV related *Betacoronavirus* and diverse *Alphacoronavirus* members found in western old-world☆

Meriadeg Ar Gouilh[a,g,*], Sébastien J. Puechmaille[b,c,d], Laure Diancourt[a], Mathias Vandenbogaert[a], Jordi Serra-Cobo[e], Marc Lopez Roïg[e], Paul Brown[h], François Moutou[f,1], Valérie Caro[a], Astrid Vabret[g], Jean-Claude Manuguerra[a], on behalf of the EPICOREM consortium[2]

[a] *Institut Pasteur, Unité Environnement et Risques Infectieux, CIBU, Infection et Epidemiologie, 75015, Paris, France*
[b] *Greifswald University, 17489, Greifswald, Germany*
[c] *University College Dublin, Belfield, Dublin 4, Ireland*
[d] *Chauves-souris Aveyron, 12310, Vimenet, France*
[e] *IRBIO & Departament de de Biologia Evolutiva, Ecologia i Ciències Ambientals, Facultat de Biologia, Universitat de Barcelona, 08028, Barcelona, Spain*
[f] *Ecole Nationale Vétérinaire d'Alfort, 94704, Maison-Alfort, France*
[g] *Normandie Université, EA2656, Groupe de Recherche sur l'Adaptation Microbienne, 14000, Caen, France*
[h] *French Agency for Food, Environmental and Occupational Health Safety (ANSES), Avian and Rabbit Virology Immunology and Parasitology Unit (VIPAC), Université Européenne de Bretagne, Ploufragan/Plouzané laboratory, 22440, Ploufragan, France*

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ABSTRACT

The emergence of SARS-CoV and MERS-CoV, triggered the discovery of a high diversity of coronaviruses in bats. Studies from Europe have shown that coronaviruses circulate in bats in France but this reflects only a fraction of the whole diversity. In the current study the diversity of coronaviruses circulating in western Europe was extensively explored. Ten alphacoronaviruses in eleven bat species belonging to the Miniopteridae, Vespertilionidae and Rhinolophidae families and, a SARS-CoV-related *Betacoronavirus* in *Rhinolophus ferrumequinum* were identified. The diversity and prevalence of bat coronaviruses presently reported from western Europe is much higher than previously described and includes a SARS-CoV sister group. This diversity demonstrates the dynamic evolution and circulation of coronaviruses in this species. That said, the identified coronaviruses were consistently associated with a particular bat species or genus, and these relationships were maintained no matter the geographic location. The observed phylogenetic grouping of coronaviruses from the same species in Europe and Asia, emphasizes the role of host/pathogen coevolution in this group.

1. Introduction

Ten years after the SARS-CoV pandemic, the emergence of the MERS-CoV reminded us that unknown coronaviruses still pose a potential threat to human health (Drosten et al., 2003; Bermingham et al., 2012). Those two emblematic coronaviruses likely emerged from interspecies transmission in the vicinity of humans, such as suspected for a growing number of other coronaviruses (*e.g.* BCoV/OC43, PRCV, 229E, NL63). This interspecies-jump capacity makes coronaviruses of particular concern to animal and public health and advocates for stronger surveillance of their circulation in wildlife. Coronaviruses are extremely diverse and circulate in many wildlife species however, diversity is most notable in bats (Tang et al., 2006; Wacharapluesadee et al., 2015). Phylogenetic relationships between coronaviruses infecting humans and those infecting bats have been extensively discussed though no direct transmission has ever been documented (Huynh et al., 2012; Ge et al., 2013; Yang et al., 2014). The ecological richness and phylogenetic diversity of bat species are fundamental drivers of coronavirus diversity and evolution in bats (Tang et al., 2006; Wacharapluesadee et al., 2015; Woo et al., 2006; Cui et al., 2007; Lau et al., 2007; Gouilh et al., 2011; Balboni et al., 2012; Drexler et al., 2014). Rhinolophids (Rhinolophidae) and their sister group the hipposiderids (Hipposideridae) have been previously shown to harbour SARS-CoV like viruses in Asia, eastern-Europe and Africa (Gouilh et al.,

☆ All authors are members or collaborators of the Epicorem consortium (http://coronavirus.fr).

* Correspondence to: GRAM - Groupe de Recherche sur l'Adaptation Microbienne, EA 2656 UNICAEN / UNIROUEN, Université de Normandie, Laboratoire de Virologie, CHU de Caen, CNR ROR - Centre National de Référence pour les Virus de la Rougeole, Rubéole et Oreillons, Av. Georges Clémenceau, 14033 Caen, Cedex, France.
E-mail addresses: meriadeg.le-gouil@pasteur.fr, meriadeg.legouil@normandie-univ.fr (M. Ar Gouilh).

[1] Retired.

[2] Membership of the EPICOREM Consortium can be found here: http://coronavirus.fr.

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2011; Li et al., 2005, 2006; Tong et al., 2009; Lau et al., 2010; Quan et al., 2010; Drexler et al., 2010; Rihtarič et al., 2010; Lelli et al., 2013). The Rhinolophidae family geographic range extends from Asia to southern Europe and Africa. Consequently, rhinolophids in the western Europe could harbour betacoronaviruses, including SARS-CoV like viruses. Therefore, SARS-CoV phylogroup may circulate up to the western limit of the region.

To date, several studies have reported coronaviruses circulating in bats in Europe but none have describe the presence of SARS-CoV closely related coronaviruses in France, Spain or in the western limit of Europe (Lelli et al., 2013; CBEM et al., 2010; Falcón et al., 2011; Kohl and Kurth, 2014; Goffard et al., 2015). The aims of the present study were (i) to get a wider picture of coronaviruses genetic diversity circulating in representative bats species living in the western Palearctic and (ii) to explore the presence of SARS-CoV related viruses in the region. As bat coronaviruses are shed in faeces, sampling consisted mainly of guano collection. This sampling strategy allowed us to, minimize the impact of sampling on bat populations under study (*i.e.* In accordance with wildlife conservation principles and in order to minimize biases), and to focus surveillance at key transmission points and ecological interfaces.

2. Materials and methods

2.1. Sampling

Permits to carry out the sampling were obtained from the French Direction Régionale de l'Environnement, de l'Aménagement et du Logement (Arrêté n○ 2009–11) and the Spanish authorities: Departament de Medi Ambient i Habitatge (Generalitat de Catalunya), Conselh Generau d'Aran, Conselleria de Medi Ambient i Territori (Govern de les Illes Balears) and Departamento de Agricultura, Ganadería y Medio Ambiente (Gobierno de Aragón). From 2008–2016, more than 1500 faecal samples were collected from 26 Rhinolophidae, Vespertilionidae, Miniopteridae and Molossidae bat species. Regions with a great diversity of bat species (central and southern France, northern and north-eastern Spain and Balearic Islands) including swarming sites an maternity colonies, were particularly targeted as they were more likely to harbour a greater viral diversity. The western limit of the study area (western Brittany) and sites harbouring species that had not yet been extensively studied, were also targeted. While most of the study sites were located in France and Spain, a few samples were also opportunistically collected from other countries in the region such as Tunisia and Morocco. These sampling sites fall within three major climatic zones representative of the western Palearctic: the temperate oceanic (Atlantic coast, Brittany and north Spain), the Mediterranean (north-east Spain, Balearic islands) and the humid continental (central-southern France and north-east Spain). Bat species were identified using both morphological characters and acoustic data and confirmed by cytochrome b (Cyt-b) sequencing (Puechmaille et al., 2007). Most faecal samples were collected under roosting bats (n = 1186, 76% - *Rhinolophus ferrumequinum* in LF6, LF9, LS7; *Myotis emarginatus* in LF6; *Myotis myotis* in LS11 and ambiguous specimen, Table 1) while others were obtained directly from captured individuals. During captures, all manipulations were conducted in accordance with Eurobats (www.eurobats.org) guidelines. Trapping sessions were conducted using harp-trap, flip-net or hand-net, and fresh faeces were collected from clean cotton bags in which bats were temporarily isolated. Sampling under roosting bats was carried out after bats had left for foraging at dusk; clean sheets of paper were deposited on the floor under the colony's roost and fresh faeces were collected within 2–10 h. All samples were preserved in cold, antibiotic supplemented, universal transport medium for virus preservation or RNA later (Ambion) for RNA preservation.

2.2. Molecular methods, detection and characterization of coronaviruses

Extractions were performed following the manufacturer's instructions with the exception that 7 µl of linear polyacrylamide (Sigma) were added to the sample before the lysis step instead of the RNA-carrier supplied in the kit. Nucleic acids were eluted in 60 µl RNAse-free saline buffer, 7 µl were immediately used for reverse transcriptase (RT) reaction using ssIII-RT (Life tech) and hexamers. Five µl of RT product were then used as template in a 25 µl semi-nested PCR reaction resulting in the amplification of a 440 / 220 (first PCR / nested PCR) nucleotide fragment of the RNA-dependent-RNA-Polymerase (RdRp, nsp12) coding region. The homemade semi-nested PCR protocols and primers designed to detect a broad range of coronaviruses were described previously (Gouilh et al., 2011) and allow to obtain fragments of the polymerase (nsp12) ranging from 121 to 393 nucleotides after primer and quality trimming. Briefly, the first PCR (PCR 1) used BatCoV pol 15197 (forward: 5′-GGTTGGGAYTAYCCWAARTGTGA-3′) and Bat-CoV pol 15635 (reverse: 5′-CCATCRTCMGAHARAATCATC-ATA-3′) primers; the second semi-nested PCR (PCR 2) used BatCoV pol nested 15419 (forward nested primer: 5′-GCNAATWSTGTNTTTAA-CAT-3′) and the PCR 1 reverse primer. For both PCRs (PCR 1 and the semi-nested PCR 2) the pcr programs were composed of 3 min of denaturation at 94 °C, followed by 40 cycles including 30 s at 94 °C, 30 s at 50 °C (with a touch-down of 0.7 °C per cycle during the first 10 cycles) and 30 s at 72 °C. The final extension was performed at 72 °C for 8 min. Bat Cyt-b sequences were amplified by PCR (Puechmaille et al., 2007) for all coronavirus-positive samples in order to confirm the host species and for a representative number of coronavirus-negative samples, to evaluate co-roosting. The PCR products were revealed by electrophoresis on 2% agarose gels and were sequenced using Big-Dye v1.1 chemistry on an ABI-3730XL sequencer. Resulting chromatograms were trimmed, analysed and assembled using the CLC Main Workbench software v7 (Qiagen) and cleaned sequences were submitted to a BLAST analysis (www.ncbi.nlm.nih.gov/blast/Blast.cgi) in GenBank.

2.3. Phylogenetic analyses

Trimmed original sequences were aligned with a set of sequences summarizing the genetic diversity of coronaviruses using MAFFT (Katoh and Standley, 2013). Preliminary phylogenetic analyses in maximum likelihood were done using PhyML, implemented in seaview (Gouy et al., 2010; Guindon et al., 2010). The main phylogenetic analyses were performed under a Bayesian statistical framework implemented in BEAST (version 1.8.3) (Drummond and Rambaut, 2007), using the model that fits best the data according to the corrected Akaike Information Criterion (AICc) obtained in Jmodeltest2 (Darriba et al., 2012). The general time reversible model of substitution was used, with a gamma distribution and a proportion of invariant sites (GTR + I + G). The coalescent (constant size) model was specified as tree prior and a relaxed molecular clock with an uncorrelated lognormal distribution was used (Drummond et al., 2006; Kingman, 1982). The MCMC (Markov Chain) was launched for 30E8 iterations to reach Effective Sampling Size (ESS) values above 200.

3. Results

This study revealed a great diversity of coronaviruses in bats in the Western Palearctic (Fig. 1; cf. Table S1 for GenBank accession numbers). New coronaviruses were detected in France, Spain, Tunisia and Morocco (Fig. 2). Among the 1551 samples tested, 212 (13.6%) were found positive for coronavirus, representing 10/26 (42%) species of bats and 20/39 (51%) of localities (Table 1). When considering species sampled at a given site with significant sampling size (n > 30), the prevalence ranged from 8.8% (*i.e. Alphacoronavirus* EPI4 in *Myotis nattereri* in LF5, Pont-château, Loire Atlantique, France) to 37.9% (*i.e. Betacoronavirus* EPI1 in *Rhinolophus ferrumequinum* in LF6, Cantoin, Aveyron, France). Identity to known coronaviruses ranged from 85% to 99% according to 393 nucleotides of the conserved nsp12 gene (Fig. 2B). Six alphacoronaviruses were found to have their closest

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Table 1
Taxonomy of bats, UTM coordinates and prevalence of coronaviruses genera and species per locality and host species.

Family	Genus	Species	Zone	UTM X	UTM Y	Locality	CoV Genus & species	Count	Pos.	Pos. per locality (%)	Pos. per species (%)
Miniopteridae	*Miniopterus*	*schreibersii*	31 T	483	491	LF1, Laissac, Aveyron, France	α, EPI 8	12	**1**	**8.33%**	**9.52%**
			31 T	465	492	LF2, Lagarde, Aveyron, France		1	0	0.00%	–
			31 T	604	486	LF3, Dions, Gard, France		1	0	0.00%	–
			31 T	418	461	LS1, Sant Llorenç Savall, Spain	α, EPI 7	9	**1**	11.11%	–
			31 T	402	457	LS2, Olesa de Bonesvalls, Spain		1	0	0.00%	–
			31 S	497	439	LS3, Inca, Majorque, Spain	α, EPI 3	6	**1**	16.67%	–
			31 T	477	461	LS4, Malgrat de Mar, Spain		8	0	0.00%	–
			31 T	312	474	LS5, Lés, Spain		2	0	0.00%	–
			31 T	310	463	LS6, Os de Balaguer, Spain	α, EPI 9	1	**1**	100.00%	–
			31 T	464	438	LS8, Palma, Majorque, Spain		1	0	0.00%	–
	Miniopterus	*maghrebensis*	29 R	736	352	LM1, Ifri'N Caid, Morocco		7	0	0.00%	0.00%
Molossidae	*Tadarida*	*teniotis*	30 T	698	454	LS9, San Pedro, Oliete, Spain	α[b]	8	**1**	12.50%	**12.50%**
Rhinolophidae	*Rhinolophus*	*euryale*	29 R	736	352	LM1, Ifri'N Caid, Morocco	α, EPI 10	3	**1**	33.33%	**33.33%**
	Rhinolophus	*ferrumequinum*	31 T	483	491	LF1, Laissac, Aveyron, France		7	0	0.00%	**14.64%**
			31 T	502	496	LF4, Lapanouse, Aveyron, France		2	0	0.00%	–
			31 T	488	496	LF6, Cantoin, Aveyron, France |	β, E PI 1 α, EPI 6	161	**61**	**37.89%**	–
			30 T	568	525	LF5, Pontchâteau, Loire-Atlantique, France		2	0	0.00%	–
			31 T	463	470	LF7, Batère, Pyrénées-Orientales, France	α, EPI 4	3	**1**	33.33%	–
			30 T	547	528	LF8, Pluherlin, Morbihan, Bretagne, France		2	0	0.00%	–
			30 T	397	530	LF9, Plovan, Finistère, Bretagne, France |	β, EPI 1 α, EPI 6	705	**90**	**12.77%**	–
			29 R	736	352	LM1, Ifri'N Caid, Morocco		2	0	0.00%	–
			31 S	497	439	LS3, Inca, Majorque, Spain		4	0	0.00%	–
			31 T	411	461	LS7, Rocafort, Spain	β, EPI 1	48	**8**	**16.67%**	–
			31 S	582	442	LS10, Ferreries, Minorque, Spain		2	0	0.00%	–
			31 S	492	436	LS11, Llucmajor, Majorque, Spain	α, EPI 7	1	**1**	100.00%	–
	Rhinolophus	*hipposideros*	31 T	483	491	LF1, Laissac, Aveyron, France		8	0	0.00%	0.00%
			31 T	465	492	LF2, Lagarde, Aveyron, France		1	0	0.00%	–
			31 T	502	496	LF4, Lapanouse, Aveyron, France		3	0	0.00%	–
Vespertilionidae	*Barbastella*	*barbastellus*	30 T	547	528	LF10, Pluherlin, Morbihan, Bretagne, France		1	0	0.00%	0.00%
	Eptesicus	*serotinus*	31 T	604	486	LF3, Dions, Gard, France	NA	2	**1**	50.00%	**20.00%**
			31 T	490	495	LF11, Lacalm, Aveyron, France		2	0	0.00%	–
			31 T	316	471	LS16, Senet, Spain		1	0	0.00%	–
	Eptesicus	*isabellinus*	32 S	599	402	LT1, Zaghouan, Tunisia		2	0	0.00%	0.00%
	Hypsugo	*savii*	30 T	698	454	LS9, Oliete, Spain		1	0	0.00%	0.00%
	Myotis	*alcathoe*	31 T	465	492	LF2, Lagarde, Aveyron, France		1	0	0.00%	0.00%

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Table 1 (*continued*)

Family	Genus	Species	Zone	UTM X	UTM Y	Locality	CoV Genus & species	Count	Pos.	Pos. per locality (%)	Pos. per species (%)
			31 T	463	470	LF7, Batère, Pyrénées-Orientales, France		1	0	0.00%	–
	Myotis	*bechsteinii*	31 T	483	491	LF1, Laissac, Aveyron, France		2	0	0.00%	0.00%
			30 T	568	525	LF5, Pontchâteau, Loire-Atlantique, France		9	0	0.00%	–
	Myotis	*blythii*	31 T	463	470	LF7, Batère, Pyrénées-Orientales, France		2	0	0.00%	0.00%
			31 T	504	488	LF13, Creissels, Aveyron, France		1	0	0.00%	–
			31 T	312	474	LS5, Lés, Spain		4	0	0.00%	–
			31 T	310	463	LS6, Os de Balaguer, Spain		1	0	0.00%	–
	Myotis	*capaccinii*	31 T	604	486	LF3, Dions, Gard, France		6	0	0.00%	**46.67%**
			31 T	488	496	LF6, Cantoin, Aveyron, France		1	0	0.00%	–
			31 S	492	436	LS11, Llucmajor, Majorque, Spain |	α, EPI 3 α, EPI 5	6	**6**	100.00%	–
			31 T	402	457	LS2, Olesa de Bonesvalls, Spain		1	0	0.00%	–
			31 T	325	465	LS12, Llimiana, Spain	α, EPI 3	1	**1**	100.00%	–
	Myotis	*daubentonii*	31 T	465	492	LF2, Lagarde, Aveyron, France	α, EPI 4	6	**2**	33.33%	**9.09%**
			31 T	604	486	LF3, Dions, Gard, France		4	0	0.00%	–
			30 T	568	525	LF5, Pontchâteau, Loire-Atlantique, France	α, EPI 4	20	**1**	**5.00%**	–
			31 T	490	492	LF12, Cruéjouls, Auvergne, France		1	0	0.00%	–
			31 T	312	474	LS5, Lés, Spain		2	0	0.00%	–
	Myotis	*emarginatus*	31 T	483	491	LF1, Laissac, Aveyron, France		7	0	0.00%	0.00%
			31 T	465	492	LF2, Lagarde, Aveyron, France		4	0	0.00%	–
			30 T	568	525	LF5, Pontchâteau, Loire-Atlantique, France		5	0	0.00%	–
			30 T	547	528	LF10, Pluherlin, Morbihan, Bretagne, France		1	0	0.00%	–
			31 T	484	495	LF14, Brénac, Aveyron, France		2	0	0.00%	–
	Myotis	*escalerai*	31 S	497	439	LS3, Inca, Majorque, Spain		8	0	0.00%	0.00%
			31 T	378	459	LS13, Orpi, Spain		2	0	0.00%	–
			31 T	463	470	LF7, Batère, Pyrénées-Orientales, France		3	0	0.00%	–
			31 T	375	459	LS14, Santa Maria de Miralles, Vilafranca, Spain		2	0	0.00%	–
	Myotis	*myotis*	31 T	604	486	LF3, Dions, Gard, France		3	0	0.00%	**11.79%**
			31 S	497	439	LS3, Inca, Majorque, Spain	NA	88	**8**	**9.09%**	–
			31 T	477	461	LS4, Malgrat de Mar, Spain	α, EPI 3	7	**4**	57.14%	
			31 T	312	474	LS5, Lés, Spain	α, EPI 5	1	**1**	100.00%	–
			30 T	568	525	LF5, Pontchâteau, Loire-Atlantique, France		18	0	0.00%	–
			31 T	310	463	LS6, Os de Balaguer, Spain |	α, EPI 2 α, EPI 9	2	2	100.00%	–
			31 T	504	488	LF13, Creissels, Aveyron, France		3	0	0.00%	–
			30 T	552	526	LF15, La Roche Bernard, Morbihan, France		17	0	0.00%	–
			31 S	492	436	LS11, Llucmajor, Majorque, Spain |	α, EPI 5 α, EPI 7	89	**12**	**13.48%**	–
			31 T	325	465	LS12, Llimiana, Spain		1	0	0.00%	–
	Myotis	*mystacinus*	30 T	568	525	LF5, Pontchâteau, Loire-Atlantique, France		3	0	0.00%	0.00%
			31 T	484	495	LF14, Brénac, Aveyron, France		1	0	0.00%	
	Myotis	*nattereri*	30 T	568	525	LF5, Pontchâteau, Bretagne, France	α, EPI 4	34	**3**	**8.82%**	**8.82%**
	Myotis	*nattereri_ssp.a*[a]	31 T	483	491	LF1, Laissac, Aveyron, France		5	0	0.00%	0.00%
			31 T	465	492	LF2, Lagarde, Aveyron, France		2	0	0.00%	–
			31 T	502	496	LF4, Lapanouse, Aveyron, France		1	0	0.00%	–
			31 T	490	492	LF12, Cruéjouls, Auvergne, France		2	0	0.00%	–
			31 T	481	492	LF16, Biounac, Aveyron, France		1	0	0.00%	–
	Myotis	*punicus*	29 R	736	352	LM1, Ifri'N Caid, Morocco		3	0	0.00%	**27.27%**

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Table 1 (*continued*)

Family	Genus	Species	Zone	UTM X	UTM Y	Locality	CoV Genus & species	Count	Pos.	Pos. per locality (%)	Pos. per species (%)
			32 S	599	402	LT1, Zaghouan, Tunisie	α, EPI 9	1	**1**	100.00%	**37.50%**
			32 S	602	402	LT2, Zaghouan, Tunisie	α, EPI 9	7	**2**	28.57%	–
	Nyctalus	*leisleri*	31 T	418	461	LS15, Sant Llorenç Savall, Spain		1	0	0.00%	0.00%
			31 T	316	471	LS16, Senet, Spain		3	0	0.00%	–
	Pipistrellus	*kuhlii*	30 T	698	454	LS9, Oliete, Spain		3	0	0.00%	0.00%
	Pipistrellus	*pipistrellus*	31 T	604	486	LF3, Dions, Gard, France		2	0	0.00%	0.00%
			31 T	484	495	LF14, Brénac, Aveyron, France		9	0	0.00%	–
			31 T	481	492	LF16, Biounac, Aveyron, France		1	0	0.00%	–
			30 T	416	531	LF17, Quimper, Finistère, Bretagne, France		3	0	0.00%	–
			31 T	480	496	LF18, Orlhaguet, Aveyron, France		6	0	0.00%	–
			31 T	480	496	LF19, Ste-Genevière/Argence, Aveyron, France		1	0	0.00%	–
			30 T	698	454	LS9, Oliete, Spain		1	0	0.00%	–
	Pipistrellus	*pygmaeus*	31 T	484	495	LF14, Brénac, Aveyron, France		1	0	0.00%	–
	Plecotus	*austriacus*	31 T	604	486	LF3, Dions, Gard, France		1	0	0.00%	0.00%
			30 T	698	454	LS9, Oliete, Spain		2	0	0.00%	–
SUB TOTAL (unambiguous specimen)								**1446**	**211**	**14.59%**	NA
Uncertain determination (pools in mixed species roots)	*Rhinolophus* *Myotis*	*ferrumequinum* *emarginatus*	31 T	488	496	LF6, Cantoin, Aveyron, France	α, EPI 6	74	**1**	**1.35%**	NA
	Rhinolophus	*ferrumequinum* *hipposideros*	31 T	465	492	LF2, Lagarde, Aveyron, France		1	0	0.00%	NA
	Myotis	*emarginatus* *nattereri_ssp.a* *daubentoni*									
	Miniopterus	*schreibersi*									
	Miniopterus *Rhinolophus*	*schreibersii* *ferrumequinum*	31 S	582	442	LS10, Ferreries, Minorque, Spain		2	0	0.00%	NA
	Plecotus *Pipistrellus* *Miniopterus* *Eptesicus* *Myotis* *myotis* *Myotis*	*austriacus* *pipistrellus* *schreibersii* *serotinus myotis* *capaccini* *daubentoni*	31 T	604	486	LF3, Dions, Gard, France		1	0	0.00%	NA
	Pipistrellus *Miniopterus* *Myotis*	pipistrellus schreibersii myotis	31 T	477	461	LS4, Malgrat de Mar, Spain	α, EPI 3	27	**1**	**3.70%**	NA
GRAN TOTAL (including pools)								**1551**	**212**	**13.67%**	NA

Samples are divided in two categories, those for which the host species has been unambiguously determined and confirmed by genetics, and those for which the confirmation was not possible (pooled sampling). Positives are bolded.
NA: Not Applicable; ND: Not Determined.

[a] nattereri_sp.a* refers to a cryptic lineage, a putative new species yet not formally described.

[b] Sequencing of this coronavirus nsp12 gene didn't provide signal of sufficient quality to characterize the species.

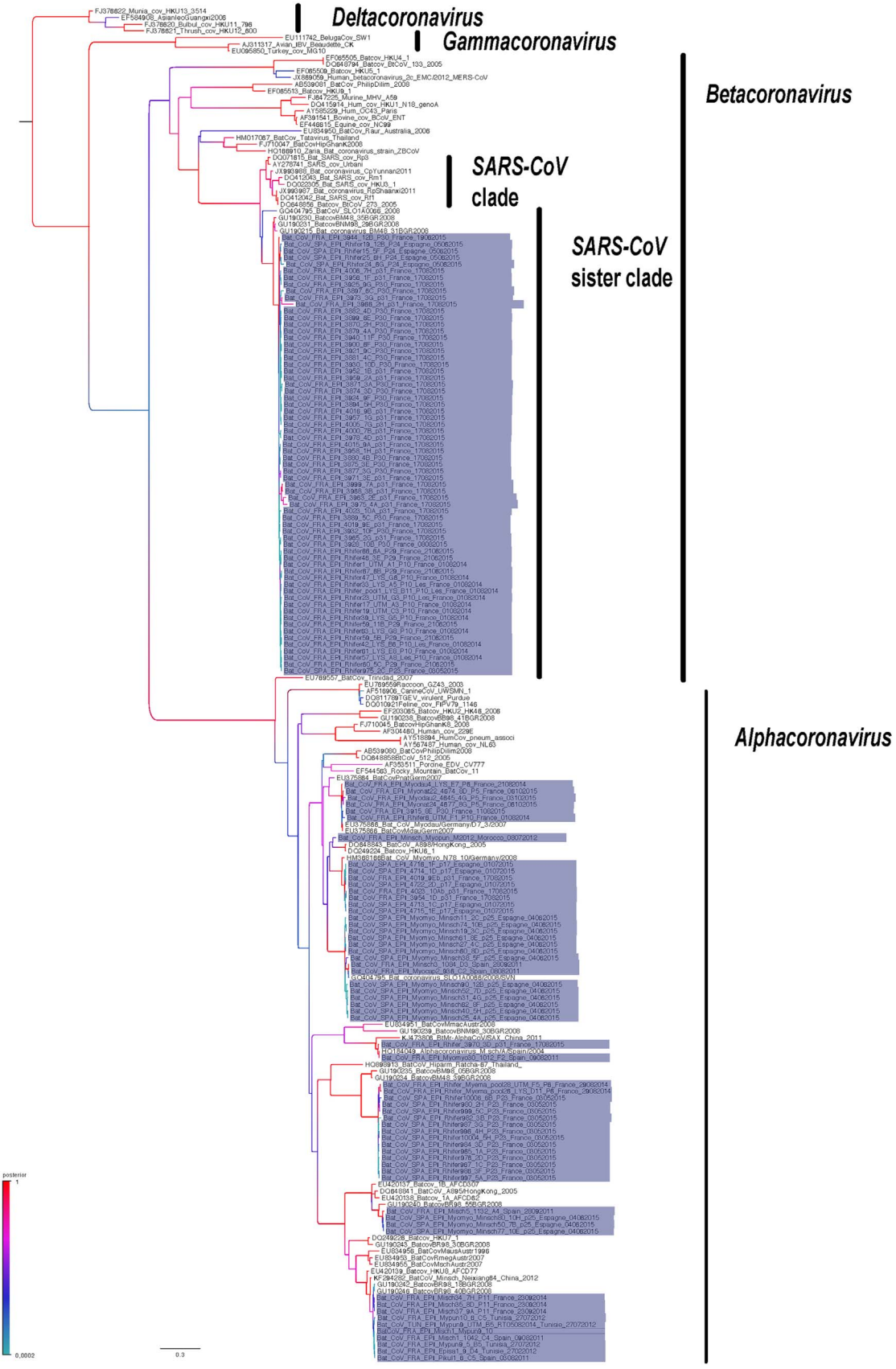

Fig. 1. Bayesian phylogeny of 127 genetic sequences representative of coronavirus strains detected in the study with sequences representing the world diversity of *Coronavirinae*. A) Nsp12 sequences were aligned using Mafft 7 (http://mafft.cbrc.jp). Statistical support (posterior probability) of nodes are depicted using a gradual color code of the tree, red indicating significant posterior probability values (> 0.95). Strain names and main information is written in taxa labels. Viruses detected in this study are highlighted in purple.

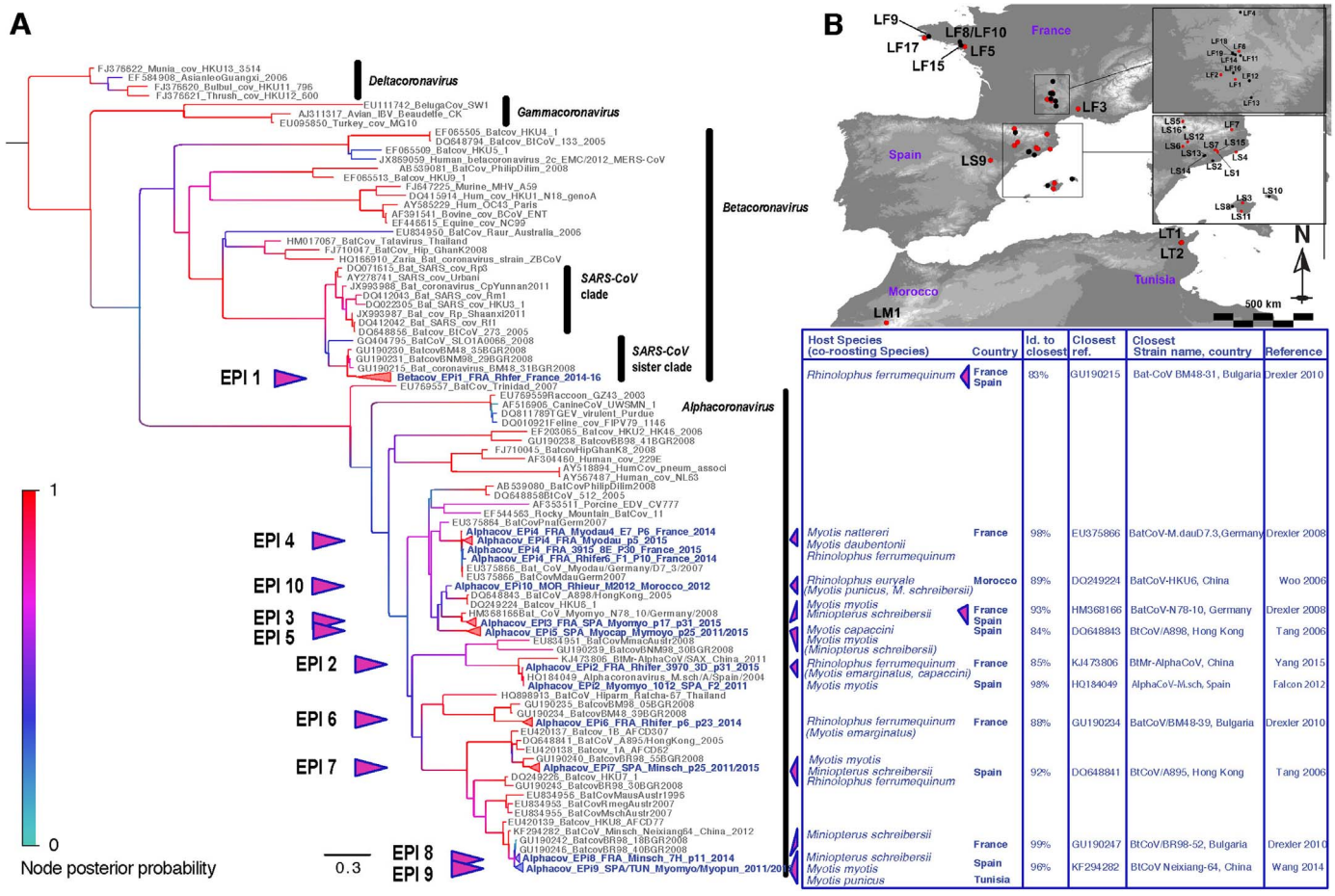

Fig. 2. Phylogenetic analysis and geo-localisation of coronaviruses detected in the Western Palearctic region. A) Bayesian phylogeny depicted in Fig. 1 (selected nodes collapsed for clarity reasons) showing clusters including a number of sequences ranging from 3 to 63 with a mean of 14 per clade. Statistical support (posterior probability) of nodes are depicted using a gradual color code of the tree, red indicating significant posterior probability values (> 0.95). Labels of viruses detected in this study are bolded and coloured in blue. Corresponding host names are indicated in the right panel, in front of each taxa reported in the study. When significant mixing of species at the roost was observed, the name of the co-roosting coronavirus-negative species is added in brackets. Country of origin and identity score (ID) to the closest reference found in GenBank were also added for each coronavirus clade detected in this study. B) Map of the study region depicting the 39 investigated sites and highlighting in red those where bat samples were found positive for coronaviruses.

described relative in Europe (in Bulgaria, Spain or Germany) whereas five were more closely related to Asian coronaviruses previously reported from China and Hong Kong S.A.R. (Fig. 2 A/B) (Woo et al., 2006; Drexler et al., 2010; Falcón et al., 2011; Gloza-Rausch et al., 2008). Alphacoronaviruses were predominantly detected in *Myotis* species (Vespertillionidae) while betacoronaviruses were associated with *Rhinolophus ferrumequinum* only (Rhinolophidae) (Table 1, Fig. 2). Phylogenetic analyses show no major contradiction between virus-host association found here and in literature (Wacharapluesadee et al., 2015; Woo et al., 2006; Drexler et al., 2010). The 121 partial nsp12 sequences analysed represent at least nine *Alphacoronavirus* species (44% pairwise nucleotide identity or less) and one putative new species of *Betacoronavirus* (*Betacoronavirus* EPI1 - "EPI" stands for EPICOREM, the acronym of the name of the project in which this study was hosted). This putative new species of *Betacoronavirus* has less than 83% pairwise nucleotidic identity to the closest reference and exhibits an intraspecific genetic diversity (*i.e.* Strains – Fig. S2). Notably, this *Betacoronavirus* EPI1 grouped with the SARS-CoV sister-clade and was detected in *Rhinolophus ferrumequinum* only, but repeatedly in different colonies from western Brittany to north-eastern Spain (*i.e.* LF6, LF9, LS7, LS11; Fig. 2, Table 1). Notably, no MERS-CoV-like virus was detected in this study. Among nine alphacoronaviruses reported here, mainly in Vespertilionidae and Miniopteridae bats, two species, tentatively named *Alphacoronavirus* EPI4 and *Alphacoronavirus* EPI7 were also detected in multiple species, including in Rhinolophidae (Fig. 2, Table 1). Interestingly, *Alphacoronavirus* EPI6 was detected in *Rhinolophus ferrumequinum* only and clustered with alphacoronaviruses previously detected in Eastern Europe by Drexler *et. al.* in a clade rooted by *Alphacoronavirus* Hiparm Ratcha detected in *Hipposideros armiger* (Hipposideridae, sister family to Rhinolophidae) in Thaïland in 2007 (Gouilh et al., 2011; Drexler et al., 2010).

4. Discussion

4.1. Prevalence and diversity

Overall prevalence and diversity in the studied sites and host species indicate a very active circulation of coronaviruses in bats in the region. According to nucleotide identity on the very conserved nsp12, half of the *Coronavirinae* species detected here (Alphacov. EPI2, EPI5, EPI7, EPI9 and EPI10) are closer to strains reported from Asia and are new for the region. Therefore, the diversity of coronaviruses in western Europe is much higher than previously described (Figs. 1 and 2 (Drexler et al., 2010; Rihtarič et al., 2010; Lelli et al., 2013; CBEM et al., 2010; Falcón et al., 2011; Kohl and Kurth, 2014; Goffard et al., 2015; Gloza-Rausch et al., 2008)). Globally, coronavirus species were mostly associated with one bat genus (or even species) and phylogenetically related to those that circulate in their host's sister species in Asia (Fig. 2). This highlights the fundamental effect of the hosts diversity, phylogeny and evolution on the contemporaneous genetic diversity of coronaviruses

found in bats (Gouilh et al., 2011; Foley et al., 2015).

Another factor that may contribute to the genetic diversity and to the evolution of coronaviruses in bats is linked to the great variations of prevalence observed between sampling sites, species or date of sampling. The prevalence reflects the circulation rate of a coronavirus. Variations or pulses of prevalence indicate a heterogeneous circulation of coronaviruses in bat colonies or bat populations and a very low prevalence may induce a bottleneck effect locally. This, in combination with genetic drift, may promote the variability of strains leading to a fast evolution of coronaviruses. Moreover, the seasonal movements of bats, the heterogeneous distribution of individuals within the species range, the sexual and the gregarious behaviours of certain species, may reinforce and even trigger the prevalence variations and their effects on the genetic evolution of coronaviruses.

Despite these important variations in prevalence observed between sites or species, most coronavirus phylogroups and putative species were detected in several distant sites within the distribution area of the host species. This is the case for alphacoronaviruses such as EPI4 and EPI6, detected in *Myotis daubentonii* and in *Rhinolophus ferrumequinum*, in several locations, respectively. Similarly, *Betacoronavirus* EPI1 was detected in *Rhinolphus ferrumequinum* across various locations from western France to Spain (Table 1). This indicates that contact rates and seasonal movements of bats ensure efficient circulation and rapid diffusion of coronaviruses within a host-species range and throughout the western Palearctic region (Fig. 2, Table 1).

The highest prevalence observed at several sites were associated with i) mixing-species roosts or ii) maternity colonies. i) At least three species: *Myotis myotis*, *Myotis capaccinii* and *Rhinolophus ferrumequinum* were co-roosting in LS11, Majorque, Spain. The colony of *Myotis myotis* sampled in that location exhibited the highest prevalence (13.5%) of alphacoronaviruses EPI5 and EPI7 detected among locations with representative sampling size (Table 1). ii) The maternity colonies of *Rhinolophus ferrumequinum* harboured the *Betacoronavirus* EPI 1 at a high prevalence both in LF6, Bretagne and LF9, Aveyron, France (Fig. 2, Table 1). These high prevalences illustrated the intense circulation of coronaviruses associated with these specific ecological contexts. Both mixed-species roosts and maternity colonies boost the viral prevalence of a given colony. When occurring concomitantly at several sites across the wide geographical range of bat species, these local boosts of prevalence may promote local-specific and fast seasonal genetic drift of coronaviruses, possibly giving rise to new viral lineages. This diversification process is illustrated here by the genetic variability of the RdRp (*i.e.* that exhibits Single Nucleotide Polymorphisms) found within several phylogroups and within putative coronavirus species (*e.g. Betacoronavirus* EPI1 and *Alphacoronavirus* EPI4 - Fig. S2).

In addition, colonies of mixed-species where several species of *Alphacoronavirus* co-circulate and where individuals may be co-infected represent the ideal ecological context for evolution mediated by recombination. Several coronaviruses are known to recombine frequently and this molecular mechanism is a main driving-force in their evolution (*e.g.* HcoV-OC43, HcoV-NL63) (Kin et al., 2015, 2016; Pyrc et al., 2006; Dominguez et al., 2012). Unfortunately, due to unique and short size region used for detection in this study, this hypothesis was not tested.

4.2. Bat coronavirus host specificity and spill-over

Besides these general patterns that illustrate the contribution of prevalence variation and genetic diversity to the genetic evolution of coronaviruses in bats, our data also provide evidence of relative coronavirus/host association and potential spill-over capacities of these viruses. Several *Alphacoronavirus* species, were identified in different species of bats (*e.g.* a given *Alphacoronavirus* species infecting several species of bats). This attests that inter-species jump may (although rarely) occur in a favourable ecological context such as when different species of bats share the same roost, a behaviour called co-roosting. This in turn may promote the spread of a coronavirus across the distribution area of the new host. This hypothetical mechanism may explain the detection of *Alphacoronavirus* EPI4 in *Myotis nattereri*, *Myotis daubentonii* and *Rhinolophus ferrumequinum* in three locations and the presence of *Alphacoronavirus* EPI5 in both *Myotis myotis* and *Myotis capaccinii* (Table 1 and Fig. 2). Furthermore, co-roosting behaviour of *Myotis myotis*, *Miniopterus schreibersii* and *Rhinolophus ferrumequinum* may also explain the detection of *Alphacoronavirus* EPI7 in these taxa that belong to different species and genera. The apparent zoonotic behaviour of these alphacoronaviruses described here contrasts with conclusions of other studies (Fischer et al., 2016) but correlates with the social behaviour of species in the genus *Myotis* that often share their roost with multiple species, and sometimes even with other genera (*e.g. Miniopterus* or *Rhinolophus*) (Barataud and Aulagnier, 2012; Crucitti, 1993). This co-roosting behaviour of *Myotis* spp. was specifically observed during the fieldwork of our study. Several *Myotis* sp. individuals were observed in close contact with *Rhinolophus ferrumequinum*, in several colonies. This frequent interspecies contact at roosts, combined with phylogenetic proximity of host species is likely to promote interspecies transmission in a context of viral diversification induced by the intense circulation of alphacoronaviruses in *Myotis* spp.

Conversely, no *Myotis* species nor other Vespertillionidae are reported here to be infected with *Betacoronavirus* EPI1 (hosted by *Rhinolophus ferrumequinum*) whereas this coronavirus is widespread in the study region and *Myotis* species are often co-roosting with *Rhinolophus ferrumequinum*. More specifically, *Myotis emarginatus* regularly forms mixed clusters with *Rhinolophus ferrumequinum* but so far, no *Betacoronavirus* has ever been isolated from the former. A possible hypothesis to explain this would be that an evolutionary trade-off maintains *Betacoronavirus* EPI1 adapted to its host species. In such a context, a spill-over to *Myotis* species, divergent by > 60 million years, would require a major change that, albeit still possible, would be unlikely to occur. In addition, the frequency of contact between *Rhinolophus* and *Myotis* may not be high enough to give this spill-over a sufficient probability to be observed as yet. Another hypothesis would point the intense circulation of diversified alphacoronaviruses in *Myotis* spp. as a trigger of a complex immunological repertoire directed toward alphacoronaviruses that may, to some extent, provide partial cross-protection against infection by *Betacoronavirus* EPI1. Given the behaviour of *Myotis emarginatus*, the species of the genus *Myotis* that is the most frequently observed roosting with *Rhinolophus*, this species may play the role of intermediate host for coronaviruses transmission between *Myotis* and *Rhinolophus* and would be the first species to test for an eventual *Betacoronavirus* inter-species jump from *Rhinolophus* to *Myotis*. Unfortunately, our sampling of *Myotis emarginatus* was limited and the occurrence of such a spill-over between the two species should be further investigated.

Another illustration of the possible correlation between limited interaction of host species and the likelihood of coronaviruses spill-over, is the specific association of *Alphacoronavirus* EPI6 with *Rhinolophus ferrumequinum* observed here (Table 1, Fig. 2). Despite the fact that alphacoronaviruses are mostly found circulating in numerous species of Miniopteridae and Vespertillionidae, our phylogenetic analyses and the ecological context suggest a strict association between *Alphacoronavirus* EPI6 and Rhinolophidae, a familly usually associated with betacoronaviruses. Indeed, this association between these Alphacoronaviruses and Rhinolophidae can be extended to the whole clade rooted by *Alphacoronavirus* Hiparm Ratcha described in 2007 in Hipposideridae bats in Thailand. This clade has been detected in Asia and in eastern and western Europe in Rhinolophoidea only, and thus represents, to date, a unique example of coevolution between a clade of alphacoronaviruses and this bat super family (Gouilh et al., 2011; Drexler et al., 2010; Foley et al., 2015).

5. Conclusions

Findings exposed here show that the methods used in the study is

performant for environmental surveillance in various ecological settings. This study also demonstrates that, beyond the high diversity of alphacoronaviruses harboured by bats, SARS-CoV sister-clade members are currently circulating widely in Western Europe. Albeit *Betacoronavirus* appeared restricted to *Rhinolophus ferrumequinum*, most alphacoronaviruses detected here are zoonotic. Further studies are needed i) to better understand this difference of host specificity between the two groups, ii) to investigate the evolution patterns of this *Betacoronavirus* clade in bats in the Western Palearctic and iii) to estimate more precisely the likelihood of spill-over of these viruses through molecular epidemiology and gain-function testing. The SARS-related *Betacoronavirus* EPI1 exhibits notable diversity across time and space which suggests a fast evolution. This therefore advocates for sustained surveillance and for intensifying studies on these coronaviruses so as to get a better understanding of their pattern of circulation in wildlife. This should be in consideration of conservation prerogatives and human activities, albeit no direct spill-over to domestic animal nor human has yet been documented.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2018.01.014.

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