Combining host genetic structure and serology to investigate rabies-related lyssaviruses in the greater mouse-eared bat (Myotis myotis)

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Abstract

European bat lyssavirus 1 (EBLV-1) is widespread and frequent in European bats, particularly of the genus Eptesicus. However, other species can be seropositive, suggesting a complex ecology still mostly unknown. In Italy, EBLV-1 antibodies are described since 2012 in South Tyrolean Myotis myotis. This study provide phylogenetic evidence for either the current or the recent movement of M. myotis from continental Europe to South Tyrol across the Alpine valleys, which might allow for the introduction of LYSVs. Serological analyses confirmed antibodies against EBLV-1 in this bat across the entire region, showing marked seasonal pattern and a sharp peak of positivity in late summer. No statistical difference was detected between the maternity colonies investigated in either the likelihood for infection or the antibody titres. Indeed, South Tyrolean populations of M. myotis showed no significant genetic differentiation using nuclear and mitochondrial markers, supporting the existence of a regional meta-population and a low philopatric behaviour. This structure, never described for M. myotis elsewhere, well explains the spread and maintenance of LYSVs trough the movement of female bats between colonies. Since no virus was detected in the study, we cannot exclude that our serological data don't derive from a cross-reaction with an unknown LYSV. However, because all LYSVs can cause clinical rabies in humans, this study has a strong impact on public health regardless of the viral species actually circulating. In addition, the unravelling of the peculiar genetic structure of South Tyrolean M. myotis is crucial to inform conservation strategies for this endangered species.

Keywords

Lyssavirus, bats, *Myotis_ myotis* , genetic, population

Introduction

Today, the role of bats as important reservoir of zoonotic viruses is so widely accepted as highly discussed (Joffrin, Dietrich, Mavingui, & Lebarbenchon, 2018; Wang & Anderson, 2019; Wilkinson & Hayman, 2017). Among these pathogens, the association between vampire bats and rabies has been long known, along with the evidence that several bat species might harbor and transmit rabies virus (RABV) in the Americas, as well as rabies-related lyssaviruses (LYSVs) worldwide (Johnson, Aréchiga-Ceballos, & Aguilar-Setien, 2014; Shipley et al., 2019; Velasco-Villa et al., 2017). Interestingly, while RABV is responsible for all bat-associated human cases in the Americas, this virus is not found in chiropters from the rest of the globe, where up to other 14 of the 16 known LYSVs have been described instead (Shipley et al., 2019; Walker et al., 2018).

In Europe, six rabies-related lyssaviruses (RRLVs) have been described, namely *European bat 1 lyssavirus*

(EBLV-1), *European bat 2 lyssavirus* (EBLV-2), *Bokeloh bat lyssavirus* (BBLV), *West Caucasian bat lyssavirus* (WCBV), *Lleida bat lyssavirus* (LLEBV) and *Kotalahti bat lyssavirus* (KBLV), with the last one still waiting for official classification from the International Committee on Taxonomy of Viruses (ICTV) (Walker et al., 2018). Among these, EBLV-1 is the most frequently reported, is widespread in the continent and, more recently, in Great Britain (Middlemiss, 2019). Notably, EBLV-1 has been associated with spillover events to non-flying mammals and human cases (Dacheux et al., 2009; Müller et al., 2004; Shipley et al., 2019; Tjørnehøj, Fooks, Agerholm, & Rønsholt, 2006). Serotine bats have been long considered the reservoir for EBLV-1, because the majority of cases are reported in *E. serotinus* and *E. isabellinus.* However, the discordance between the genetic structure of *E. serotinus* and EBLV-1 isolates in France suggests that other species might be involved in the spread of the virus (Troupin et al., 2017). This hypothesis is further supported by serological studies showing exposure of several other bat species, including the grater mouse-eared bat (*Myotis myotis*) in Spain, France, Germany, Croatia and Italy (Leopardi et al., 2018; Picard-Meyer et al., 2011; Schatz et al., 2014; Serra-Cobo et al., 2013; Šimić et al., 2018). Although this bat species seems to mostly move locally between summer and winter roosts, longer transboundary migrations have also been recorded (Hutterer et al., 2005), which might contribute to virus dispersal. However, the relationship between the home range of *M. myotis* and the circulation of EBLV-1 has never been investigated, also because the pattern of movements is not clearly defined and the virus is yet to be characterized from this species. In particular, the lack of viral detection in *M. myotis* prevents any conclusion about its role in the epidemiology of EBLV-1, as sero-positivity might also reflect cross-reactivity with yet unknown related LYSVs (Kuzmin et al., 2008; Wright et al., 2008). Indeed,*Myotis* bats have frequently been associated with other LYSVs, including EBLV-2 in *M. daubentonii* and *M. dasycneme* , BBLV in *M. nattereri* and the newly described KBLV in *M. brandtii* .

In this study, we investigated the genetic structure of the greater mouse-eared bat collected from five maternity colonies in South Tyrol, the northernmost region in Italy bordering the Austrian territories of North Tyrol, to investigate the connectivity between them and, indirectly, the effect of the Alps as natural barrier for animal dispersal. We further tested for potential sex-biased dispersal and assessed the fidelity of females to their natal roost (defined as female philopatry), features that are common in temperate bats (Kerth, Mayer, & König, 2000; Kerth, Mayer, & Petit, 2002; Moussy et al., 2015, 2013). We therefore interpreted serological data obtained from these colonies, along with genetic information and environmental parameters, in the attempt to understand infection dynamics of RRLV in *Myotis*bats within the study area, even in the absence of molecular characterization (Peel, McKinley, et al., 2013).

Materials and methods

Animal sampling

In 2016 we investigated five maternity colonies of greater mouse-eared bats located in the roof of five active churches of South Tyrolean valleys, the northernmost region in Italy. From each colony, a mean number of 32 individuals were captured within their roost during the day, manually or using hand nets according to the structure of the roof and the position of the colony, and placed in individual cotton bags to rest for 10-15 minutes. All animals were inspected and measured to determine the species, age, sex and physiological status. Relevant measurements included the length of the upper tooth row (CM^3), which is critical to resolve morphological taxonomy of our target species *M. myotis* against the sibling*M. blythii,* which has sporadically been reported in the area (Arlettaz, Ruedi, & Hansser, 1991). From each animal, we collected a tissue sample from the right patagium using a 3 mm biopsy punch and 20-100 µl of blood from the uropatageal vein using a 300 µl insulin syringe with a 30 G needle. Biopsies and blood samples were used for genetic and serological studies, respectively. All procedures were performed under physical restraint for a maximum of 6 minutes/individual; after sampling, animals were given water to prevent dehydration and were placed in a ventilated cotton bag for small animal transport in groups of 15, until release within the roost. All roosts were sampled twice around mid-May and mid-September, before and after the birth pulse, respectively. A total of 318 blood samples and 338 uropatageal biopsies were collected for the purpose of the study. All personnel working with bats was vaccinated against rabies and showed a protective antibody

titer throughout the duration of the project (WHO, 2018).

Genetic structure of M. myotis from South Tyrol

DNA was extracted from wing biopsies using the DNeasy blood and tissue commercial kit (QIAGEN) according to the manufacturer's instructions. The partial cytochrome b (cytb) was amplified as described elsewhere (Irwin, Kocher, & Wilson, 1991), in order to confirm the identification based on morphological parameters. Only samples genetically confirmed as*M. myotis* (n=166) were included in the following analyses.

The genetic structure of the target population was determined combining analyses based on nuclear DNA (bi-parental inheritance) and mitochondrial DNA (maternal inheritance), in order to account for sex-biased gene flow. In addition, we used only data from females sampled from each of the five colonies during the same week in May (Table 1), thus avoiding possible issues related to animal movements during the reproductive season. On the other hand, we considered the sampling of adult males within maternity colonies as occasional and excluded these individuals (n=11 sampled in May) from analyses.

Nuclear genetic structure was investigated using microsatellites available for this species in the literature (Castella, Ruedi, & Excoffier, 2001) (Table S1). Forward primers were labelled with florescent dyes (MWG-Operon) and PCR amplification was carried out in three multiplex mixtures containing 10-20 ng of template DNA, using the Qiagen multiplex kit (QIAGEN) (Table S1). The amplification protocol was as follows: initial denaturation step at 95°C for 15 minutes; 35 cycles of denaturation for 30 seconds, annealing at 60°C, 55°C or 50°C for 90 seconds for mix 1, 2 and 3 respectively, and extension at 72°C for 1 minute; final extension step at 72°C for 10 minutes. PCR products diluted 1:100 were analyzed on an ABI PRISM® 3130xl automatic sequencer (Applied Biosystem, Foster City, CA). The molecular size of microsatellite alleles was evaluated by using GeneMapper4.0. The reliability of all microsatellites was tested for each locus in terms of the presence of null alleles, the coefficient of inbreeding and the deviation from Hardy-Weinberg equilibrium (HWE) using a Chi-square test implemented in Micro-checker 2.2.3.

Two hypervariable domains of the mitochondrial control region (CR) were used to investigate the genetic structure at the mitochondrial level. In detail, the full control region of the mitochondrion was PCR-amplified in two overlapping fragments of about 1200 bp, using the Platinum TAQ kit (Invitrogen), according to the manufacturer's instructions. Primers were developed using reference sequences from the mitochondrion of*M. myotis* (Table S2). The amplification protocol was as follows: initial denaturation step at 94°C for 2 minutes; 40 cycles of denaturation for 30 seconds, annealing at 51°C or 56°C for 30 seconds respectively for PCR1 and 2, and extension at 72°C for 2 minutes; final extension step at 72°C for 5 minutes. PCR products were sequenced on an ABI PRISM® 3130xl genetic analyzer. Sequences were trimmed to obtain two fragments of the hypervariable HVI and HVII domains. Haplotypes for the concatenated HVI and HVII were inferred in MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

Genetic information obtained for the nuclear and mitochondrial DNA were used to investigate the genetic population structure of *M. myotis*within South Tyrol. Analyses were carried out assuming each bat belonged to a distinct population based on its roost of sampling. We first determined the genetic variability existing within each separate roost. In particular, we calculated allelic richness across loci (A) and expected heterozygosity (H_E) from microsatellites data using GeneAlex, while the number of haplotypes (N), gene (h) and nucleotide (π) diversity of mitochondrial sequences were calculated in Arlequin 2.0 (Castella et al., 2001; Ruedi et al., 2008). In addition, we used MEGA 6 to determine mean nucleotidic distance between individuals from different roosts based on the concatenated HVI and HVII. We then assessed the genetic differentiation between populations at both the nuclear and mitochondrial level, as expressed by the fixation index (F_{ST}) calculated using Genealex and Arlequin 2.0 respectively. We considered little difference for F_{ST} values ranging from 0 to 0.05, moderate from 0.05 to 0.15 and great for values above 0.15 (Balloux & Lugon-Moulin, 2002). We tested the hypothesis of genetic isolation by distance (IBD) by plotting linearized F_{ST} values=F_{ST}/(1-FS) (Slatkin, 1995) against the ln(geographical distance) using a Mantel test implemented in GeneAlex (Rousset, 1997). In particular, we used geographical 2D distances calculated along the valleys' bottom rather than Euclidean distances, as they are likely closer to real distances covered by bats when

flying.

For mitochondrial data only, we also investigated the phylogenetic relationship between haplotypes through Maximum Likelihood (ML) phylogenetic analyses implemented in PhyML 3.0 software using the general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites (with four rate categories, G4) and a heuristic SPR branch-swapping search (Dereeper et al., 2008). One thousand bootstrap replications were performed to assess the robustness of individual nodes. Mean nucleotide distance between and within clades thus identified was calculated in MEGA 6 (Tamura et al., 2013).

Phylogenetic relationship of South Tyrolean M. myotis with European individuals based on mitochondrial haplotypes (HVII)

To investigate the phylogenetic relationship between South Tyrolean*M. myotis* and individuals from neighboring areas we used sequences only from the HVII region of the mitochondrion, which has been widely used throughout Europe (Bogdanowicz, Lesiński, Sadkowska-Todys, Gajewska, & Rutkowski, 2013; Bryja et al., 2010; Castella et al., 2001; Furman et al., 2014; Ruedi & Castella, 2003; Ruedi et al., 2008). Sequences from this study were trimmed to include the sole HVII region and were aligned with representative sequences of different haplotypes reported in Europe for this bat species using Mafft with the G-INS-I setting (Katoh, Misawa, Kuma, & Miyata, 2002) (Table S3). Phylogenetic relationship between individuals was determined using ML tree implemented in PhyML 3.0 software using the general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites (with four rate categories, G4), a heuristic SPR branch-swapping search and 1000 bootstrap replications to assess the robustness of individual nodes (Dereeper et al., 2008). To obtain the best representation of the haplotype diversity in our study area, phylogenetic analyses were run using all available samples, thus including adult males and youngsters in their first year of age captured during the reproductive season, either in May or September (Table 1).

Serology and statistical analyses

Blood samples were pre-diluted 1:30 and were analyzed for the presence of antibodies against EBLV-1 using a modified Rapid Fluorescent Focus Inhibition Test (RFFIT), in which EBLV-1b was used as challenge virus (Leopardi et al., 2018; Serra-Cobo, Amengual, Carlos Abellán, & Bourhy, 2002). Samples were analyzed on a three-fold dilution basis and titers were calculated through the Reed-Muench method and expressed as LogD50/ml. Samples were considered positive when able to inhibit viral growth at a minimum dilution of LogD50/ml [?]1.95.

We then tested how individual and environmental parameters influence both the likelihood of being seropositive and the serological titer of neutralizing antibodies against LYSVs in the target species *M. myotis.* In particular, the variables tested included geographic (altitude and coordinates), seasonal (before and after the birth pulse), demographic (age and sex) and genetic parameters (relative presence of individuals showing haplotypes clustering to distinct clades, using as a reference mitochondrial haplogroups defined by Ruedi et al, 2008). Full statistical analyses are reported as supplemental material (Supplementary Methods). Briefly, we applied a population averaged models using Generalized Estimating Equations (GEE) and a linear mixed model (LMM) to investigate the effects of all variables, respectively on the likelihood of showing neutralizing antibodies and on their titer (Dohoo, Wayne, & Stryhn, 2009; Liang & Zeger, 1986; West, Welch, & Galecki, 2007). For both types of models, we treated individuals sampled within the same colony as a cluster, thus assuming that all observations obtained within the same colony are correlated. Accordingly, we performed a robust estimation of the variances of the regression coefficients for the qualitative analysis and included a random-intercept for colony in the LMM (Rao et al., 2014; Ying & Liu, 2006). To assess the goodness of proposed models, we used the Quasi-likelihood under the independence model Criterion (QIC) and the Area under the ROC Curve (AUC) for GEE models, or the Akaike Information Criterion (AIC) and residual analysis for LMM models (Dohoo et al., 2009; Littell, Milliken, Stroup, Wolfinger, & Oliver, 2006; Samur, Coskunfirat, & Saka, 2014) considering a p-value <0.05 as significant.

Results

Description of colonies and samples

Five colonies of greater mouse-eared bats were investigated in South Tyrol in 2016 (Figure 1). All colonies were located in the roof of active churches and covered almost the whole region to the border with Austria. The distance between colonies, calculated along bottom valleys in order to reflect the real route of animals, ranged from 29.7 (roosts one-three) to 125.8 kilometers (roosts three-four) (Figure 1). All roosts hosted maternity colonies from late March to late September, mostly including adult females with their offspring. Adult males were sporadically sampled in all locations, particularly during the month of September (Figure S1). All colonies hosted between 1000 and 2000 animals, with the exception of the northernmost site, where we only found around 200 individuals (colony three, Figure 1). *M. myotis*was often the most abundant species within roosts. However, co-roosting with the sibling species *M. blythii* was confirmed in all locations, especially during the mating season in September, when the relative frequency of the two species widely differed between colonies (Figure S2). In addition, a segregated aggregation of about 100 *M. marginatus* was recorded within colony two in May.

Population genetic structure

Individual samples of *M. myotis* suitable for genetic analyses were 120 for the microsatellite analysis and 112 samples for the mitochondrial analysis, as detailed in Table 1. Data were obtained from 25-30 female bats sampled in May from four out of five colonies, while no more than nine samples were collected from colony three due to its small size and scarce accessibility. In order to secure the robustness of genetic analyses, we then run an additional set of tests using a reduced dataset including only nine individuals for each colony. These analyses showed consistent results with the main tests (Supplementary Methods).

Among the 15 microsatellites markers published for *M. myotis*(Castella & Ruedi, 2000), 12 were selected to carry out this study as they i) could be successfully amplified in most samples, ii) showed variability within the populations, iii) had no evidence for null allele and iv) respected the Hardy Weinberg equilibrium (Table S1). Genotyping of all individuals allowed for the detection of 109 distinct alleles, with an overall mean of 9.5 alleles across loci, ranging from 6.83 to 10.58 within each of the investigated roost (Table 2). Genetic analyses based on allele frequency for the 12 selected microsatellites supported low genetic differentiation between different roosts (F_{ST} = 0.030, SE 0.006). Indeed, pairwise F_{ST} values between colonies ranged between 0.009 and 0.036 (mean 0.0193) and showed significant although little differentiation only for the small colony three (p-value = 0.001) (Table 3). This same colony also showed the lowest mean allelic richness across loci (Table 2).

Regarding genetic analyses on the mitochondrial DNA, we aligned sequences of 413 bp, among which 20 sites were variable among the South Tyrolean dataset, seven within the HVI and 13 within the HVII regions, defining nine distinctive haplotypes included in two main clades (Figure 2, Table S4). Mean nucleotide distance was 0.12% within groups and 4.5% between groups. The most common haplotype was Hc_01, found in 66% of all individuals and in all colonies, with frequency ranging between 41% and 75% in different locations. The overall frequency of the other haplotypes varied between 0.9% and 17%, with five haplotypes found in a single colony, one found in two colonies and two found in four colonies (Table S4). In each colony, we found three to seven distinct haplotypes, with colony two showing the highest variability (n=7) and the highest number of private alleles (n=3) (Figure 3, Table 2, TableS4). Indeed, mean gene and nucleotide diversity confirmed to be higher within this colony (Table 2). In turn, colony two showed the highest mean nucleotide distance from other colonies based on sequences from HVI+HVII (Table 3). Similarly to what was observed for microsatellites, our data support a very limited geographical structuring in the area, with F_{ST} values ranging between -0.013 and 0.084 (mean 0.02), and showing significant although low differentiation only between colonies 1 and 2 (F_{ST}: 0.084; p value: 0.019) (Table 3). The Mantel test detected a no sign for isolation by distance based on either mitochondrial (R = 0.287, p=0.052), or nuclear markers (R= 0.011, p=0.43) (Figure S3).

Phylogenetic analyses of M. myotis based on HVII sequences

Genetic sequences referring to the sole HVII domain of the mitochondrial CR region belonged to 10 distinct

haplotypes, among which six were newly described and one was previously described in *Myotis blythii* only (Table S5).

Phylogenetic analyses that considered our haplotypes with the ones already described across Europe showed they all clustered within haplogroups A, B or C as described by Ruedi et al. (2008) (Figure 3). Ninety-five percent of individuals showed haplotypes clustering within haplogroup B, including the widespread haplotype H12 and six other haplotypes described for the first time, named H-ST16-1/6. Four out of six of these haplotypes were unique to a single colony, one was exclusively found in an adult male, and one was found in two different colonies and an adult male (Figure 3, Table S5). In addition, six individuals from three colonies (4.1%) shared the pan-European haplotype H1, belonging to the haplogroup A. Finally two individuals from two different colonies carried the genetic signature of haplogroup C, previously found in North-Western Italy and Switzerland, among which one haplotype was newly described and another one was previously described from a *M. blythii* . Crucially, both these individuals were youngsters, so that they were not included in the determination of the genetic structure.

Haplotypes belonging to more than one haplogroup were found in all colonies, with the exception of the southern colony five, with representatives from groups A, B and C described only in colony three, much closer to the border compared to other colonies (Figure 3, Table S5). Interestingly, adult males included in these analyses only carried signatures belonging to haplogroup B (Figure 3, Table S5).

Circulation of rabies-related lyssaviruses

Serological analyses performed in 2016 showed neutralization of EBLV-1b in 119 out of 270 (44%) individual blood samples from South Tyrolean*M. myotis,* with serological titers ranging from 2 to 2.4 LogD50/ml (Table 4). In addition, 46 samples (17%) from different roosts and seasons showed viral inhibition below the determined cutoff for the method. Observed sero-prevalence and specific antibody titers increased after the birth pulse, occurring at the end of May across the whole population (Table 4). Despite the percentage of seropositive individuals varied from 0 to 26.5% among colonies in early May, the seasonal increasing trend was confirmed in all colonies (Table 4, Figure 4). Overall, animals under 1 year of age showed a higher percentage of positivity and higher titers compared to adults; however, this result might be confounded by the fact that we found only few adult animals in September, when sero-prevalence has been proven considerably higher in all colonies (Table 4, Figure S1). Indeed, taking into account the correlation among bats within the same colony, results from multivariable analyses suggest that the influence of seasonality alone might explain the qualitative (QIC=173.1630, AUC=90.57) and quantitative (AIC=-104.9) serological pattern found in the dataset (Supplementary methods). Overall, these analyses confirmed the higher probability to find seropositive bats in autumn as compared to spring (94.17% vs 9.55%), also showing a mean significantly higher serological titer (2.32 vs 2.11 LogD50/ml) (Table 5, Figure 5) and regardless of the age category. Indeed, we found no significant difference in serological titers between youngsters and adults in September (Figure 5).

Discussion

This study describes the combined use of population genetics and serological analyses to investigate the infection dynamics of a rabies-related lyssavirus (RRLV) in the greater mouse-eared bat in South Tyrol, the northernmost region in Italy. In particular, serological tests were specifically directed towards EBLV-1, even if the circulation of a highly related virus cannot be completely ruled out due to the lack of virus identification in this study and, more in general, in this species.

In this context, our aim was to define the spread of EBLV-1 (or its related RRLV) in the area and to interpret serological data from different locations using the connectivity between roosts derived from genetic analyses as a proximate measure for the likelihood of viral transmission. In addition, phylogenetic analyses were performed to address the possible movement of individuals across the Alps, which could be related with the dispersal of lyssaviruses that, despite being widely circulating in Europe, have never been described in Italy (Leopardi et al., 2018; Shipley et al., 2019). Our study has focused on maternity roosts, which are easier to locate compared to groups of adult males, and provide higher chances for virus amplification as a consequence of a bigger colony size, more frequent and closer interactions among individuals and due to the

yearly replenish of susceptible individuals after the birth pulse (Drexler et al., 2011).

The detection of antibodies against EBLV-1 in South Tyrolean maternity colonies of greater mouse-eared bats dates back to 2012, despite no mortality has ever been related to LYSV circulation so far (Leopardi et al., 2018). The presence of antibodies in healthy bat colonies is peculiar in the ecology of LYSVs, and has been extensively described elsewhere (Amengual, Bourhy, Lopez-Roig, & Serra-Cobo, 2007; David T S Hayman et al., 2010; Robardet et al., 2017; Suu-Ire et al., 2017). Indeed, LYSVs are widely known for their pathogenicity in non-flying mammals, invariably lethal when symptoms develop (Fisher et al., 2018). In this study, we confirmed that all the investigated colonies are exposed to the virus, as they show highly specific neutralizing antibodies. Antibody titer were lower compared to other mammals; similar pattern has been already described in bats for other pathogens, both in the field and after experimental infection, and might be related with the high efficiency of their innate, non-antibody-mediated pathway for viral control (Baker, Schountz, & Wang, 2013). In addition, we detected a significant variation of sero-positivity across a single reproductive season, supporting seasonal variation in the circulation of LYSVs in all the locations investigated and, overall, an increasing sero-prevalence in late summer compared to spring. These data are consistent with similar findings in *Myotis* and serotine bats (Amengual et al., 2007; Robardet et al., 2017; Serra-Cobo et al., 2013), and could relate to an increased virus transmission after the birth pulse, as suggested for other viruses as a consequence of the large uptake of susceptible individuals (Drexler et al., 2011). Indeed, higher antibody titers were found during late summer, which could be a sign of seroconversion due to viral exposure. This result differs from recent evidences determined for EBLV-2 in *Myotis daubentonii,* for which it was suggested a peak in virus transmission later during the autumnal swarming, when males and females aggregate for mating (Horton et al., 2020). However, this is rather predictable, considering that bats are highly variable in their ecology, so that differences in life history traits including hibernation, colony formation, parturition and mating are likely to determine peculiarities in virus dynamics as well (D. T S Hayman et al., 2013). Despite peaks in transmission might depend upon host ecology, most species follow strict seasonal patterns which might affect prevalence of viruses (D. T S Hayman et al., 2013); thus, we suggest that comparison between data of sero-prevalence detected from different years or locations should always account for differences in the sampling period. In this context, 100% peak of sero-prevalence recorded in this study is exceptional compared to previous evidences in the same host species (Amengual et al., 2007; Schatz et al., 2014; Serra-Cobo et al., 2002; Šimić et al., 2018) and is likely related to a very late sampling, when adults were already dispersing for mating. In addition, it is wise to consider to what extent the strong effect of seasonality might hide the influence of other factors, including the geographical, demographical or climatic data. Indeed, although preliminary analyses suggested that the youngest animals were more likely to be serologically positive compared to adults, the effect of age lost significance when accounting for the dependence on seasonality. This is likely related to non-homogeneous sampling across age classes of different sampling campaigns.

None of the five roosts investigated was more likely to be infected, suggesting that EBLV-1 or a related cross-reacting virus is circulating across the area with no geographical restriction. Indeed, genetic data derived from South Tyrolean *Myotis myotis* are consistent with a single meta-colony, with movement of individuals between colonies likely favoring viral transmission. Despite several studies already documented regional panmixia of *M. myotis* at the nuclear level, we also found lack of structuring based on mitochondrial DNA, strengthening the hypothesis that population across the whole South Tyrol are highly connected. In addition, F_{ST} values inferred from nuclear and mitochondrial markers were similar, suggesting a much lower philopatric behaviour than previously described in Germany, France and Switzerland despite a similar spatial scale (Castella et al., 2001; Petri, Pääbo, Von Haeseler, & Tautz, 1997), and supporting regular movements of individuals between colonies, similarly to what observed in the serotine bat (*Eptesicus serotinus*) (Moussy et al., 2015). The potential use of several roosts in the area was also supported by occasional recapture in the big colony two of adult females which were tagged elsewhere, despite a structured capture-recapture study has still to be performed to quantify inter-colonial movements. Interestingly, colony two showed the highest diversity in the area, also supporting the mixing of bats in this spot, characterized by the biggest population size and by a central position within the study area. However, such a higher diversity might also

be explained by the historical aggregation of bats in this territory from different colonies already existing in neighbouring zones; indeed, the church was completed only in the early 19th century, well after the remaining roosts included in the study.

A meta-population structure for *Myotis myotis* is described here for the first time, providing critical information to unravel dynamics of LYSVs in this bat species. Indeed, previous mathematical models suggested this bat was unable to sustain the circulation of EBLV-1 alone, supporting that sero-positivity in the Balearic Islands was likely associated with inter-species transmission from the highly vagile species *Miniopterus scheibersii* (Colombi et al., 2019; Pons-Salort et al., 2014). In South Tyrol, the greater mouse-eared bats form very large maternity colonies within churches, where the interaction with other species is strongly limited compared to the cave dwelling populations typical of the Mediterranean area. In the area target of our study, colonies mostly consist of *M. myotis,*with *M. blythii* being sporadically found throughout the season and a small population of *M. emarginatus* recorded in a single roost from May to July. No co-roosting with *Miniopterus schreibersii* have ever been recorded in the studied colonies (Drescher, 2004). However, the yearly detection of seropositive individuals highly supports maintenance of the virus within the population (Leopardi et al., 2018). In this context, the meta-population structure seen in our sampling area as opposed to the close population model in the Balearic Islands could explain virus maintenance in *M. myotis* even in the absence of *M. schreibersii* . Indeed, several studies suggest that the exchange of individuals between roosts may be one of the main factors favoring the persistence of viruses across the whole population, sometimes allowing for local fade out of infection with subsequent reintroduction (Blackwood, Streicker, Altizer, & Rohani, 2013; Colombi et al., 2019; Horton et al., 2020; Pons-Salort et al., 2014).

Despite being largely described across Europe, EBLV-1 has never been detected in Italy (Leopardi et al., 2018; Shipley et al., 2019). Indeed, the large majority of individuals included in our sample carried a mitochondrial signature typical of the Italian peninsula, confirming the Alps as major barrier for bats, including *M. myotis* (Ruedi & Castella, 2003; Ruedi et al., 2008). However, our data also confirmed that individuals belonging to the major European clade A and to the clade C, previously reported only across the border between Switzerland and North-Western Italy, are present in South Tyrol, suggesting that transboundary admixture of animals is possible. Indeed, haplotypes from all three clades were found in the small colony three, located just on the border with Austria, which also showed a weak but significant sign of nuclear fixation. While it is possible that these results are due to continental individuals reaching this location during dispersal, coexistence of several divergent matrilines in this population may also result from the persistence of ancestral polymorphism during coalescence. As colony three is located in the oldest church in the area, dating back to 1337, it is likely that the current genetic pattern is shaped by both present- time and recent- past demographic events. Either way, these data support that South Tyrolean valleys might act as narrow corridors across the Alps, allowing for the movement of individuals from the north. In particular, the dispersal of bats across the Alps could either provide or have provided in the recent past a corridor for the introduction of LYSVs from continental Europe, where they are widespread. Regarding EBLV-1, the origin of the current lineage has been dated back to 1400 (Troupin et al., 2017), so that introduction in Italy could be related to both ongoing or recent admixture of animals from South Tyrol and other northern areas, where the virus has been confirmed virologically. The spread of viruses across the home range of bat populations has already been described for other pathogens, including lyssaviruses, henipaviruses, coronaviruses and astroviruses (Halczok et al., 2017; Leopardi et al., 2016; Olival et al., 2020; Peel, Sargan, et al., 2013). Unfortunately, the lack of viral characterization in this bat species prevented us to compare the geographical structuring of LYSVs and *Myotis myotis* , which could help to explain the role of this bat in the dispersal and evolution of EBLV-1 (Carver & Lunn, 2020; Olival et al., 2020). Challenges in the detection of bat viruses are not peculiar to our study or to LYSVs, but are likely related with low prevalence and short shedding period of viruses in these animals and, more generally, in wildlife (Wilkinson & Hayman, 2017). In this context, antibodies are easier to detect and persist longer, so that serology is widely used as a proxy for virus circulation in wildlife to model mechanisms of virus persistence within bat population even prior to virus detection (Gilbert et al., 2013; Peel, McKinley, et al., 2013).

Regarding the genetic approach used in this study, we were able to amplify 12 microsatellites described by

Castella et al., (2001), and developed a new protocol for the amplification of the complete control region of the mitochondrion. This included sequencing of two hypervariable regions (HVI and HVII), which were successfully amplified and concatenated from most samples. Compared to previous studies which had used the sole HVII, the additional presence of the HVI fragment allowed to identify a higher variability, suggesting that our approach might have a slightly better resolution for genetic analyses. South Tyrolean *Myotis* bats showed variability in all but one microsatellite (C113), confirming results already obtained by Berthier et al (Berthier, Excoffier, & Ruedi, 2006). In general, mean diversity within colonies was similar to what has already been described in other studies, both at the nuclear and mitochondrial levels (Castella et al., 2001, 2000; Ruedi & Castella, 2003; Ruedi et al., 2008). Our analyses showed more heterogeneity for the parameter "nucleotide diversity" compared to the "gene diversity" calculated within different colonies, ranging between 0.001 to 0.013 and from 0.431 to 0.783, respectively (Table 2). Indeed, gene diversity only considers the number of detected haplotypes while the nucleotide diversity also takes into account the degree of their divergence. Thus, the ten-fold higher nucleotide diversity in colonies two and three is likely related to the admixture of haplotypes that belong to different phylogenetic clades, as shown in other studies (Castella et al., 2001; Ruedi & Castella, 2003; Ruedi et al., 2008). Similarly to other studies, we determined genetic structuring of *M. myotis* based on both nuclear and mitochondrial markers, in order to test for female philopatry, which is generally supported for this species (Castella et al., 2001). However, we found comparable results from microsatellites and mitochondrial sequences, thus disproving the expected pattern in our sample. These unexpected results could be either related to peculiar ecology of South Tyrolean populations or to lower spatial scale compared to other studies on the same species.

In conclusion, we provide serological evidence for the circulation of a RRLV in the South Tyrolean population of *M. myotis,* antigenically related to EBLV-1. Unlike the Mediterranean populations, *M. myotis* in northern Italy is a strictly house dwelling species, where adult females aggregate in church roofs between April and September to form large maternity colonies reaching over 2000 individuals, with a remarkable chance for human encroachment (Zahn, 1999). As all LYSVs can cause clinical rabies in humans, this study has a strong impact on public health, regardless of the viral species actually circulating in the study area. Fortunately, we founded a strong seasonal pattern, suggesting the highest risk for human exposure might be limited in time, easily predictable and likely associated with animal birth. In this context, preventive measures to limit human exposure by limiting access to the colony during most critical times could be beneficial to both humans and animals, which is imperative considering the ecological importance of this endangered species.

Genetic data supported animal movements between colonies, potentially favoring viral dispersal across the host population home range and likely allowing for viral maintenance in the area. In addition, genetic data from this study provide important information on the ecology of the species, suggesting that females might be more faithful to the natal area rather than to the natal roost. Besides being crucial to inform epidemiological modeling for disease dynamics, these results have relevant implications for the conservation of this endangered species, suggesting for example that monitoring and protection should be performed at the regional rather than local level, as animal movements between roosts might confound fluctuations in colony sizes.

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Data Accessibility

All sequences for the entire Control Region of the mitochondrion generated for the study are available in Genbank, with accessions MT254225 - MT254403. Specimen level data including species, sampling location (coordinates), sex, age class, microsatellite genotypes, and extrapolated sequences for HVI and HVII of the mitochondrion are available at Dryad (dataset to be deposited).

Author Contributions

Stefania Leopardi: research design, fieldwork, laboratory and computational analyses, paper writing

Marzia Mancin: research design, statistical analyses, paper writing

Barbara Zecchin, Angela Salomoni and Bianca Zecchin: laboratory analyses

Pamela Priori: fieldwork

Dino Scaravelli: research design and fieldwork

Paola De Benedictis: funding, research design and coordination, review of the manuscript

Tables

Table 1. Sample size for serological and genetic analyses used during the study.

Colony	Nuclear Variability	Nuclear Variability	Mitochondrial variability	Mitochondrial variability	M
	A[1]	**H_E**[2]	**N**[3]	**h**[4]	π
1	10.58 (0.84)	0.81 (0.02)	4	0.431 +/- 0.109	0
2	10.00 (0.88)	0.80 (0.03)	7	0.783 +/- 0.065	0

Colony	Nuclear Variability	Nuclear Variability	Mitochondrial variability	Mitochondrial variability	M
3	6.83 (0.63)	0.75 (0.03)	3	0.555 +/- 0.165	0
4	10.17 (0.88)	0.80 (0.03)	3	0.467 +/- 0.087	0
5	9.50 (0.87)	0.79 (0.03)	3	0.435 +/- 0.111	0

The number is based on the total of *M. myotis* sampled from each colony, as *M. blythii* were excluded from these analyses.[1]AM: Number of adult males included in the sample.

Table 2: Genetic variability of colonies under study

[1]Allelic richness, [2]expected heterozygosity across microsatellite loci; [3]Number of haplotypes, [4]gene diversity and[6]nucleotide diversity based on the concatenated HVI+HVII fragments of the mitochondrion's control region, calculated using the T92 model for nucleotide substitution. The standard error is indicated between brackets. Standard deviation is indicated as +/-.

Table 3 . Pairwise differentiation between colonies

			1	2	3	4	5
Nuclear DNA	**F_{ST}**	**1**		0.293	0.002	0.348	0.243
		2	0.010		0.001	0.095	0.119
		3	0.029*	0.036*		0.001	0.001
		4	0.009	0.011	0.033*		0.137
		5	0.010	0.011	0.033*	0.011	
Mitochondrial DNA	**F_{ST}**	**1**		0.019	0.309	0.332	0.615
		2	0.084*		0.273	0.070	0.061
		3	0.008	0.019		0.285	0.455
		4	0.002	0.050	0.022		0.825
		5	-0.013	0.064	-0.007	-0.033	
	nucleotidic distance	**1**		0.002	0.002	0.001	0.001
		2	0.010		0.002	0.002	0.002
		3	0.008	0.011		0.001	0.001
		4	0.004	0.008	0.006		0.001
		5	0.004	0.008	0.006	0.001	

Values for nuclear and mitochondrial pairwise fixation index (F_{ST} - Wright, 1951) between individuals of different roosts are shown below the diagonal with the associated p-value (based on 1000 permutations) indicated above the diagonal. Significant values are marked with*. Nuclear indexes are based on analyses of 12 microsatellites. Mitochondrial indexes are calculated using sequences of concatenated HVI and HVII fragments. Between colonies mean nuclear distance is indicated below the diagonal as calculated with MEGA 6 using T92 model of nucleotide substitution with the standard error indicated above the diagonal.

Table 4. Serological analyses based on the RFFIT test against EBLV-1

Month	Roost	Observed seroprevalence (+/n) [1]	titer (mean) (LogD50/ml)	Dubious (n)[2]	AF [+/n (%)]	Y [+/n (%)]	AM [+/n (%)]
May	1	26.5 (9/34)	2.1	12	9/34 (26.5)		
	2	10.3 (6/58)	2.1	12	5/50 (10)	0/1	1/7 (14.3)
	3	<25% (0/8)		4	0/7	0/1	
	4	<10% (0/33)		5	0/32	0/1	
	5	7.1 (2/28)	2	6	2/21 (9.5)	0/3	0/4
	mean	10.5 (17/161)	2.06				
Sept	1	100 (9/9)	2.3		3/3 (100)	4/4 (100)	2/2 (100)
	2	100 (42/42)	2.3		4/4 (100)	33/33 (100)	5/5 (100)
	3	100 (3/3)	2.3			1/1 (100)	2/2 (100)
	4	84.6 (22/26)	2.3	4	7/9 (77.8)	7/7 (100)	8/10 (80)
	5	89.7 (26/29)	2.4	3	3/3 (100)	22/24 (91.7)	2/2 (100)
	mean	93.5 (102/109)	2.32				
Overall	Overall	44 (11/270)	2.22	46	33/163 (20.2)	67/75 (89.3)	20/32 (62)

[1]In case of negative results for all samples, we indicated the prevalence as below the limit of detection as calculated based on the sampling size in relation to the size of the colony.

[2]Number of samples showing signs of virus inhibition between 40-50%, which is below the cutoff value for the determination of titer. AF: adult females; Y: animals below 1 year of age; AM: adult males

Table 5: Mean values for the seasonal prevalence and titre of antibodies against LYSVs predicted from GEE and LMM models.

season

Predicted sero-prevalence

Error

Lower

Upper

spring

0.09551

0.04306

0.03823

0.2190

autumn

0.9417

0.03535

0.8206

0.9828

season

Predicted mean antibody titre

Error

Lower

Upper

spring

2.1106

0.03682

2.0376

2.1835

autumn

2.3235

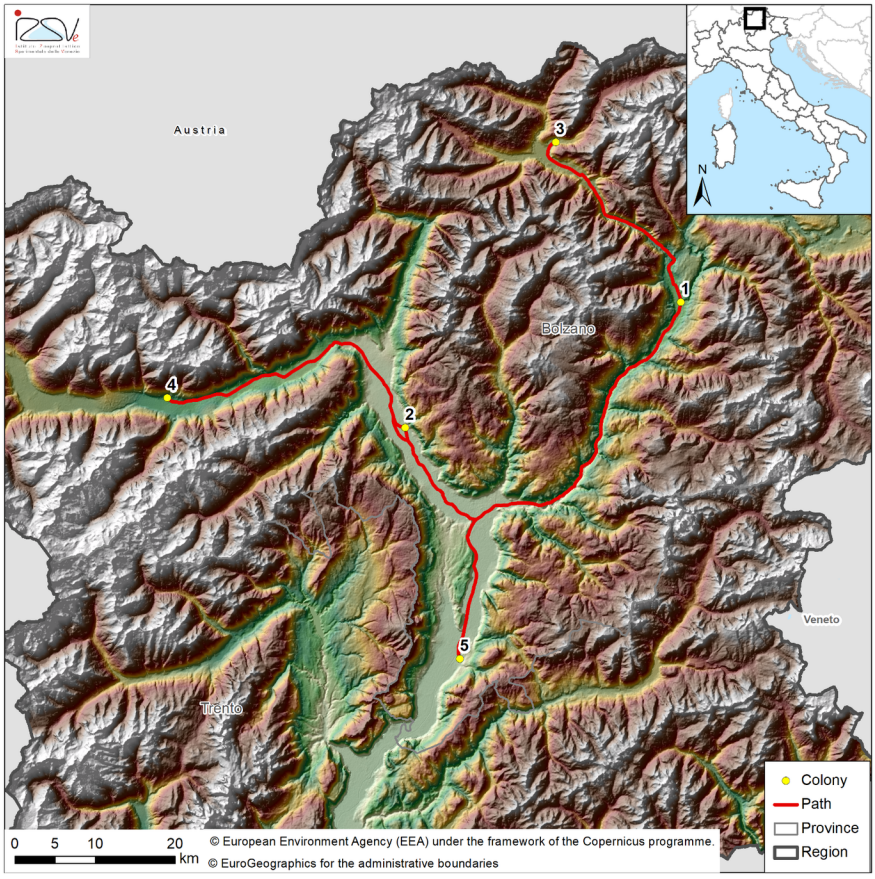
0.01658

2.2907

2.3563

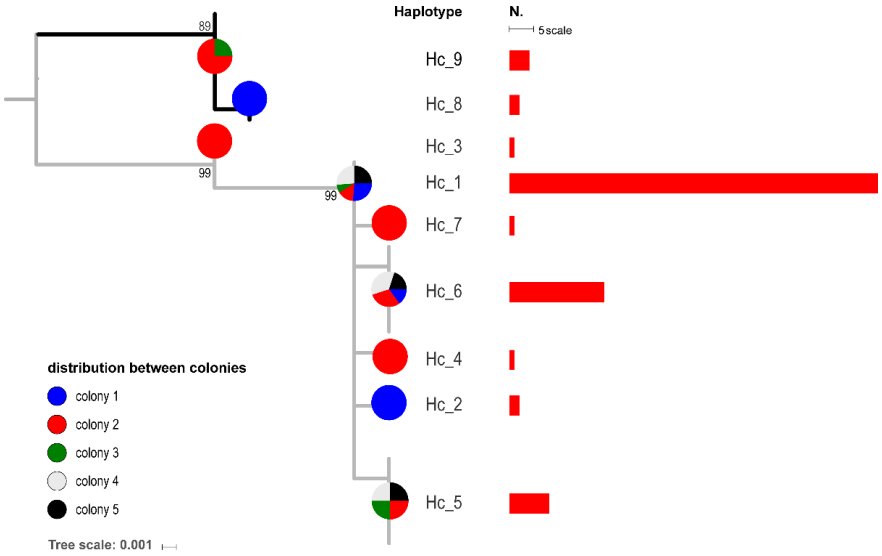
Figures

Figure 1. Geographical location of maternity colonies of*M. Myotis* included in the study



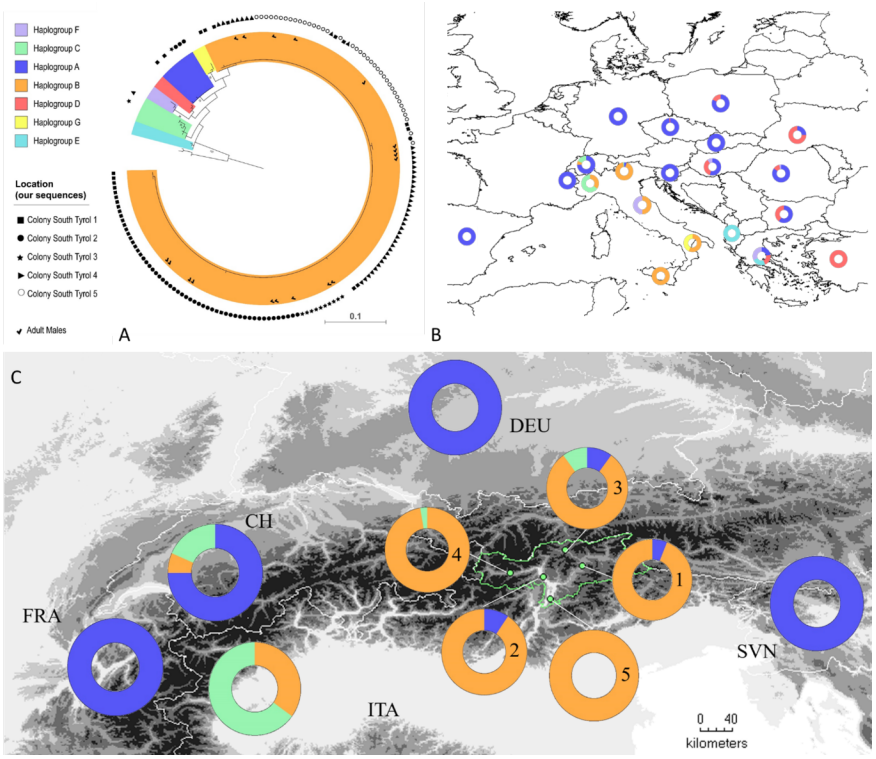
The location of each roost investigated during 2016 in South Tyrol is shown as a yellow circle within the map, numbered from one to five as indicated throughout the text. Possible routes taken by bats to move between colonies are shown in red, determined along the valley's bottoms. The map was created using European Digital Elevation Model version 1.1 under the framework of the Copernicus program.

Figure 2. Phylogenetic relationship between haplotypes identified for the concatenated HVI-HVII.



A maximum of five sequences for haplotypes have been used to build ML tree using phyML. Boostrap values above 70 are shown in the tree. The figure shows 9 haplotypes, denoted as Hc 1-9, grouped in two main clades, indicated as grey (B) and black (A) continuous lines. The distribution of each haplotype between the five colonies under study is shown as a pie chart, in which colonies are represented in blue (1), red (2), green (3), grey (4) and black (5). The total number of individuals corresponding to each haplotype is indicated as a bar chart beside the tree.

Figure 3.Phylogenetic analyses based on the HVII of the mitochondrial CR.



Sequences are classified in Haplogroups according to Ruedi et al (2008) shown with different colors maintained across panels A-C (A: blu; B:orange;C: green; D:red; E:light blu; F: violet; G:yellow). Panel A: ML Phylogenetic tree customized using iToll. Sequences from this studies are shown using different symbols according to sample location, as indicated in the table. Adult males are shown as check signs. Panel B. distribution of hlotypes in Europe, according to this study and data the literature (Bogdanowicz et al., 2013; Bryja et al., 2010; Castella et al., 2001; Furman et al., 2014; Ruedi et al., 2008; Ruedi and Castella, 2003). Panel C: Zoom of haplotypes described in the study area and bordering ones.

Figure 4. Distribution of the observed sero-prevalence in different colonies according to the sampling season

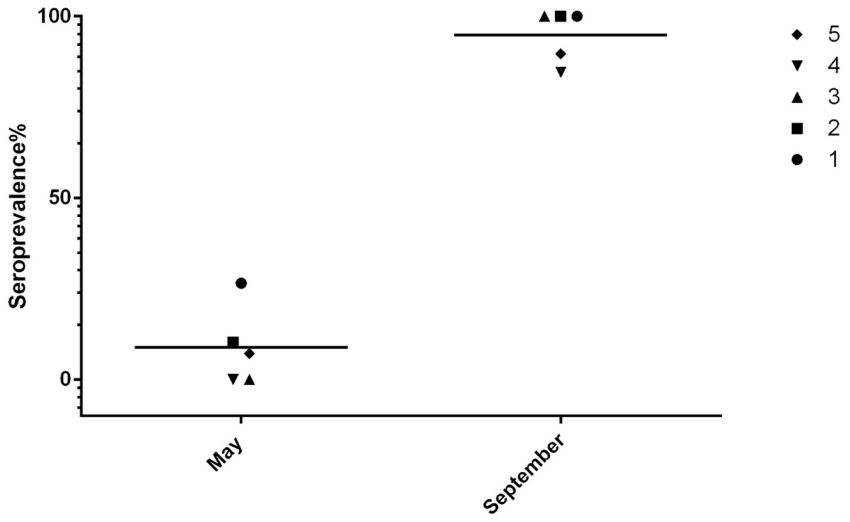


Figure 5. Distribution of antibodies titer for season and for age in autumn