Comparative phylogeography and asymmetric hybridization between cryptic bat species

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	pointing to an earlier expansion of E. isabellinus northwards. We found no evidence of mitochondrial introgression between species, but nuclear markers identified a male-mediated ongoing asymmetric hybridization from E. isabellinus to E. serotinus (28% hybrids in E. serotinus and 5% in E. isabellinus) in the contact zone. Although none of the bats studied tested positive for Lyssavirus RNA, the asymmetric hybridization supports the potential for the recently suggested interspecific transmission of EBLV-1 from E. isabellinus into E. serotinus.

Original article

COMPARATIVE PHYLOGEOGRAPHY AND ASYMMETRIC HYBRIDIZATION BETWEEN CRYPTIC BAT SPECIES

Running title: Hybridization between cryptic bats

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Keywords: *Eptesicus*; *isabellinus*; *serotinus*; Approximate Bayesian Computation; hybrids.

ABSTRACT

Cryptic speciation and hybridization are two key processes that affect the origin and maintenance of biodiversity and our ability to understand and estimate it. To determine how these two processes interact, we studied allopatric and sympatric colonies of two cryptic bat species (*Eptesicus serotinus* and *E. isabellinus*) with parapatric distribution in the Iberian Peninsula. These species are the main reservoir for the most commonly rabies virus found in bats in Europe: the European Bat *Lyssavirus* type 1 (EBLV-1). We used mtDNA and nuclear microsatellite markers to confirm the taxonomic status of both species and to show a more pronounced and geographically-based genetic structure in *E. isabellinus* than in its sibling *E. serotinus.* Using Approximate Bayesian Computation (ABC) we inferred rapid range expansion in both species after the Last Glacial Maximum until reaching their present distributions. ABC analysis also supported interspecific differences in genetic diversity and structure, pointing to an earlier expansion of *E. isabellinus* northwards. We found no evidence of mitochondrial introgression between species, but nuclear markers identified a male-mediated ongoing asymmetric hybridization from *E. isabellinus* to *E. serotinus* (28% hybrids in *E. serotinus* and 5% in *E. isabellinus*) in the contact zone. Although none of the bats studied tested positive for *Lyssavirus* RNA, the asymmetric hybridization supports the potential for the recently suggested interspecific transmission of EBLV-1 from *E. isabellinus* into *E. serotinus*.

INTRODUCTION

Given rising threats from anthropogenic climate and land-use changes, it has never been more important to understand biodiversity (Leadley, 2010) and the extinction and speciation processes that drive its origin and maintenance (Hewitt, 2001). Cryptic speciation, the process of evolutionary divergence in the absence of morphological differentiation, has been described in a variety of organisms and ecosystems, and has important implications not only for biodiversity estimates but also for habitat conservation, wildlife management (Bickford et al., 2007), pest control and epidemiology (de Vienne et al., 2013; Doña et al., 2017). Nevertheless, the genetic and ecological interactions between sibling species are not well understood (Struck et al., 2017), particularly when they are found in sympatry. A useful approach is the analysis and comparison of the recent phylogeographic history of the distinct lineages and inspection of their particular responses to environmental/climatic changes (e.g. glacial cycles) by analyzing how expansion/retraction events associated with these climatic changes shaped their genetic make-up (Carstens & Richards, 2007; Richards, Carstens, & Lacey Knowles, 2007). On the other hand, hybridization (defined as the admixture of evolutionary distinct lineages) is also a fundamental evolutionary process, commonly described in plants and animals, which is important in generating biodiversity (e.g. speciation) and its conservation (Mallet, 2005). The exchange of genetic material along secondary contact zones particularly during lineage expansions is well documented and often results in increased genetic diversity and adaptability of the new gene pools (Barton & Hewitt, 1985). This contact of formerly isolated gene pools can bring about genomic introgression (Currat *et al.* 2008) or hybrid speciation (Canestrelli et al., 2016; Mallet, 2007), both recognized as major evolutionary drivers (reviewed in Abbott *et al.* 2013).

Since cryptic speciation and hybridization contribute substantially to the origin and spatial distribution of biodiversity, the ecological and evolutionary consequences of these

processes have been widely addressed (Battey & Klicka, 2017; Martinsson & Erséus, 2017; Seehausen, Takimoto, Roy, & Jokela, 2008; Soltis & Soltis, 2009). However, their interaction (hybridization between cryptic species) has received little attention. We can expect that both processes are correlated, and that hybridization is more successful between cryptic sibling species (genetically distinct but morphologically identical species) due to a higher genetic compatibility between closely related lineages, as has been found for some birds (Mallet, 2005) and plants (Maguilla & Escudero, 2016) under natural conditions. Successful hybridization between differentiated lineages is also expected to be enhanced when these cryptic lineages share similar ecological requirements and sympatric (or parapatric) distributions.

The application of molecular techniques to species identification and hybridization studies has greatly contributed to understanding these biological processes (Allendorf et al., 2001; Frankel, 1974; Fitzpatrick et al., 2012). In fact, the molecular review of traditional taxonomy has recognized often distinct evolutionary lineages within morphologically identical entities (i.e. cryptic species) unveiling important hidden diversity (Pfenninger & Schwenk, 2007). Codominant markers such as microsatellites are useful for detecting hybrids (e.g. Randi 2008; Fitzpatrick 2012), allowing the identification of groups and individual assignments through clustering algorithms (Bohling, Adams, & Waits, 2013; Vähä & Primmer, 2006). Multi-marker approaches (for example, those combing microsatellites with mitochondrial DNA [mtDNA] sequences) in particular, provide powerful tools to address phylogeographic questions and to reconstruct intra- and inter-specific differentiation processes (Dool et al., 2013; Mallet, 2005; Mallet, Besansky, & Hahn, 2016; Mitchell, Muehlbauer, & Freedberg, 2016). In fact, the distinct genetic information provided by each marker, together with the recent application of Approximate Bayesian Computation (ABC) approaches (Beaumont, Zhang, & Balding, 2002) to statistically compare alternative

evolutionary history scenarios, are improving our understanding of the evolutionary processes behind the origin and geographical patterns of present biodiversity.

Bats are a particularly suitable group for studying cryptic speciation, hybridization and their interactions due to their high proportion of cryptic species and their rich and complex social and interspecific relationships (Altringham, 2011). Molecular studies have unveiled an unexpectedly high proportion of cryptic diversity within the group, and the majority of the 191 new bat species recognized from 1992 (Koopman, 1993) to 2005 (Simmons, 2005) were described within already known morphological complexes. In the Iberian Peninsula (hereafter Iberia), new cryptic lineages are found in up to 20% of the traditional morphologically-defined species (Ibáñez, García-Mudarra, Ruedi, Stadelmann, & Juste, 2006). On the other hand, hybridization, and particularly mtDNA introgression, has been reported in bats across families and habitats worldwide [e.g. *Rhinolophus* (X. G. Mao, Zhu, Zhang, & Rossiter, 2010); *Myotis* (Berthier, Excoffier, & Ruedi, 2006); *Eptesicus* (Artyushin *et al.* 2009); *Uroderma* (Hoffmann *et al.* 2003); *Epomophorus* (Nesi *et al.* 2011)]. The widespread mating behaviour of swarming, which involves the gathering of multi-species in a single site to breed, may facilitate -at least in some cases- this interspecific genetic exchange (Bogdanowicz *et al.* 2012).

In Iberia, the most divergent lineages were found within the serotine bat (Ibáñez et al., 2006). One lineage corresponded to *Eptesicus serotinus* (Schreber, 1774), a species common across Europe (C Moussy et al., 2015) and occupying the Northern half of Iberia, and another to *E. isabellinus* (Temminck, 1840), distributed along the southern half of Iberia and Northern Africa. A molecular revision of the genus *Eptesicus* confirmed the species status of the latter lineage (Javier Juste, Benda, Garcia-Mudarra, & Ibáñez, 2013). Both species show different environmental preferences with a narrow and overlapping zone in central Iberia (Santos et al., 2014), which is the only area in which both species are in contact. This contact zone is acting

as both the northernmost limit of the distribution for *E. isabellinus* and the southernmost limit of the distribution of the *E. serotinus*. Both species can only be distinguished morphologically by putative differences in their fur brown color (being generally darker in *E. serotinus*) and show wide overlap in all measurements (Juste et al., 2017). This bat complex is also considered the main natural reservoir of the European Bat *Lyssavirus* type-1 (EBLV-1), endemic to Europe and an important vector of rabies in humans and other mammals (P. Mingo-Casas et al., 2017). *Eptesicus* bats account for more than the 95% of the rabid bats detected in Europe. Population structure of EBLV-1 is mostly driven by geographical factors, but each of the two *Eptesicus* bats seems to host distinct lineages of EBLV-1 in Iberia (Sonia Vázquez-Morón, Juste, Ibáñez, Berciano, & Echevarría, 2011). The presence of a contact zone raises the possibility of viral exchanges with possible epidemiologic implications.

In this study, we sampled allopatric and sympatric colonies of the bats *E. serotinus* and *E. isabellinus* across Iberia, focusing on the contact zone of these sibling species. We aimed to i) reconstruct and compare the recent evolutionary histories of the two species, ii) assess the biological interactions between them, and iii) determine potential patterns of EBLV-1 transmission. We specifically tested the hypothesis of interspecific gene flow between these two cryptic bat species based on the combined information from mtDNA and nuclear microsatellite markers.

MATERIAL AND METHODS

Sampling design

We searched for *Eptesicus* bat colonies along the North-South gradient across Spain during spring (the reproductive season) from 1998 to 2013, mainly through inspecting potential refuges at dawn. Once the colony was identified, bats were mist-netted in one night and 2 mm-diameter wing membrane biopsies were collected according to Worthington-

Wilmer & Barratt (1996) together with oropharyngeal swabs from each bat before they were released shortly after capture. Tissue samples were kept in 96% ethanol at -20ºC until processed in the lab and swabs stored in an RNA preservation lysis buffer (Casas, Powell, Klapper, & Cleator, 1995) for later virus checking.

A total of 347 *Eptesicus* bats were captured and sampled from 19 breeding colonies across Iberia. Of these, 107 individuals from six maternity colonies were identified (based on their dark brown fur) in the field as presumably *E. serotinus*; two colonies (PCR and CTJ) were located in the contact zone, and the remaining four (CHA, TUD, CAN and UGA) were found throughout the northern non-overlapping area. All were stable maternity colonies with the exception of TUD, which was comprised of adult males only. The remaining 240 bats were identified as *E. isabellinus,* belonging to five breeding colonies within the contact zone (JAR, GAR, COR, SER and TOR) and eight colonies located in the allopatric area in Southern Iberia (AZN, ORG, ALC, HOR, BOQ, POS, USO and TRJ) (Figure 1, Tables 1 and 2). The taxonomic identification based on morphology was subsequently confirmed genetically using the numerous molecular diagnostic characters, particularly in the mtDNA, found between both lineages (Juste et al., 2013).

Prior to DNA extraction, the tissue was digested with proteinase K and total DNA was extracted following a standard phenol/chloroform protocol (Sambrook, Fritsch, Maniatis, Harbor, & Slatkin, 1989). The colonies were considered sympatric if a colony of the sibling species was found within 50 km distance, given that both species are sedentary (Dietz, Nill, & Helversen, 2009).

Mitochondrial DNA analysis

A section of the hyper-variable II region (HVII) of the mitochondrial control region (CR) was amplified in 107 *E. serotinus* and 240 *E. isabellinus* using the primers CSBC_F

(Wilkinson & Chapman, 1991) and H607_R (Wilmer, Moritz, Hall, & Toop, 1994) annealing in the conserved box C and the $tRNA^{PHE}$ gene, respectively. See Supporting Information Table S1 for PCR conditions and primers. DNA sequences are uploaded to GenBank (accession numbers MH443793-MH444139).

Phylogenetic relationships between haplotypes were reconstructed with PAUP* 4.0b10 (Swofford et al., 2001) using the Maximum Parsimony criterion (MP). Gaps were treated as "missing" and bootstrap support for the nodes was obtained after 2000 iterations. Mitochondrial DNA diversity was described based on the number of segregating sites (S), number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π) and average number of nucleotide differences (k). All descriptors were estimated per species and colony using the software DnaSP 5.10 (Librado & Rozas, 2009). To estimate genetic differentiation between populations we used Φst (Holsinger & Weir, 2009) calculated in ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010), as the analogue to Fst for microsatellites. Due to differences in the effective population sizes between mitochondrial (HVII) and nuclear (microsatellites) markers, we recalculated Φ_{ST} based on Crochet (2000) ($\Phi_{ST'}$) to compare it with F_{ST}-values (see below). Pairwise Φ_{ST} distances estimated between colonies of both species were analyzed to evaluate the relative importance of isolation by distance (IBD). A Mantel test was performed through the Isolation by Distance Web Service (http://ibdws.sdsu.edu/~ibdws/) (Jensen, Bohonak, & Kelley, 2005) using the log-transformed geographic distances between colonies obtained from GeoDataSource (http://www.geodatasource.com/distance-calculator).

For each species, three possible grouping designs of the distribution of genetic variability were evaluated in a hierarchical molecular analysis of variance (AMOVA) (L Excoffier, Smouse, & Quattro, 1992) implemented in ARLEQUIN v.3.5.1.2 (LAURENT Excoffier & Lischer, 2010). We compared the following hypotheses: 1) no spatial genetic

variation throughout the whole species range; 2) the genetic variability is mainly divided into colonies in allopatry and sympatry; and 3) the genetic variability of the species is divided into four groups based on geographic criteria (see below). A median-joining network of haplotypes was constructed for each species using Network 4.6.1.2 (Bandelt, Forster, & Röhl, 1999). The resulting networks were simplified by cutting superfluous median vectors and pruning unnecessary links for *E. isabellinus* with the MP option of the software.

Microsatellite genotyping and data analysis

Individuals were genotyped using 12 microsatellites originally designed for the bats: *Plecotus auritus* (PAUR05, Burland *et al.* 1998), *Myotis myotis* (D15, Castella & Ruedi 2000; NN8, Petri *et al.* 1997), *Myotis bechsteinii* (B22; Kerth *et al.* 2002), *Eptesicus fuscus* (EF1, EF14, EF15, EF20, EF4, EF6, *Nyctalus noctula* (P213), and *Thyroptera tricolor* (TT20, Vonhof *et al.* 2001). All indirectly labeled using a M13 extension (Schuelke, 2000). See Supporting Information Table S1 for PCR conditions. Microsatellites were selected to avoid linkage disequilibrium among loci, using GENEPOP on the web (Rousset, 2008), and null alleles by using the EM algorithm implemented in FREENA (Chapuis & Estoup, 2007). Microsatellite datasets are available through the Mendeley data depository (DOI: http://dx.doi.org/10.17632/tpxc5dfs7v.1#file-b0e6021e-88c3-417d-b612-62c44dc06cc7). Hardy-Weinberg Equilibrium (HWE) was then tested per species, locus and population using Markov Chain Monte Carlo (MCMC) simulations in GENEPOP on the web (Rousset, 2008). Observed (H_O) and (non-biased) expected (H_E) heterozygosity were estimated using all loci per population with GENETIX 4.05.2 (Belkhir *et al.* 2004) and the inbreeding coefficient (F_{IS}) using FSTAT v.2.9.3.2 (Goudet, 1995). Genetic structure was quantified with global F_{ST} and 95% confidence intervals and population pairwise F_{ST} values in GENETIX 4.05.2.

Individual-based assignment tests were run in STRUCTURE 2.3.2 (Falush, Stephens, & Pritchard, 2007) both to estimate the genetic structure within each species separately and to determine whether there was admixture between them. The algorithm implemented in STRUCTURE infers the number of genetically differentiated groups (K) and identifies pure and admixed individuals based on individual membership coefficients (*q*-coefficients). The selection of K was based on the second-order rate of change of log probability of the data between successive values of K (Evanno, Regnaut, & Goudet, 2005) as implemented in STRUCTURE HARVESTER (Earl & von Holdt 2011). We applied this method using the admixture (independent allele frequency model) model and 10 replicates for each K (K ranging from 1 to 5) and ran simulations based on 100,000 MCMC iterations, collecting data every 100 steps. The first 10,000 steps were discarded as burn-in to estimate the convergence of the chains. CLUMPP v.1.1.2 (Jakobsson & Rosenberg, 2007) was used to obtain the averaged individual *q*-coefficients. We also used a Discriminant Analysis of Principal Components (DAPC) (Thibaut Jombart et al., 2010), a method used to find group subdivision that maximizes differences between groups while minimizing variation within them. The DAPC were run using the *Adegenet* package in R (T. Jombart, 2008). We used the function *find.clusters* to run successive K-mean clustering with increasing number of clusters (max.n.clust=10) and applied the Bayesian Information Criterion (BIC) to select the model that best represents the number of groups.

Identifying hybrids

The program NEWHYBRIDS 1.1 beta (Anderson & Thompson, 2002) was used to identify potential hybrids. Runs were performed using Jeffrey's prior and a burn-in period of 20,000 repetitions followed by 100,000 repetitions of sampling. As suggested by the authors, we also ran an additional analysis with uniform priors to remove any possible bias due to low frequencies of alleles (Anderson & Thompson, 2002). The results of the STRUCTURE and

NEWHYBRIDS analyses were combined to define hybrids, following the criteria defined by Burgarella *et al.* (2009), to obtain the highest proportion of correctly identified hybrids. Accordingly, an individual was classified as hybrid only if satisfying two conditions: a) the sum of q-values was higher than 0.75 for all hybrid categories (F1 hybrids and backcrosses) in NEWHYBRIDS and b) it was assigned to one of the two *Eptesicus* species with a q-value lower than 0.90 in STRUCTURE.

Approximate Bayesian Computation model-based inference of evolutionary history

Alternative evolutionary scenarios for the two cryptic *Eptesicus* species were tested using the Approximate Bayesian Computation (ABC) approach implemented in DIYABC v2.1.0 (Cornuet et al., 2014). ABC analyses were carried out with the combined mtDNA and microsatellite datasets, and included all samples genotyped for the microsatellite markers. For this analysis, populations of each species were grouped into two broad geographical groups, one group at the Centre of the Iberian Peninsula, that included for each species the populations in the contact zone, and a second allopatric group at either the North (for *E. serotinus*) or the South (for *E. isabellinus*) of the Iberian Peninsula (see Figure 1). Separate analyses were run to determine for each species the time of population split [pre (Scenario 1.1 and 3.1) or post (Scenario 1.2 and 3.2) Last Glacial Maximum (LGM)] and changes in demographic history (Scenario 2.1 and 4.1= null model of no change in population size since LGM; Scenario 2.2 and 4.2= post-LGM expansion of both populations; and Scenario 2.3 and 4.3= post-LGM population expansion followed by more recent decline in sympatric population due to interspecific competition). Both species' dataset were then combined to compare evolutionary histories and to inspect for gene flow between species, extent and direction (Scenario 5.1= gene flow from *E. serotinus* to *E. isabellinus* sympatric populations; Scenario 5.2= gene flow from *E. isabellinus* to *E. serotinus* sympatric populations; and Scenario 5.3= no gene flow between the species). We set pre-LGM split times at 10^4-10^6

generations ago, and post-LGM split times at 10-10^4, following the assumption that generation time in bats is likely to be around 2 years (e.g. Kerth *et al.*, 2002; Flanders *et al.*, 2009). We fixed effective population sizes (*Ne*) in non-demographic analyses as equal across populations, ranging between 10^3 and 10^6. In the demographic history analysis *Ne* varied between populations and across time, depending on the scenario (see Supporting Information S2 for detailed information on scenarios and parameters).

Microsatellite loci were assumed to follow a Generalized Stepwise Mutation model (GSM) and mean mutation rate was bounded between 10^{-3} and 10^{-4} (Balloux & Lugon-Moulin, 2002; Thibaut Jombart et al., 2010). The mutation rate of HVII was set at 10^{-7}-10^{-6} following the premise that the mutation rate of the CR is 10 fold faster than mtDNA genes/regions, like the Cytochrome *b* (see Nabholz *et al.* 2008 for mutation rates of Cytochrome b for mammals). Based on the results of jModelTest, the mutation model was set as Kimura-2-parameters. The percentage of invariant sites was adjusted to 30% for *E. serotinus*, while the shape of the gamma distribution was adjusted to 0.5 for *E. isabellinus*. The majority of available summary statistics were included in all runs, but statistics specifically relevant for demographic changes, Tajima's D for the mtDNA and Mean Garza-Williamson's M for the microsatellite datasets, were only included in the demographic history analyses. A total of 10^6 simulations per scenario were tested in each analysis. The posterior probability of scenarios was estimated using a weighted polychotomous logistic regression. We checked model performance and empirically evaluated the power of the model to discriminate among scenarios and to determine confidence in scenario choice.

***Lyssavirus* testing**

To inspect bats for *Lyssavirus* infection, oropharyngeal swab were collected from all captured specimens and deposited in a buffer designed for viral RNA preservation and cold

stored until processing. In the lab, *Lyssavirus* RNA was searched for by a nested reverse transcription PCR targeting 260 bp of the viral nucleo-protein gene using published primers, methodology and conditions (S. Vázquez-Morón, Avellón, & Echevarría, 2006). A positive control was run alongside samples to probe the sensitivity of the method and discard false negatives.

All sampling procedures were approved by the Ethical committee of the EBD- CSIC.

RESULTS

Mitochondrial genetic diversity and structure

The HVII fragment of the CR selected and amplified in all samples was the sequence just before the series of repeats that was 282 bp long in *E. serotinus* and 274 bp in *E. isabellinus*. Sequences were trimmed to the shortest fragment, resulting in an alignment of 277 bp in analyses involving the two species plus outgroup. GenBank accession numbers: MH443793 to MH444139 (all-sequences alignment available in Supporting Information S3). The morphology-based identification was confirmed genetically by the tree topology obtained that showed all samples divided into two groups corresponding to the two species of bats (Supporting Information Figure S4). A total of 17 and 24 haplotypes were identified in the HVII fragment in *E. serotinus* and *E. isabellinus*, respectively. In both species, genetic diversity varied substantially among colonies regardless of their geographic location, their proximity to the edge of the distributional range, or if the colony was in contact or not with the other species (Figure 2, Table 2, Supporting Information Table S5). Overall values of genetic diversity were similar in both species. However, two colonies of *E. isabellinus* (AZN and HOR) were monomorphic with a unique haplotype shared by all their individuals (Figure 2, Table 2, Supporting Information Table S5).

Genetic differentiation values (Φ_{ST}) were relatively high for the two species, but slightly lower for *E. serotinus* (average Φ_{ST}=0.43 / $\Phi_{ST'}$ = 0.16) than for *E. isabellinus* (average Φ_{ST}=0.64 / $\Phi_{ST'}$ = 0.31). Genetic differentiation between colony pairs was overall high and greater than 0.100 in all *E. serotinus* pairwise comparisons (Supporting Information Table S6). The lowest value of genetic differentiation was found between pairwise comparisons of *E. isabellinus* colonies: AZN-HOR (Φ_{ST}=0.000) and SER-USO (Φ_{ST}=0.029). Mantel's tests showed that IBD was not significant in either *E. serotinus* (Z = 16.0292, r = 0.0379, p = 0.57) or *E. isabellinus* (Z = 94.7963, r = 0.5255 p = 0.99). The decomposition of the variation through the AMOVA analyses revealed that over half of the variation was found 'within populations' for *E. serotinus*, and under all scenarios the 'between populations' component explained less than 7% of the total variability found in the species (Supporting Information Table S7). For *E. isabellinus*, instead, the 'within populations' variation component was much lower (< 25%), and the 'between groups' component instead, was relatively important for all designs (> 25%). In summary, the AMOVAs indicated that genetic variability is more geographically structured in this species (Supporting Information Table S7). This conclusion was also supported by the haplotype networks (Figure 2). The network of *E. serotinus* showed no structure between groups of colonies whereas the haplotypes of *E. isabellinus* appeared in two geographically separate groups (East and North of Spain). These groups were connected by the most frequently sampled haplotype that shows a star-like topology.

Nuclear genetic diversity and structure

A total of 77 *E. serotinus* and 231 *E. isabellinus* were genotyped for the 12 selected microsatellites. Loci EF6 and EF15 were subsequently removed because they showed high frequency (>0.05) of null alleles in 10 and 16 of the 19 colonies, respectively. There was no evidence of linkage disequilibrium among the remaining 10 microsatellite loci (Table 2,

Supporting Information Table S8). Only one colony of *E. serotinus* (CTJ) and the colonies JAR, SER and USO of *E. isabellinus* showed significant deviations from HWE (Table 2, Supporting Information Table S8). All colonies had lower genetic diversity than expected under HWE, as also reflected in their positive F_{IS} indexes, with the exception of TRJ. The overall significant genetic structure (F_{ST}= 0.132; 95% CI: 0.083-0.203) was caused by the pairwise comparisons between *E. isabellinus* and *E. serotinus* (mean: 0.281; min: 0.202, max= 0.367), whereas intraspecific pairwise comparisons between colonies showed quite shallow genetic structure in both species (*E. serotinus:* overall F_{ST}= 0.047, 95% CI= 0.027-0.069; *E. isabellinus*: overall F_{ST}= 0.011, 95% CI= 0.003 - 0.016). The multi-species Bayesian analysis of population genetic structure clearly differentiated between *E. isabellinus* and *E. serotinus* and divided all genotyped individuals into two clusters (K=2) in agreement with the taxonomic distinction of two species (Figure 3). When STRUCTURE was run for each species separately, *E. isabellinus* did not show any intraspecific genetic structure (K=1), whereas *E. serotinus* individuals were divided into two clusters (K=2) and individuals from the CTJ, PCR and TUD colonies were mostly admixed (Figure 3B). A strong interspecific genetic structure was also observed with DAPC (Figure 3C) differentiating *E. serotinus* and *E. isabellinus*.

Results from NEWHYBRIDS and STRUCTURE identified hybrid individuals in both species, though the frequency was clearly uneven (28% of *E. serotinus* versus 5% of *E. isabellinus* bats). All hybrids identified according to the most restricted criteria were found in the contact zone. The five hybrid individuals in *E. isabellinus* were found in three out of the five colonies that were sampled in the contact zone (BOQ: n= 3, COR: n=1 and GAR: n=1, 15.79%, 5.26%, and 7.14% of the sampled individuals in each colony respectively). On the other hand, the proportion of hybrids was remarkably high in the only two colonies of *E. serotinus* sampled in the zone of sympatry, CTJ (n=7 out of 19, 36.84%) and PCR (n=2 out of

9, 15.38%), while no hybrids were found in any of the allopatric colonies. A detailed report of the proportion of hybrids within each colony is found in Supporting Information Table S9.

ABC model-based inference placed both species' population split times as post-LGM and the older population as the allopatric population (*E. isabellinus:* posterior probability >0.99, error rates<0.0001; *E. serotinus:* posterior probability >0.99, error rates<0.0001). In the case of *E. isabellinus*, the central, sympatric population was colonized from the southern populations at approximately 6,250 years ago (ya) (75% Credible Intervals: 2,000-9,800 ya), while the central *E. serotinus* population was colonized from the northern population at around 2,600 ya (75% CI: 1,400-5,100 ya). The combined model indicates that contact between the sympatric central populations of the two species has allowed the hybridization through gene flow from *E. isabellinus* to *E. serotinus* (posterior probability=0.870), with rates of admixture estimated at 0.252 of the *E. serotinus* population (75% CI: 0.193-0.325; Figure 4). Interestingly, this is a very similar value to the hybridization rate found in the NEWHYBRIDS analysis. Demographic history models indicate that both populations of *E. isabellinus* have expanded more than 100 fold post-LGM, while in the case of *E. serotinus* the allopatric (northern) population expanded by just under 10 fold and the sympatric (central) population by nearly 100 fold. None of the populations have subsequently decreased in size following contact with their cryptic sister species (*E. serotinus:* posterior probability=0.722, error rates=0.118; *E. isabellinus:* posterior probability=0.950, error rates=0.086; see Supporting Information S2 for DIYABC outputs).

Finally, no positive results were found in the PCR screening for EBLV virus in the saliva swabs of any of the sampled bats.

DISCUSSION

Cryptic speciation and hybridization are evolutionary processes that have contributed substantially to the origin and distribution of biodiversity. Nevertheless, the interaction between these two processes has been largely overlooked, in particularly in mammals, even though hybridization between cryptic species is expected to be relatively high (Mallet, 2005). Using genetic markers (mitochondrial and nuclear) with different modes of inheritance we show differences in the recent evolutionary histories of two cryptic bat species (*E. serotinus* and *E. isabellinus*) in Iberia, and provide strong evidence for asymmetric hybridization between sympatric populations.

Overall mitochondrial genetic diversity values were similar in both species. These values were close to those reported for *E. serotinus* from other parts of Europe (C Moussy et al., 2015) and slightly higher than those of the American congener, *E. fuscus* (Neubaum, Douglas, Douglas, & O'Shea, 2007). Still, genetic diversity varied substantially between colonies and two allopatric colonies of *E. isabellinus* were completely monomorphic at the mtDNA level (AZN and HOR). The diversity values showed no differences between colonies in the contact zone (which also represents the North and South edge-of-range of the two species) and the more central populations (Figure 2), contrary to the expected pattern of reduced diversity in peripheral populations (Bridle & Vines, 2007). In fact, colonies of *E. isabellinus* near the distribution limit were neither scarcer nor smaller than colonies at the center of the species distribution. The lack of pattern indicates that the species' edge-of-range may be recent and that diversity values may result instead from prevailing environmental conditions or the evolutionary history of each colony. For instance, the two monomorphic colonies were located in recently built concrete bridges and their lack of diversity probably reflects random fixation of alleles during recent colony formation due to founder effect. *E. serotinus*, on the other hand, seemed to be rarer in the contact zone, and despite intense search efforts, only two maternity colonies (PCR and CTJ) and a few vagrant males were found in

413 the area. Nevertheless, these two colonies at the edge of the species range did not show lower
414 values of genetic diversity. Interestingly, the social structure of the colony does not seem to
415 determine colony diversity either because the only colony of bachelor males sampled (TUD)
416 showed similar values as the other maternity colonies.

417 Mitochondrial and nuclear genetic differentiations confirm the species status of *E.*
418 *serotinus* and *E. isabellinus* (Javier Juste et al., 2013), although genetic structure varied
419 considerably between markers, as well as between species. In fact, whereas most of the
420 variation in *E. serotinus* was found within colonies, *E. isabellinus* showed twice as much
421 differentiation among colonies and this variation component was geographically structured
422 with a distinction between a group of Southern (Andalusian) colonies and a group of colonies
423 along the Northern swath in the Central Plateau that are separated by the Sierra Morena
424 mountains (Figure 2). This pattern matches that found in bats by Juste *et al.* (2009), as well as
425 in plants (Nieto Feliner, 2014) and amphibians (Martínez-Solano, 2004). Levels of
426 differentiation between colonies are similar to levels found in other continental colonies of *E.*
427 *serotinus* (C Moussy et al., 2015), and are typical of sedentary or short-distance migrating
428 bats (Caroline Moussy et al., 2013). Differentiation among colonies was mostly high in all
429 mtDNA pairwise comparisons, and can be associated with the strong roost fidelity of females
430 (J Juste et al., 2009). This mtDNA structure contrasts with the results of the microsatellite
431 dataset that, apart from supporting the specific distinction of the two bats, show a weak
432 genetic structure, indicating male-mediated gene flow between colonies. Clustering analyses
433 did not reveal any pattern in either of the two bats, likely due to the overall weak genetic
434 structure (Latch, Dharmarajan, Glaubitz, & Rhodes, 2006). The differences between mtDNA
435 and nDNA markers stress the different roles played by females and males in relation to the
436 genetic structuring of the populations. Dispersing males are responsible for gene flow
437 between populations, while the philopatric behavior of females results in strong genetic

structure at the mtDNA level. These sex-biased differences are considered typical for most bat species (Altringham, 2011), and have been described in several species, including *Rhinolophus ferrumequinum* (Flanders et al., 2009), *Myotis myotis* (Ruedi et al., 2008), *Miniopterus schreibersii* (Ramos Pereira, Salgueiro, Rodrigues, Coelho, & Palmeirim, 2009) and *Myotis escalerai,* in the Iberian Peninsula (Razgour et al. 2015). The colonies JAR, SER and USO of *E. isabellinus* and CTJ of *E. serotinus* were not in Hardy-Weinberg equilibrium, suggesting population sub-structuring or deviation from random mating. These colonies were all located at the edge of the distribution areas, which may explain the deficit of heterozygotes combined with a potential Wahlund effect due to possible sampling of different family groups within colonies (Hansen, Nielsen, & Mensberg, 1997).

The best supported recent evolutionary history scenarios based on the ABC analysis using the joint mDNA and nDNA dataset suggest that both bats expanded in Iberia after the LGM from their respective refuges: *E. serotinus* expanded southwards and *E. isabellinus* northwards. According to the results, the refuge in the case of *E. isabellinus* was probably located in the southwestern corner of the Peninsula as suggested by Juste *et al.* (2009). There is no information on the refuge of *E. serotinus,* but the lack of genetic structure, even across Europe (Moussy et al., 2015, Troupin et al., 2017), suggests a rapid expansion of *E. serotinus* as new suitable habitats opened in Europe. In Iberia, ABC inference indicates a slightly older expansion for *E. isabellinus*, which would explain its relatively deeper geographic structure based on the mtDNA marker. Interestingly, neither of the two bats seems to have experienced population declines either during the expansion or after the contact. The expansions could have been reciprocally limited by the presence of the other species due to competitive exclusion, eventually resulting in the present parapatric distribution along an East-West axis in the center of the Peninsula. Alternatively, the sharp environmental changes in the contact zone from the forested mountain habitats to more xeric open lands may have played a role in

463 defining the current distribution edges, given the important differences in the environmental
464 optima of the two species according to their species distribution models (Santos et al., 2014).
465 The contact zone between the two species seems to be much wider in Portugal (Rebelo,
466 2013), where the transition between Atlantic and Mediterranean environments is smoother.
467 To fully understand the current dynamics and to make reasonable predictions about shifts in
468 the species' limits, more information is needed on both patterns and processes occurring
469 within and adjacent to the contact zone (Harrison, 1986).

470 Our empirical evidence revealed no mitochondrial introgression between the two
471 species because all the colonies showed unambiguous haplotypes belonging to either one
472 species or the other. Accordingly, colonies in the contact zone did not show higher mtDNA
473 diversity, as would have been expected had they incorporated alien haplotypes. Interestingly,
474 mitochondrial introgression with the co-generic *E. nilssonii* was detected in Western
475 populations of *E. serotinus* (Artyushin *et al.,* 2009) and probably associated to its post-glacial
476 expansion. However, the nuclear markers identified in our study an ongoing male-mediated
477 asymmetric hybridization from *E. isabellinus* to *E. serotinus* (28% in *E. serotinus* and 5% in
478 *E. isabellinus*) in the contact zone. Asymmetric hybridization has been also recently reported
479 in the species complex of *Myotis myotis* and *M. byithii*, two bat species roosting in mixed
480 maternity colonies (Afonso, Goydadin, Giraudoux, & Farny, 2017). Asymmetry in the genetic
481 exchange is relatively common (Barton & Hewitt, 1985) and can be promoted by different life
482 history traits (e.g. sex differences in body size, mating strategies, dispersal behavior etc.). In
483 this case, however, the two cryptic bats are highly similar in their morphology, echolocation
484 characteristics and ecology (J. Juste et al., 2017). Alternatively, asymmetric introgression
485 could arise from post-mating barriers, such as sex-biased sterility and fitness differences
486 (Barton & Hewitt, 1985), or could simply be the result of differences in abundance, whereby
487 the rarer species is more likely to mate with the other species simply because hetero-specifics

488 are more common in the area. Hybridization also enables the establishment of new
489 populations, as species shift their distribution range and overlap with competing species
490 through the acquisition of local genetic adaptations (Hovick and Whitney, 2014). The
491 negative effects of species competition may be ameliorated by the combined effects of
492 demographic mechanisms (e.g. colonization/extinction of bat colonies) and severe Allee
493 effects without implying any new adaptive change in hybrids (Mesgaran et al., 2016). The
494 proportion of hybrids found in one of the colonies of *E. serotinus* (PCR) is almost double the
495 number of hybrids found in the other colony (CTJ). These differences could indicate clinal
496 variation, though more colonies need to be sampled to validate this hypothesis. The presence
497 of hybrids only in the narrow contact zone points to a recent genetic exchange rather than past
498 overlap in species distributions as suggested for other bats (Mao et al., 2010).

499 Hybridization is often related to particular social behaviors. In the case of the bat
500 *Myotis alcathoe* it has been linked to the particular swarming behavior of bats that gather to
501 mate in multi-specific ensembles in places like cave entrances (Bogdanowicz et al., 2012).
502 For other bats, like the Asian horseshoe bats, hybrids were associated with opportunistic
503 winter mating with torpid females (Mao et al., 2010). For the European serotine, as for the
504 American *E. fuscus*, it is still unknown when mating takes place (Maarten J. Vonhof,
505 Strobeck, & Fenton, 2008). Detailed data on mating behavior are needed to fully understand
506 the possible relationship between hybridization and specific aspects of their social life.

507 EBLV-1 is the zoonotic rabies virus most frequently detected in bats in Europe, and
508 both *Eptesicus* species are considered its main reservoirs (Sonia Vázquez-Morón et al., 2008).
509 *Lyssavirus*-positive bats were not found in this study, in line with previous results for
510 *Eptesicus isabellinus* showing viral RNA presence in only 2.8% of more than a thousand bats
511 tested (Sonia Vázquez-Morón et al., 2008). *E. serotinus* and *E. isabellinus* host distinct and
512 characteristic EBLV1 virus strains, each with a different phylogenetic history (Sonia

Vázquez-Morón et al., 2011). A recent study has reported the first detection of an EBLV-1 of the strain considered typical of *E. isabellinus* in an *E. serotinus* bat from North-Eastern Spain (Patricia Mingo-Casas et al., 2018). The sequence of this virus is close to a known southern sequence of the virus, which suggests a recent transmission from *E. isabellinus* to *E. serotinus,* in agreement with the direction of the asymmetrical hybridization described in our study. The process of viral transmission across bat species is not well understood, but most probably requires close contact between the bats (Echevarría, Avellón, Juste, Vera, & Ibáñez, 2001; Sonia Vázquez-Morón et al., 2008). Whether the transmission of the virus strain was in fact promoted by the asymmetric hybridization and whether this host exchange in the EBLV-1 strains is widespread are questions in need of further investigation.

Conclusions

The herein described hybridization between *E. serotinus* and *E. isabellinus* is a new example to add to the surprisingly short list of hybridizing bat species (reviewed in Bogdanowicz *et al.*, 2012). Nevertheless, the extent of hybridization reported in the literature might be an underestimation because, like in our case study, hybrids are frequently impossible to distinguish morphologically from the parental species (Mallet, 2005). Interestingly, out of the less than twenty hybridization events (including introgression) reported for bats, the majority involves pairs of cryptic but not necessarily sister species, from the large *Pteropus* fruit bats (Webb & Tidemann, 1995) to the tiny European *Pipistrellus* (Sztencel-Jabłonka & Bogdanowicz, 2012). This pattern is pointing to an association of hybridization with other evolutionary processes linked to cryptic species, such as parallelism or stasis (Struck et al., 2017), although with exceptions such as the cryptic species complex of European *Plecotus* that maintain totally isolated their genetic pools even in sympatry (Andriollo et al., 2018). This particular case could result from the high level of differentiation between these lineages (J. Juste et al., 2004), since it is expected that recently diverged taxa have less chance of

538 incompatibilities or genetic breakdown when their genomes are assembled together, making a
539 successful hybridization between them probably more attainable, especially in secondary
540 contact zones. Nevertheless, the relationships between hybridization and level of
541 differentiation still need more studies to be properly understood.
542
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557

RESUMEN

Los procesos de especiación críptica e hibridación son determinantes para el origen y mantenimiento de la biodiversidad, así como para nuestra capacidad de conocerla y estimarla. En este trabajo estudiamos colonias en alopatría y simpatría de dos especies crípticas de murciélagos (*Eptesicus serotinus* y *E. isabellinus*) con distribución parapátrica en la península ibérica. Se sabe que estas especies suponen el principal reservorio para los virus de la rabia más comunes en murciélagos en Europa: *Lyssavirus* tipo 1 de los murciélagos europeos (EBLV-1). Utilizamos ADN mitocondrial y microsatélites para confirmar la taxonomía de ambas especies y para mostrar que en *E. isabellinus* existe una mayor estructura genética correlacionada con la distribución geográfica que la encontrada en *E. serotinus*. Datamos una expansión de rango rápida en ambas especies tras el ultimo máximo glacial hasta que alcanzaron su actual área de distribución, utilizando para ello métodos basados en la computación de aproximación bayesiana (ABC). Estos análisis también confirman diferencias interespecíficas en la diversidad genética y estructura, lo que sugiere una expansión hacia el Norte de *E. Isabellinus* anterior a la de la especie hermana. No encontramos introgresión del ADN mitocondrial entre especies, aunque el análisis de los microsatélites identificaron una hibridación asimétrica actual de *E. isabellinus* hacia *E. serotinus* en la zona de contacto (28% de híbridos en *E. serotinus* y 5% en *E. isabellinus*). A pesar de que ninguno de los especímenes analizados portaban ARN de *Lyssavirus*, la hibridación asimétrica detectada en este estudio justifica el potencial de transmisión del EBLV-1 de *E. isabellinus* hacia *E. serotinus*.

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Titles and legends to figures.

Figure 1. Sampled colonies of *Eptesicus serotinus* (triangles and red) and *E. isabellinus* (circles and blue) in the Iberian Peninsula. See Table 1 for acronyms. The contact zone is marked with a shaded ellipse and the colonies within it have light colors.

Figure 2. Left: Histograms showing the distribution of mtDNA nucleotide diversity (π) by colonies for 1a) *Eptesicus serotinus* and 1b) *E. isabellinus*. Histograms of populations included in the contact zone are shown in light red (*E. serotinus*) and light blue (*E. isabellinus*), otherwise in full color. Right: Median-Joining network between HVII haplotypes of the two species of bats: 2a) *Eptesicus serotinus* and 2b) *E. isabellinus*. Circles are proportional to the number of individuals presenting each haplotype. Similarly, light red and light blue circles correspond to the contact zone. The little red dots are reconstructed or missing haplotypes and each red bar in the connecting lines represent a change. Dashed lines in 2b) divide geographic regions.

Figure 3. Population structure of *Eptesicus serotinus* and *E. isabellinus* in Spain. Bar plots showing the inferred group assignment of all bats sampled from *E. serotinus* and *E. isabellinus* based on the STRUCTURE analysis and grouped by colony for all individuals (A) and considering only *E.serotinus* individuals according to their mtDNA signature (B). Discriminant Analysis of Principal Components (DAPC) based on 10 microsatellites (C). The two main groups correspond to *Eptesicus serotinus* (right) and *E. isabellinus* (left).

Figure 4. Patterns of post-Last Glacial Maximum range expansion by *Eptesicus serotinus* (red triangles) and *E. isabellinus* (blue squares) in the Iberian Peninsula based on ABC inference. The allopatric populations of each species are marked with circles or polygons and the sympatric populations with ellipses. Direction of range expansion is marked with straight

946 arrows, indicating the median estimated time of expansion. Gene flow between species is
947 marked with a curved arrow, indicating hybridization rates.

948

949 **List of Supporting Information**

950 - **Supporting Information S1.** PCR amplification conditions for mitochondrial and
951 microsatellite markers.
952 - **Supporting Information S2.** Analyses and Results of DIYABC.
953 - **Supporting Information S3.** Nexus file containing all sequences of the section of the
954 hyper-variable II region (HVII) of the mitochondrial control region (CR) used in this
955 study (available online).
956 - **Supporting Information Figure S4.** Phlylogenetic reconstruction based on HVII of
957 *Eptesicus isabellinus* and *E. serotinus* using a Maximum Parsimony (MP) criterion.
958 - **Supporting Information Table S5.** Mitochondrial diversity in *Eptesicus serotinus*
959 and *E. isabellinus*.
960 - **Supporting Information Table S6.** Pairwise genetic distances between colonies of *E.*
961 *serotinus* and *E. isabellinus*.
962 - **Supporting Information Table S7**. Two and four grouping designs of Molecular
963 analyses of Variance (AMOVA).
964 - **Supporting Information Table S8.** Test of Hardy-Weinberg Equilibrium and genetic
965 diversity found across species, colonies and loci for *Eptesicus serotinus* and *E.*
966 *isabellinus.*
967 - **Supporting Information Table S9.** Number of individuals identified as pure
968 *Eptesicus isabellinus*, pure *E. serotinus*, hybrids or unclassified by NewHybrids,
969 Structure and the combined criterion following Burgarella et al. (2009).

970

971

TABLES

Table 1 - Colonies of *Eptesicus serotinus* and *E. isabellinus* sampled in this study. See Figure 1 for the spatial layout of locations.

Species (W)	Type	Code	Locality (Province)	Lat. (N)	Long.
E. serotinus	Allopatry	CAN	O Caneiro (A Coruña)	43.6007	-8.0549
		CHA	Chaín (Pontevedra)	42.3517	-8.5180
		TUD	Tudela del Duero (Valladolid)	41.5843	-4.5811
		UGA	Ugao (Vizcaya)	43.1786	-2.9028
	Contact Zone	CTJ	Casatejada (Cáceres)	39.9812	-5.7035
		PCR	Pozo del Rey (Cáceres)	40.0684	-5.4680
E. isabellinus	Allopatry	ALC	Alcalá del Río (Sevilla)	37.5185	-5.9748
		AZN	Aznalcollar (Sevilla)	37.5340	-6.3017
		ORG	Órgiva (Granada)	36.8719	-3.4728
		POS	Posadas (Córdoba)	37.7968	-5.1052
		TRJ	Puente Trajano (Sevilla)	37.0326	-5.9272
		HOR	Horcajo de los Montes (Ciudad Real)	39.2905	-4.5516
		USO	Puente Río Uso (Toledo)	39.7341	-5.0570
		BOQ	Boquerón (Ciudad Real)	39.4928	-4.5422
	Contact Zone	COR	Corrinche (Cáceres)	39.7754	-5.7137
		GAR	Garrovillas (Cáceres)	39.7543	-6.4375
		JAR	Jaraicejo (Cáceres)	39.7517	-5.8490
		SER	Serradilla (Cáceres)	39.7916	-6.1320
		TOR	Torrejón el Rubio (Cáceres)	39.8298	-6.0351

1001 **Table 2** - Mitochondrial [number of haplotypes (***h***), haplotype diversity (***Hd***), nucleotide
1002 diversity (***π***), number of polymorphic sites (***S***)] and nuclear diversity [observed (***Ho***) and
1003 expected (***He***) heterozygosity] across all loci and by colony.

1004

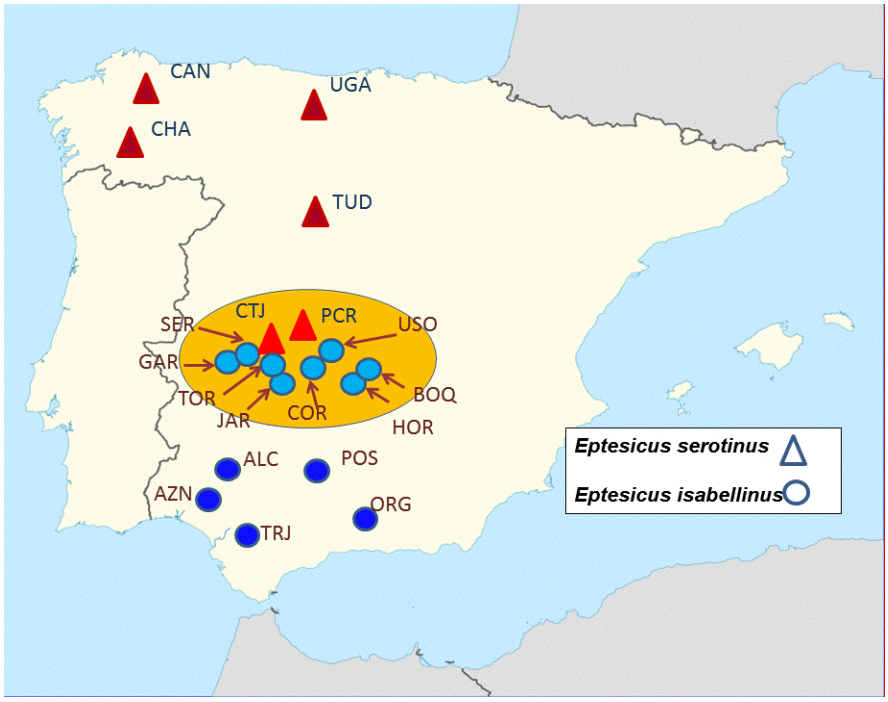
1005

Eptesicus serotinus

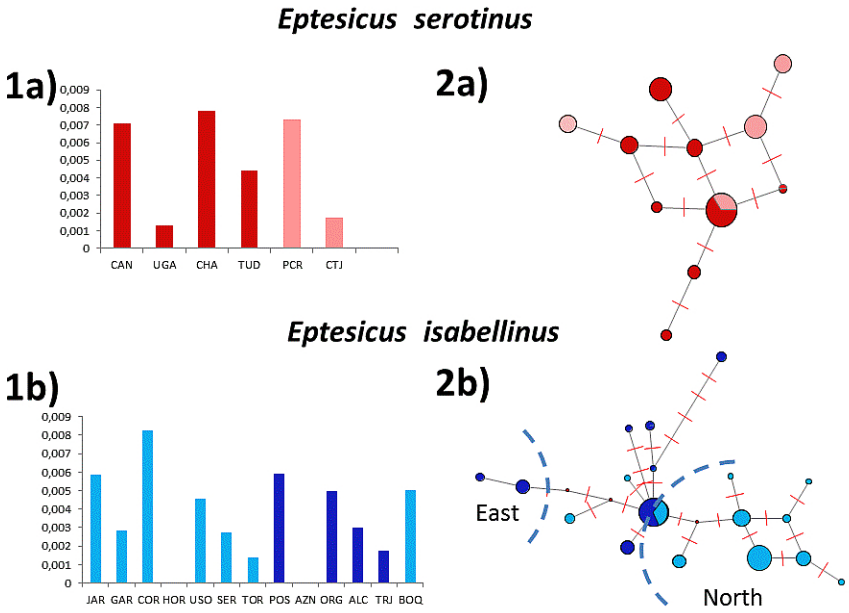
	Mitochondrial					*Nuclear*				
Colony	***N***	***h***	***Hd***	***π***	***S***	***N***	***He***	***Ho***	***Overall***	***Fis***
CHA	19	8	0.795	0.008	12	11	0.4676	0.4128	0.3355	0.123
TUD	15	5	0.781	0.004	4	14	0.5439	0.4859	0.1676	0.111
PCR	19	4	0.45	0.007	12	13	0.6956	0.6352	0.9143	0.097
CTJ	20	3	0.647	0.007	5	19	0.6164	0.3355	0.0295	0.02
CAN	20	3	0.468	0.002	2	17	0.4092	0.4094	0.6164	0
UGA	14	2	0.363	0.001	1	3	0.53	0.5167	1	0.05
Total	107	17	0.894	0.008	16	**77**	0.628	0.57	0.5105	0.052

Eptesicus isabellinus

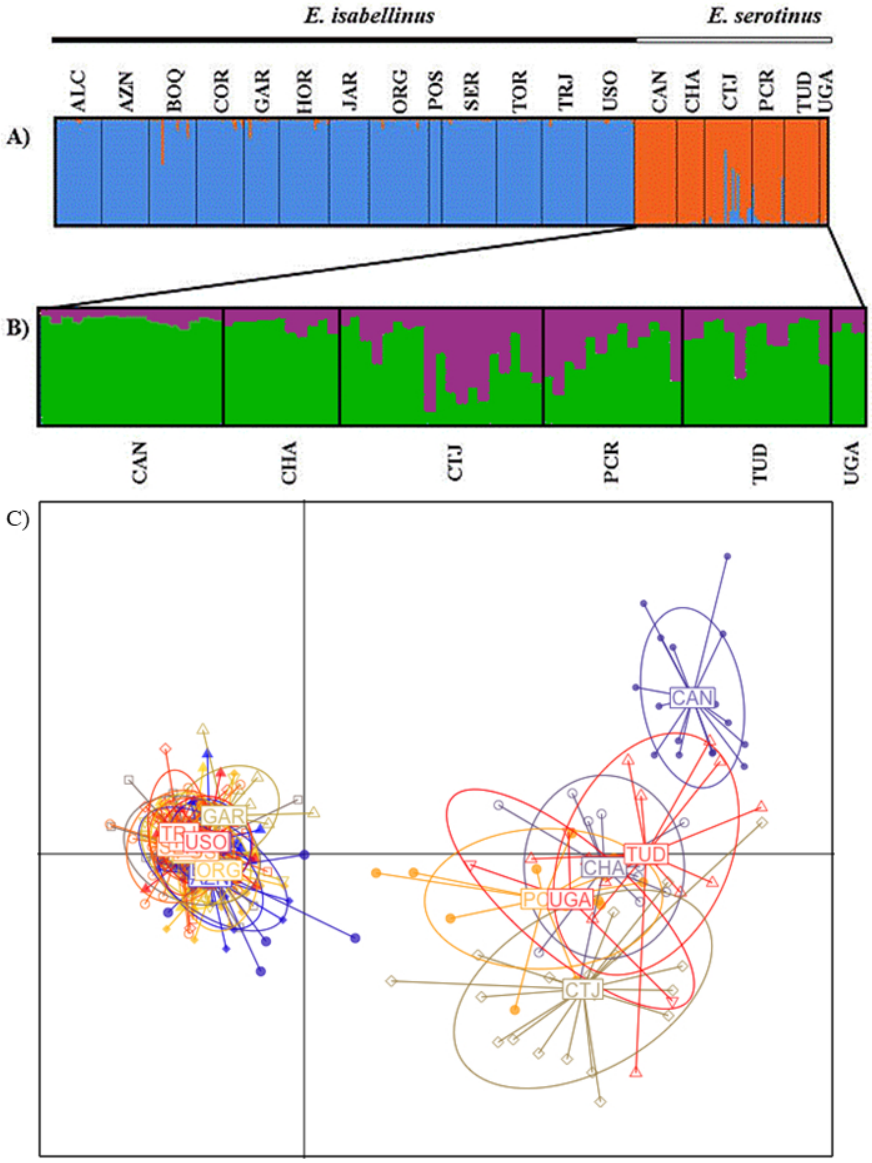
	Mitochondrial					*Nuclear*				
Colony	***N***	***h***	***Hd***	***π***	***S***	***N***	***He***	***Ho***	***Overall***	***Fis***
JAR	17	5	0.735	0.006	6	16	0.7161	0.6397	0.0035	0.111
AZN	18	1	0	0	0	19	0.7012	0.6473	0.1547	0.084
ORG	24	3	0.598	0.005	4	24	0.6846	0.6367	0.1746	0.07
ALC	20	4	0.5	0.003	4	18	0.7038	0.6547	0.0705	0.071
GAR	14	2	0.264	0.003	3	14	0.7206	0.6892	0.7209	0.046
COR	19	2	0.456	0.008	5	19	0.695	0.6918	0.8417	0.005
HOR	20	1	0	0	0	20	0.6989	0.6887	0.1778	0
POS	11	6	0.727	0.006	6	5	0.6934	0.67	0.9682	0.036
USO	20	3	0.353	0.005	11	19	0.6711	0.6499	0.013	0.032
SER	22	4	0.571	0.003	3	22	0.6822	0.6082	0.036	0.111
BOQ	19	5	0.713	0.005	9	19	0.6883	0.681	0.0547	0.011
TOR	17	2	0.382	0.001	1	18	0.6874	0.6603	0.6647	0.041
TRJ	19	2	0.491	0.002	1	18	0.6886	0.6918	0.2941	-0.005
Total	240	24	0.840	0.013	27	231	0.70	0.66	0.3211	0.049



Sampled colonies of Eptesicus serotinus (triangles and red) and E. isabellinus (circles and blue) in the Iberian Peninsula. See Table 1 for acronyms. The contact zone is marked with a shaded ellipse and the colonies within it have light colors.

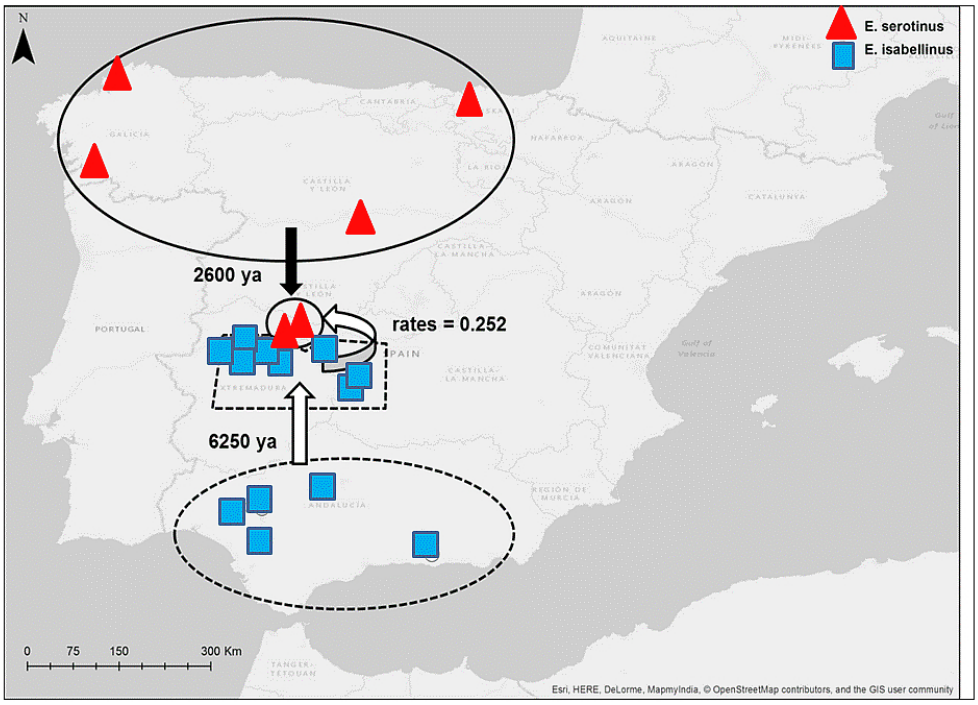


Left: Histograms showing the distribution of mtDNA nucleotide diversity (π) by colonies for 1a) Eptesicus serotinus and 1b) E. isabellinus. Histograms of populations included in the contact zone are shown in light red (E. serotinus) and light blue (E. isabellinus), otherwise in full color. Right: Median-Joining network between HVII haplotypes of the two species of bats: 2a) Eptesicus serotinus and 2b) E. isabellinus. Circles are proportional to the number of individuals presenting each haplotype. Similarly, light red and light blue circles correspond to the contact zone. The little red dots are reconstructed or missing haplotypes and each red bar in the connecting lines represent a change. Dashed lines in 2b) divide geographic regions.



Population structure of Eptesicus serotinus and E. isabellinus in Spain. Bar plots showing the inferred group assignment of all bats sampled from E. serotinus and E. isabellinus based on the STRUCTURE analysis and grouped by colony for all individuals (A) and considering only E.serotinus individuals according to their mtDNA signature (B). Discriminant Analysis of Principal Components (DAPC) based on 10 microsatellites (C). The two main groups correspond to Eptesicus serotinus (right) and E. isabellinus (left).

190x254mm (96 x 96 DPI)



Patterns of post-Last Glacial Maximum range expansion by Eptesicus serotinus (red triangles) and E. isabellinus (blue squares) in the Iberian Peninsula based on ABC inference. The allopatric populations of each species are marked with circles or polygons and the sympatric populations with ellipses. Direction of range expansion is marked with straight arrows, indicating the median estimated time of expansion. Gene flow between species is marked with a curved arrow, indicating hybridization rates.