EUROBATS Projects Initiative

Migration in European bat populations: Estimating genetic relationships between French and Spanish populations of the endangered Greater Noctule Bat, *Nyctalus lasiopterus*

Study report (June 2019)

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I would like to thank the technicians from LEM for assistance with all the laboratory work. I am particularly grateful for the help given by Juan Luis Garcia, and all his time spent teaching me. José María Gasent, specialist technician, have also brought an essential help for the microsatellites' treatment. Moreover, Alejandro Centeno, professor and researcher at Cadiz University, has given his precious time to explain how to interpret the nuclear DNA results. Thanks to him, we could gather French and Spanish data together to make a comparative study.

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1. Introduction

As part of an initiative project for the Agreement on the Conservation of Populations of European Bats, a genetic study about the Greater Noctule (*Nyctalus lasiopterus*) has been conducted in the Evolutionary Department of Doñana Biological Station (LEM-EBD), led by C. Ibáñez. This project was launched by the French Mammal Society (SFEPM) in collaboration with the LEM-EBD. A Master student, Capucine Szilas – with the help of Javier Juste, researcher of the Bats Research Group, and Juan Luis García Mudarra, laboratory technician – has been taught how to extract, amplify and sequence DNA in order to analyse various samples and estimate the relationships between French and Spanish Greater Noctules populations. We wanted to explore the possibility that the Pyrenees mountains could form a geographical barrier to migration. Mitochondrial DNA-based information has enabled us to study the gene diversity as neutral changes in the nucleotide composition (haplotypes) and its spatial structuring to reconstruct the historical relationships among populations. On the other hand, the study of microsatellites markers produced complementary information. The high variability in these markers yielded estimates of gene diversity and population structure that correspond to more recent events such as present gene flow between populations. In this way, the genetic population structure of *N. lasiopterus* in France and the relationships with the Iberian populations have been studied, as well as the Pyrenees role in the population structure.

2. Materials and Methods

a) Study area

This study was focused on two European countries, France and Spain. More precisely, data from three Spanish populations – La Rioja, Segovia, Los Alcornocales Natural Park – were available to make a comparison with French populations (Fig.1). Six groups from different localities of *N. lasiopterus* from France were determined for this study, as well as three individuals found dead in windfarms (Tab.1).

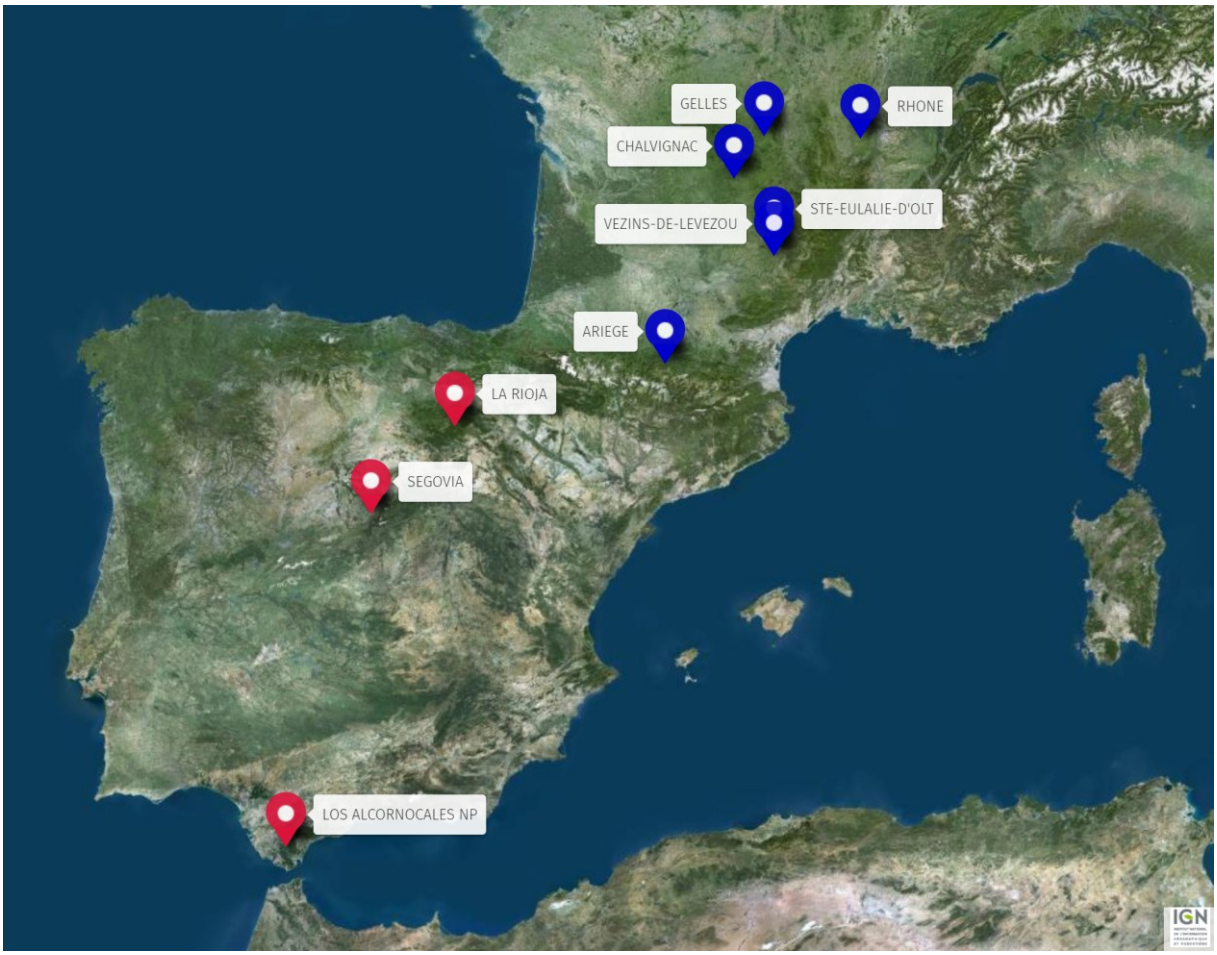

Figure 1 – Map of the study area, created with openstreetmap.fr website. The blue pointers represent the French groups and the pink ones represent the Spanish groups.

b) Mitochondrial DNA markers

As explained in the previous report (March 2019), we used two fragments of the control region: the hypervariable domain 1 (HV1) and 2 (HV2) (fig. 2).

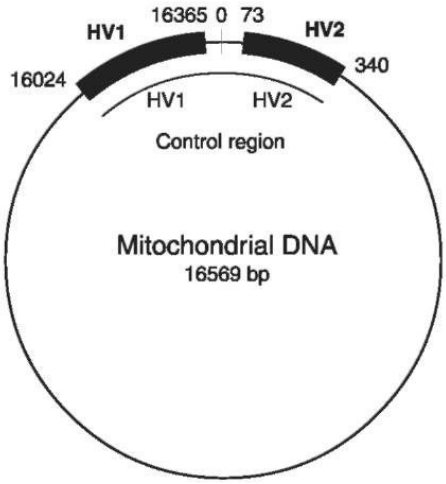

Figure 2 – Illustration of the control region of mtDNA, with HV1 and HV2 in black.

The 2 markers were amplified and sequenced with the ABI Prism 3130xl device in LEM. The sequences were edited, aligned and cropped with the Geneious® software. We described the mitochondrial fragments by considering the haploid genotype obtained after sequencing, which is called “haplotype”. It is formed by the same length-sequence corresponding to each fragment and that can be found for each individual. Haplotypic and nucleotide diversity was calculated by comparing differences in sequences. Population structure was evaluated by performing a Molecular Variance Analysis (AMOVA) which is used to analyse the variance of gene frequencies, taking into account the mutations between molecular haplotypes. We also calculated the differentiation indexes “ϕ_{ST}”. Median-joining networks were built based on pairwise ϕ_{ST} matrixes.

c) Microsatellites markers

11 microsatellites loci were used for this analysis, as it has been done in a previous study in Spain (Santos *et al.*, 2016). They were genotyped thanks to the ABI Prism 3130xl sequencer and with the help of the Geneious® software. Allelic diversity was determined and population structure was analysed.

3. Results

a) Laboratory results

After finding the best protocols to extract DNA and all the other lab processes, explained in the previous report (March 2019), we could use the DNA of 89 individuals found in France (Tab.1).

Table 1 – French groups resulting from the samples available and the success of their extraction, amplification and sequencing. Between parenthesis is the "*département*" code for each French locality. Groups 4, 6 and 7 are in italics to underline that they were represented by less than 10 individuals, which is unrepresentative of the real colony.

	Group 1	**Group 2**	**Group 3**	***Group 4***	**Group 5**	***Group 6***	***Group 7***	**Total**
Locality	Vézins-de-Lévézou, Aveyron (12)		Ste-Eulalie-d'Olt, Aveyron (12)	*Bordes-sur-Lez, Ariège (09)*	Saint-Laurent-de-Chamousset, Rhône (69)	*Chalvignac, Cantal (15)*	*Gelles, Puy-de-Dôme (63)*	France
Nb samples females	24	0	0	*2*	13	*0*	*3*	43
Nb samples males	0	10	15	*5*	0	*7*	*9*	46
Type	11 swabs 13 punches	6 swabs 4punches	6 swabs 9 punches	*1 swab 7 punches*	13 swabs 0 punch	*0 swab 7 punches*	*0 swab 5 punches*	37 swabs 49punches
Estimated population	150		50-60		30		*150*	
Total	24	10	15	*7*	13	*7*	*5*	89

b) Mitochondrial DNA

i. Genetic diversity

With DNAsp software, we measured various descriptors of sequences diversity.

According to Arlequin software, we found the differentiation index Φ_{ST} (between 0 and 1) which is like the F_{ST} for nuclear DNA, except the fact that it considers the sequences. An index closed to 0 represents a low genetic differentiation whereas an index closed to 1 means a high differentiation.

Table 2 – Global haplotypes diversity of populations within each country.

	HV1		**HV2**	
	France	**Spain**	**France**	**Spain**
Nb sequences	97	57	59	57
Nb haplotypes	18	14	12	13
Haplotypes diversity	0,858	0,845	0,697	0,820
Φ_{ST}	0,274	0,449	0,538	0,477
Global Φ_{ST}	0,404		0,610	

ii. Genetic structure

AMOVA enabled to know how the genetic variation was partitioned. With HV1 fragment, 60% of the variation was found within populations, whereas 29% was among population within the countries. The rest (11%) was among the countries. For HV2 fragment, there was almost the same amount of variation within populations and among populations (39% and 40%). 21% of variation was found among the countries.
To represent the relationships between the different groups, we used the method of Median-joining network. Haplotype networks represent the relationships among the different haploid genotypes observed in the dataset. Each internal node represents hypothetical ancestors.
Concerning HV1 fragment (fig.3), we obtained a star-shaped structure, which usually reveals the recentness of a haplotype and a demographic expansion. After remodelling the network with maximum of parsimony, we found one common haplotype between central France and La Rioja. In reality, we found no similar haplotype between individuals from the two countries. Nevertheless, the sequences were very close. Four possible lineages can be observed for France with two central haplogroups: Lévézou together with Ste-Eulalie and Ariège. A haplotype found a lot in Vézins-de-Lévézou and Ste-Eulalie was still the most commonly found and that could mean that it was more recent.
Concerning HV2 fragment (fig.4), there were no sharing haplotype. Again, a haplotype found a lot in Vézins-de-Lévézou and Ste-Eulalie was the most commonly found.
Spain and France showed no clear connexion and thus must be quite differentiated.
Both markers both showed French Greater Noctules haplotypes grouped in 3 or 4 clusters, which represent evolutionary events. We could also observe 3 clusters for Spanish haplotypes. However, there were not much geographic structure within them, because all regions were represented to a certain extent in each group.
We considered that HV2 had been the subject to fewer mutations than HV1, perhaps because of the control region structure. Thus, HV2 should represent haplotypes from an older time than HV1, because of the different mutation rate.

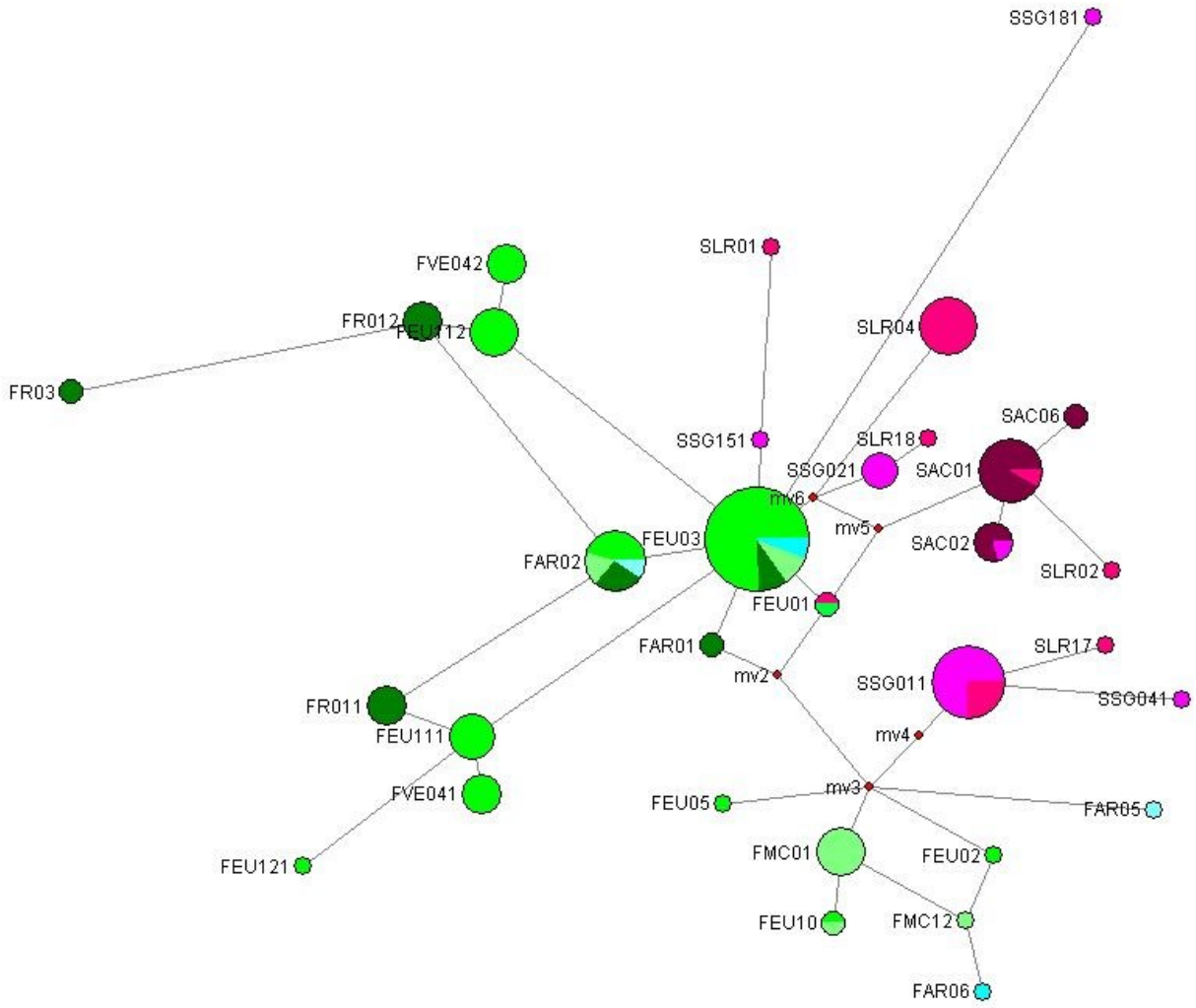

Figure 3 – Median-Joining network build with Network 5.0.1.1. Haplotypes determined with **HV1 fragment** are here represented with circles. Green colours correspond to French groups and pink colours correspond to Spanish groups. The first letter of the haplotypes names is related to the country (‘F’ for France and ‘S’ for Spain). FVE : Vézins-de-Lévézou (bright green) – FEU : Ste-Eulalie-d’Olt (bright green) – FAR : Ariège (light blue) – FR : Rhône (dark green) – FMC : North Massif Central (light green) – SLR : La Rioja (red) – SSG : Segovia (pink) – SAC : Los Alcornocales (dark purple).

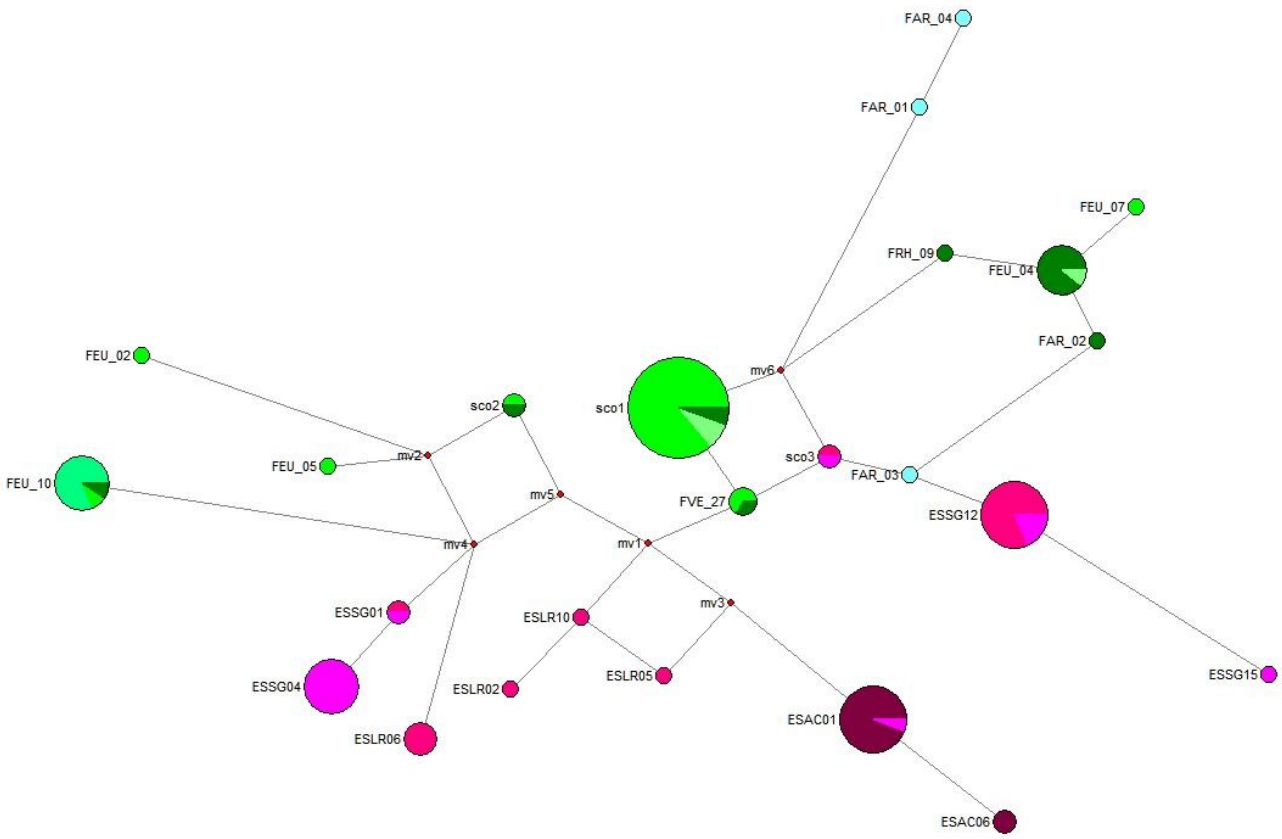

Figure 4 – Median-Joining network build with Network 5.0.1.1. Haplotypes determined with **HV2 fragment** are here represented with circles. Green colours correspond to French groups and pink colours correspond to Spanish groups. The first letter of the haplotypes names is related to the country ('F' for France and 'S' for Spain). FVE : Vézins-de-Lévézou (bright green) – FEU : Ste-Eulalie-d'Olt (bright green) – FAR : Ariège (light blue) – FR : Rhône (dark green) – FMC : North Massif Central (light green) – SLR : La Rioja (red) – SSG : Segovia (pink) – SAC : Los Alcornocales (dark purple).

c) Microsatellites

iii. Genetic diversity

Alleles information for all the markers were reported from Geneious® and pooled together with the Spanish data. All the differences between individuals, markers and population have been measured. Some information can be found in Table 3.

Table 3 – Allelic diversity from all the populations studied with their code number. The private alleles are the ones that are not found in a unique population, for each marker. Here, we noted the mean number of private alleles for all the 11 microsatellites. The "population" from Rhône (RH) is in red to underline the lack of samples for the analysis. The DNA quality was too poor to get more genotyped microsatellites.

Population	Locality	Sample size	Mean nb of different alleles	Mean nb of private alleles
VE	Vézins-de-Lévézou	24	9,455 ± 1,082	0,818 ± 0,296
EU	Ste-Eulalie-d'Olt	11	7,364 ± 0,717	0,091 ± 0,091
AR	Ariège	7	6,091 ± 0,530	0,091 ± 0,091
RH	Rhône	2	1,636 ± 0,364	0
MC	Massif Central	3	3,818 ± 0,296	0
MC_G	Gelles	5	5,000 ± 0,195	0,182 ± 0,122
MC_C	Chalvignac	7	6,818 ± 0,506	0
AC	Alcornocales	17	7,909 ± 0,719	0,455 ± 0,312
SG	Segovia	20	9,000 ± 1,095	0,636 ± 0,279
LR	La Rioja	20	9,091 ± 1,246	0,455 ± 0,247
Total	10	116	6,509 ± 0,329	0,273

iv. Genetic structure

Differentiation indexes F_{ST} have been calculated globally and pairwise. The global F_{ST} was equal to 0.013 (France alone: 0.001 and Spain alone: 0.004). A tree has been built based on the pairwise F_{ST} matrixes with the phenetic method of Neighbour-Joining (fig.5).

Figure 5 – Neighbour-Joining tree for all the populations, based of F_{ST} measured with microsatellites.

STRUCTURE software allowed us to build graphs to represent each individual assignment to a genetic group, based on their genetic differences found in each microsatellite marker. Statistics computed with STRUCTURE HARVESTER indicated that the most likely genetic distribution of the studied individuals was in K = 2 groups (green and red). Each individual had a certain probability to belong to one of the two groups. We made a first simulation where there was no *a priori* on the locality where each individual was sampled (fig.6). In a second simulation, we indicated the 7 individuals from Ariège as a single existing population and ran the model with this locality *a priori* (fig.7).

Table 4 – Populations numbers found on the STUCTURES graphs (fig. 6 and 7) between parenthesis. Information about each population are indicated, including the locality and number of individuals. The isolated individuals were found dead at the foot of wind turbines.

N° population on graph	Population	Country	Nb individuals
1	Chalvignac (Massif Central)	France	7
2	Gelles (Massif Central)	France	5
3	Vézins-de-Lévézou (males et females)	France	24
4	Massif Central (isolated individuals)	France	3
5	Ariège (Pyrenees)	France	7
6	Rhône	France	2
7	Los Alcornocales	Spain	17
8	La Rioja	Spain	20
9	Segovia	Spain	20

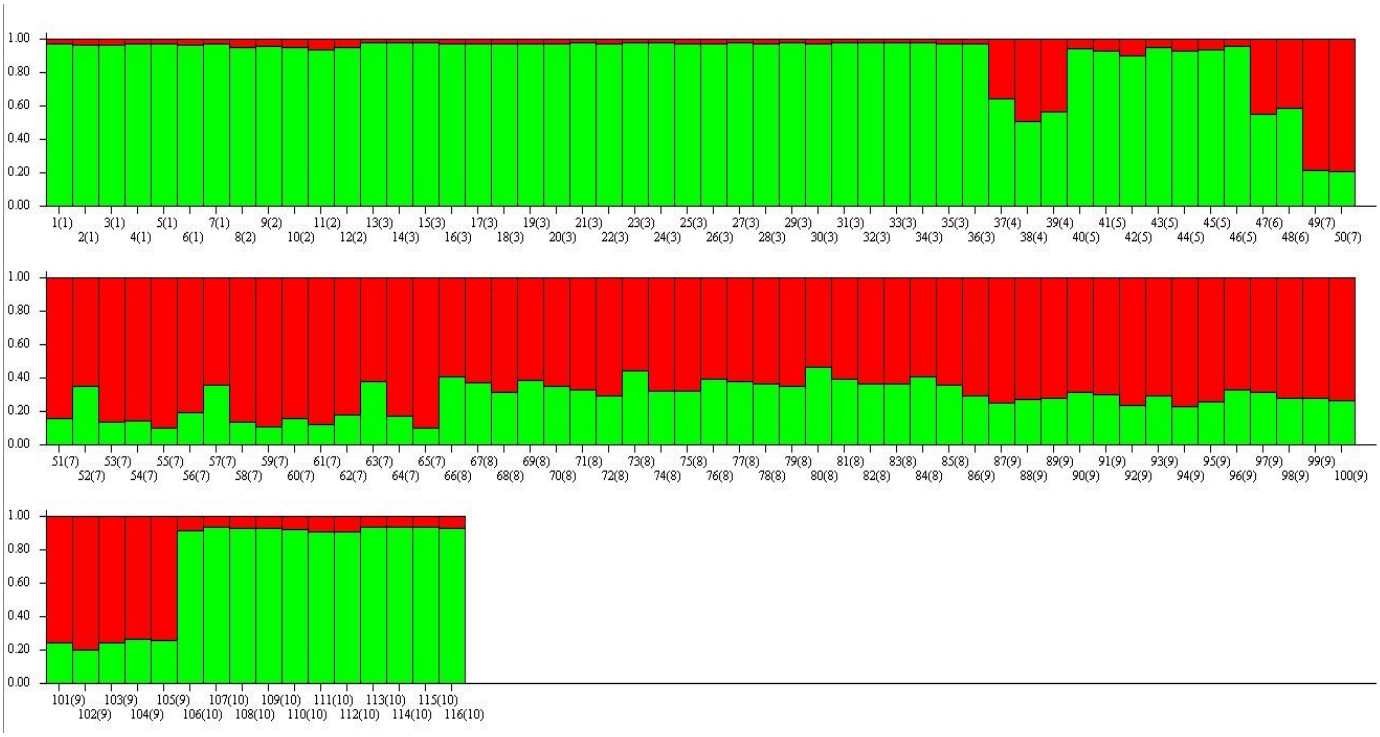

Figure 6 – Graph of the 1st simulation generated by CLUMPAK software, representing each individual's probability to belong to one of the two groups (K = 2), green or red. Individuals are reported from 1 to 116. Between parenthesis is the population number (see tab.4). There was no *a priori* about the population localities in this simulation.

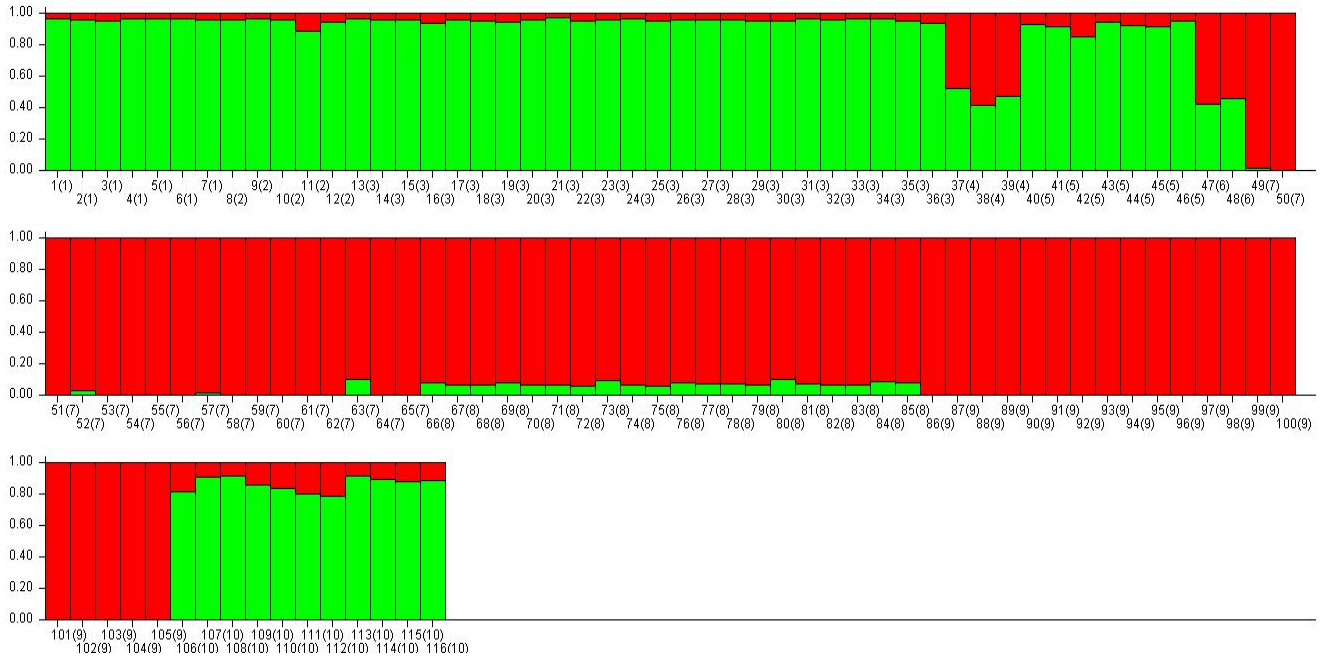

Figure 7 – Graph of the 2nd simulation generated by CLUMPAK software, representing each individual's probability to belong to one of the two groups (K = 2), green or red. Individuals are reported from 1 to 116. Between parenthesis is the population number

(see tab.4). There was an *a priori* on the Ariège population, close to the Pyrenees, in order to measure its influence on the probabilities of belonging to each group.

4. Discussion

By collecting biological samples from animals and thanks to population genetics, it is possible to study some aspects of the species that are hard to observe directly on the field. The present study allowed us to gather multiple information about gene diversity, structure within and among groups, and current gene flow. Nevertheless, the conclusions need to be considered with caution since the data set was not complete. We were aiming to improve knowledge about the Greater Noctule bat by estimating genetic relationships between individuals from France and Spain, taking into account the potential influence of the Pyrenees mountains, a terrestrial path from a country to another.

a) Results summary

i. Mitochondrial DNA

We didn't observe the same evolutionary history depending on the localisation of the DNA fragment on the circular brand. Indeed, evolutionary forces may differ among sites in a same genome. Mutation rates could have been different along the sequences and this could be due to the structure of the control region in mtDNA. From the results obtained, we supposed that HV2 fragment was showing a more ancient history because of the lower genetic diversity compared to HV1. There were more different haplotypes with HV1 fragment and they were shared by more individuals in different colonies. A more recent connexion between populations could have been the reason, or it could have been a differentiation between populations with a common origin.

<u>Within France</u>:

The males from Ste-Eulalie-d'Olt and from Ariège were more genetically diversified. The haplotypes within Ariège group were more different and exclusives, which meant that **males from various localities could have formed the actual colony**.

Males from Chalvignac had exclusive haplotypes too and other ones shared with the two colonies of males, as well as with individuals from Vézins-de-Lévézou.

Males and females from Vézins-de-Lévézou and Gelles were the less diversified. But there were only 5 samples from Gelles and thus weren't representative. **A low differentiation within a colony can be due to the recentness of this one**, in which case the differences on the genome have not yet appeared at the mitochondrial level. Females from Saint-Laurent-de-Chamousset (Rhône) had exclusive haplotypes and shared others with males from Ste-Eulalie and Ariège. Other unknown colonies could have brought these exclusive haplotypes.

Vézins-de-Lévézou and Ste-Eulalie-d'Olt:

Ste-Eulalie-d'Olt is located at about 20 km from Vézins-de-Lévézou. These two groups constituted the biggest pools of DNA and thus were the most representatives for the analysis. There were about 10 males in Vézins-de-Lévézou group and were probably juveniles from the females of this colony.

Males and females from Lévézou shared haplotypes together. They had some common haplotypes with males from Ste-Eulalie, showing that they were potentially related. Males from Ste-Eulalie remained a lot more genetically diversified.

Mitochondrial DNA being only transmitted maternally, our data could not confirm that females from Lévézou were mating with males from Ste-Eulalie. If it was the case, males from Lévézou could integrate the colony in Ste-Eulalie after dispersion. Nevertheless, there have been others genetic contributions from different colonies.

Between Spain and France:

Within Iberian Peninsula, the colony from Los Alcornocales Natural Park (south) was the most isolated from the rest of the colonies. **Spanish populations showed no clear connexions with French ones and didn't share haplotypes**.

Haplotypic diversity was equal between France and Spain. **The mitochondrial differentiation suggested a genetic isolation between the two countries, but not a totally different origin. Some individuals could have a common origin, but the origins could be diverse within a population**. Looking at the median-joining network combining all the populations studied, the colony from Segovia seemed connected to the groups from Massif Central: Gelles and Chalvignac. Also, Segovia population didn't seem so distant to Vézins-de-Lévézou and Ste-Eulalie. These two French colonies from Massif Central need a larger sampling to attest their relationship with all the other studied populations.

Ariège colony (Pyrenees):

Even if we had only 7 DNA samples from this colony, the results were remarkable and need a deeper inquiry in the future, with a more important sampling. Information that can be deduced from the genetic analysis are very useful to answer to the geographical barrier issue formed by the Pyrenees mountains. Within our sampling, haplotypes were diversified and most of them were unique, showing a certain differentiation of the group of Ariège. **The colony is likely older than the others and has been isolated for a while**. By observing the network (HV1, fig.4), a link between Ariège and the Spanish colonies could have existed. With HV2, the group was a bit close to the colonies from La Rioja and Segovia, in the north of Spain so geographically close. With HV1, these populations were more distant. **This result could mean that they were linked in the past and have been progressively differentiated with time**.

ii. Nuclear DNA

Population structure deduced from microsatellites analysis showed a more recent information about exchanges occurring between groups. Nuclear DNA is indeed transmitted by both parents and undergo recombination.

Diversity:

Allelic diversity of each group didn't vary a lot between countries. It seemed linked to the sample size more than the colony in itself. All the populations didn't show a lot of private alleles, which means that they didn't have unique haplotypes that other individuals didn't have in different groups.

The differentiation index F_{ST} revealed a low differentiation and structure. **Hence, an important gene flow seemed to currently occur in *N. lasiopterus* colonies**.

Genetic distances:

By looking at the tree based on differentiation indexes, Spanish colonies were gathered together as a genetically closer group. It confirmed again a genetic isolation between the two countries. Ste-Eulalie and Vézins-de-Lévézou populations were the closest to the Spanish ones, indicating a potential genetic link between them. Although Ariège group was the geographically closest to the Spanish colonies, it was genetically distant to all of them. It was less distant to the other French populations but still showed high genetic differentiation compared to other French groups between themselves.

Population structure:

With the STRUCTURE software, individuals have been assigned to a group depending on their genetic similarity. If an individual would have been assigned to a different group than the one where it had been collected, it would potentially have been a migrant (Frankham *et al.,* 2002). Two simulations have been done with the software: the first one without any *a priori* about the existing colonies, the second with an *a priori* on the Ariège group. It was most likely to have 2 genetically different entities (K). After running the software, we could visualize each individual's probability to belong to one of these 2 entities (fig.6 and 7). We could clearly see that all the individuals from the French populations were assigned to one same group (green) and the Spanish populations were assigned to the other (red). It was even clearer with the second simulation (fig.7), showing the influence of the Ariège group on genetic partitioning. Individuals from Ariège had a high probability to belong to the green group (corresponding to France). **The colony in Ariège could have been isolated in the past but is now clearly linked to French populations and individuals don't seem to communicate with Spanish ones. This colony needs a proper sampling to attest this hypothesis.**

Nevertheless, few individuals had an almost equal probability to belong to one of the 2 groups. They were the ones from St-Laurent-de-Chamousset, Rhône (2 individuals) and three individuals found dead in windfarms. It was not impossible that these bats hit the wind turbine in a migratory journey. **Migration behaviour could have explained the hybridization, but it remained hypothetic.** Individuals from Rhône could be related to Spanish colonies. However, a very small sampling of the female colony in Rhône was used for this study and hence needed additional DNA samples for a further analysis.

b) Conclusions

If French and Spanish populations were exchanging individuals, a more important gene flow was expected among the studied groups. **Mitochondrial and nuclear DNA allowed us to observe a clear genetic differentiation between French and Spanish populations**. On one side, mtDNA showed us a "capture" of an ancient unknown time period of the species history, within the study area. The two DNA fragments of the control region were probably giving information about different time periods. Haplotypes were not so different in terms of sequence but Spain and France

didn't share any similar haplotype. Phenetic trees and networks allowed us to see that there could have been a common origin for some individuals from the two countries.
Keeping in mind the representativity of the DNA samplings available for the present study, low differentiation could be due to the fact that the colonies are relatively recent and that they have a common origin. Otherwise, dispersion or migration by males or females (or both) could be the reason to high gene flow. There could have been recent demographic changes among colonies, that have not yet been influenced by philopatry. The female return to their birth location for the breeding season can play an important role in genetic differentiation (Moussy et al., 2012).

Microsatellites showed a clear structure, partitioning the two countries, on both sides of the Pyrenees, reinforcing the geographical barrier hypothesis. Without such a barrier, this kind of structure should have been observed if other factors were limiting gene flow, as habitat fragmentation (Wright et al., 2018) or philopatry (Moussy et al., 2012). This conclusion didn't mean that there were no genetic relationships between Iberian and French populations. Although they were differentiated, we cannot discard the migration hypothesis. The F_{ST} was low and we observed hybrids, thus migrations could occur. Some species are known to effectuate a facultative migration, a more flexible strategy than obligate migrants. For other species, only one part of the individuals migrates (e.g. sex-specific movements) (Moussy et al., 2012). Migratory behaviour could not be a fixed species characteristic, but a plastic behaviour influenced by environmental, ecological and social factors.

In the future, more DNA samples are necessary in order to carry out a more in-depth genetic analysis on representative colony samplings. The methodology used in this study could bring news insights about the migration of *N. lasiopterus* and perhaps confirm what here is only suggested. A proper sampling of the male colony in Ariège (Bordes-sur-Lez) would be very useful for this investigation. Other samplings from the already known and newly discovered colonies would also be quite informative. Possibly, different strategies co-exist. The choice of one of them could depend on environmental or individual conditions, which would be more or less suitable to migrate or to remain sedentary. This would allow Greater Noctules to select a more appropriate site for their various biological needs.

N. lasiopterus would need about 30 tree cavities for a 100-females colony (Popa-Lisseanu *et al.*, 2008). Its decline in Europe could be due to deforestation, reducing the amount of breeding sites. Wind turbines could also be a major threat, as we know

that breeding colonies are established close to windfarms in France. A better knowledge of its migration behaviour is a way to identify the pathways that are taken and to reduce the mortality caused by windfarms.

NB: A more detailed report in French in the form of a Master thesis, produced by C. Szilas, is available to complete this reading.

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