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### ► To cite this version:

Samantha Aguillon, Clara Castex, Avril Duchet, Magali Turpin, Gildas Le Minter, et al.. Landing on a small tropical island: Wide in-situ diversification of an urban-dwelling bat. *Global Ecology and Conservation*, 2024, 53, pp.e03030. <10.1016/j.gecco.2024.e03030>. <hal-04621368>

**HAL Id: hal-04621368**

**<https://hal.science/hal-04621368v1>**

Submitted on 24 Jun 2024

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## Landing on a small tropical island: Wide *in-situ* diversification of an urban-dwelling bat

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### ARTICLE INFO

#### Keywords:

Chiroptera  
Molossidae  
Reunion Island  
Population genetic structure  
Demographic history

### ABSTRACT

Island endemic bats are a considerable cause of conservation concerns, as islands are vulnerable ecosystems facing natural and anthropogenic threats such as growing urbanization. Here, we studied the Reunion free-tailed bat (*Mormopterus francoismoutoui*), an endemic species to Reunion Island that has adapted to urban settings. We investigated the evolutionary history of *Mormopterus* at a regional scale, as well as on Reunion Island sex-specific and seasonal patterns of genetic structure. We used an extensive spatio-temporal sampling including 1136 individuals from 18 roosts and three biological seasons (non-reproductive/winter, pregnancy/summer, and mating), with additional samples of *Mormopterus* species from neighbouring islands (*M. jugularis* from Madagascar and *M. acetabulosus* from Mauritius). Complementary information gathered from both microsatellite and mitochondrial markers revealed high genetic diversity but no signal of spatial genetic structure and weak evidence of female philopatry. Regional analysis suggests a single colonization event for *M. francoismoutoui*, dated around 175,000 years ago, and followed by *in-situ* diversification and the evolution of divergent ancestral lineages, which today form a large metapopulation. Population expansion was relatively ancient (55,000 years ago) and thus not linked to human colonization and the availability of human-constructed day-roost sites. Discordant structure between mitochondrial and microsatellite markers suggests the presence of yet-unknown mating sites, or the recent evolution of putative ecological adaptations. Our study illustrates the challenge of detailed genetic studies to provide critical insights to insular ecology and evolutionary history, and the importance of both mitochondrial and nuclear DNA in exploring *in-situ* diversification of an urban-dwelling bat, endemic to a small island.

### 1. Introduction

Bats are the most widely distributed terrestrial mammals on Earth but many species are facing population declines in response to

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<https://doi.org/10.1016/j.gecco.2024.e03030>

Received 31 July 2023; Received in revised form 3 June 2024; Accepted 4 June 2024

Available online 5 June 2024

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human activities and global change (Frick et al., 2020). Given their ability to fly long distances, bats are often the only mammals naturally colonizing islands. Indeed, more than half of known bat species live on islands, and 25 % of them are even endemic to islands (Jones et al., 2009). Island endemic bats represent 50 % of the world's threatened bats and are thus a major priority for conservation (Conenna et al., 2017; Jones et al., 2009). However, the ecology and evolution of island bats is often understudied, even though this information is critical to assess their conservation status and design management plans (Frick et al., 2020).

Once established on islands, bats face a diversity of selective pressures that influence their evolution and diversification. Indeed, due to reduced vertebrate species richness on islands with small surface areas, such insular ecosystems may lack predators or competitors and offer vacant ecological niches, which can favour the expansion of bat populations (Salinas-Ramos et al., 2020). Further, insular ecosystems often have limited food resources and can be subject to extreme events, such as volcanic activity, hurricanes or droughts, which can reduce bat populations (Calderón-Acevedo et al., 2021; Jones et al., 2001). Islands are vulnerable ecosystems that are highly susceptible to recent human-associated global changes, such as sea level rise and invasion by non-native species (Bellard et al., 2014). Because of their geographic isolation from other landmasses, island endemic bat species have limited dispersal opportunities outside islands and may be particularly exposed to the adverse effects of climate change (Festa et al., 2023). For example, a recent study on a Mediterranean island, the endemic Sardinian long-eared bat (*Plecotus sardus*, family Vespertilionidae) underwent a dramatic crash in population size, potentially due to recurrent wildfires and extreme temperatures (Ancillotto et al., 2021). Also, recent urbanization of island ecosystems could negatively affect the ecology of bat populations, although tolerance to anthropogenic activities has been described in some bat species (Jung and Threlfall, 2018; Russo and Ancillotto, 2015). Altogether, both historical and more contemporary factors can have significant implications for the long-term survival and conservation of island endemic bats.

Genetic analyses have become an essential tool for studying the ecology and evolution of island bats. However, because of complex histories including allopatric divergence, colonization, and hybridization on islands, studies have highlighted the need for the rigorous use of both mitochondrial and nuclear microsatellite markers (Kuo et al., 2015). Indeed, these markers have different evolutionary timescales, permitting the assessment of historical and contemporary population structure, as well as different inheriting modes, widely used to assess sex-specific life-history traits (Pinzari et al., 2023; Taki et al., 2021). Maternally-inherited mitochondrial DNA (mtDNA) can trace colonization histories and past divergence, and provide estimates of female site fidelity and dispersal, while polymorphic nuclear microsatellite DNA are good candidates to infer recent gene flow and can provide information for both sexes (Zhang and Hewitt, 2003).

By examining the genetic diversity and structure of island bat populations, we can infer drivers of gene flow, estimate population sizes, and understand demographic history. For example, genetic analysis of Miller's mastiff bat (*Molossus milleri*, family Molossidae) spanning Jamaica, Cuba, and the Cayman Islands suggested populations underwent bottlenecks, likely due to climate change in the early Pleistocene (Loureiro et al., 2020). Moreover, several studies have shown stronger genetic structure in the philopatric sex (mostly female), resulting from sex-biased dispersal behaviours in bat populations (Halczok et al., 2018; Jang et al., 2021; Moussy et al., 2013; Naidoo et al., 2016). In addition, bats often exhibit seasonal behaviours, in relation to change in food availability, habitat use, or reproductive cycle, and these factors may play critical roles in shaping genetic diversity patterns (Moussy et al., 2013). For example, in the little brown bat (*Myotis lucifugus*, family Vespertilionidae) and the northern long-eared bat (*M. septentrionalis*), individuals at swarming sites in autumn displayed a greater mtDNA genetic diversity than those at summering sites suggesting that swarming sites gather individuals from several summering populations (Johnson et al., 2015). Studies of genetic structure of island endemic bat species have mainly been carried out at the scale of multiple neighbouring islands (archipelago), but studies at a single-island scale, investigating sex or seasonal variation, are still limited (Ratrimomanarivo et al., 2009). Obtaining a comprehensive picture of the local genetic structure of island endemic bats requires a fine-scale sampling scheme, including material from multiple sites and from different seasonal periods, thus allowing the detection of subtle diversity patterns, especially on small islands.

The Reunion free-tailed bat (*Mormopterus francoismoutoui*, family Molossidae) is a tropical insectivorous bat endemic to Reunion Island. This volcanic *in-situ* formed island is located in the southwestern Indian Ocean (Mascarene Archipelago) and emerged from the sea about 3 million years ago (Cadet, 1980). Reunion is located 950 km east of Madagascar, which is home to the Peter's wrinkle lipped bat (*M. jugularis*), and 175 km southwest of Mauritius Island, which is home to the Natal free-tailed bat (*M. acetabulosus*). Reunion Island is shaped by a very mountainous landscape with the highest point at 3070 m (Piton des Neiges) and a still active volcano (Piton de La Fournaise at 2632 m). Although small in size (2512 km<sup>2</sup>), this very rugged landscape could represent several topographical barriers to bat dispersal.

*Mormopterus francoismoutoui* is broadly distributed on the island and roosts in natural settings, such as caves and cliff crevices. This bat species has adapted to anthropogenic settings and thrives in the lowland urbanized areas where numerous roost sites occur in buildings and under bridges (Augros et al., 2015; Goodman et al., 2008). However, little is known about how urbanization might modify life-history traits, population size, and genetic structure in this species. A recent longitudinal monitoring study of several roosts revealed highly dynamic roosting behaviours (Aguillon et al., 2023). Specifically, large female aggregations (up to 50,000 pregnant individuals) within a limited number of maternity roosts were observed synchronously during austral summer, which coincided with a female-biased sex-ratio at roost sites (Aguillon et al., 2023; Dietrich et al., 2015), and suggested female philopatry in this species. Moreover, towards the end of the austral summer, there was a decrease in roost size and a shift in sex-ratio (from female to male-biased), suggesting important seasonal sex-specific movements on the island. These details support the results of initial genetic work on this species, based on a limited number of samples ( $n = 31$ ), that suggested little genetic structure and no isolation by distance on the island (Goodman et al., 2008).

In order to investigate the evolutionary history and genetic structure of *M. francoismoutoui*, we used an extensive spatio-temporal field sampling and the complementary information of microsatellite and D-loop mtDNA markers. We first analysed the evolutionary history of this species, by examining its relationship to other regional *Mormopterus* bats on neighbouring islands (Madagascar and

Mauritius) in order to trace and date colonization events. We also explored the demographic history of *M. francoismoutoui* by testing the hypothesis of a population expansion linked to the recent urbanization of Reunion Island. We then analysed spatio-temporal patterns of genetic diversity and population structure and specifically tested female philopatry and seasonal changes in the genetic structure linked to the dynamic roosting behaviour of this species. We expected that genetic structure to be more prominent in females during summer due to important female aggregation observed during this period, and lower during the mating season because of mixing of individuals within roosts.

## 2. Material and methods

### 2.1. Field sampling

Samples were collected during different seasons at 18 roosts (coded with a 3-letter code) across Reunion Island (Fig. 1). Details of the longitudinal sampling of roosts can be found in Table 1. Six of these roosts (AOM, CIT, PBV, RAC, STJ, and TM5) were sampled once during the study, because of opportunistic sampling. The remaining roosts were sampled multiple times between October 2018 and March 2020. We collected repeat samples from eight roosts (ESA, MON, PSR, RBL, RPQ, STM, TGI, and VSP) during three biological seasons: (i) the pregnancy period (austral summer) from late October to early December 2018), (ii) the non-reproductive period (austral winter) in June and July 2019, and (iii) the putative mating period in March 2019 and 2020 (Aguillon et al., 2023, Table 1). Three roosts (EGI, RES, and TBA) were quasi-empty (only a few remaining individuals) during the non-reproductive/winter period, explaining the lack of data during this season and one roost (TRI), was not sampled in March because of logistic constraints (Table 1).

We captured bats during dusk emergence, mainly using harp traps (Faunatech Ausbat) and Japanese monofilament mist nets (Ecotone) set close to the roost exit as described in Aguillon et al. (2023). Because of difficulties in installing harp traps or mist nets at the exit of some roosts, we sometimes employed a butterfly net on an elongated pole to catch bats, by carefully approaching resting individuals during the day. After capture, bats were immediately hydrated with water using a sterile syringe and placed in a clean individual bag close to a source of warmth (hot water bottle), and processed at the capture site. We visually ascertained the sex and age of each individual. Age was determined by examining the epiphysis fusion in finger articulations that are not ossified for juveniles.

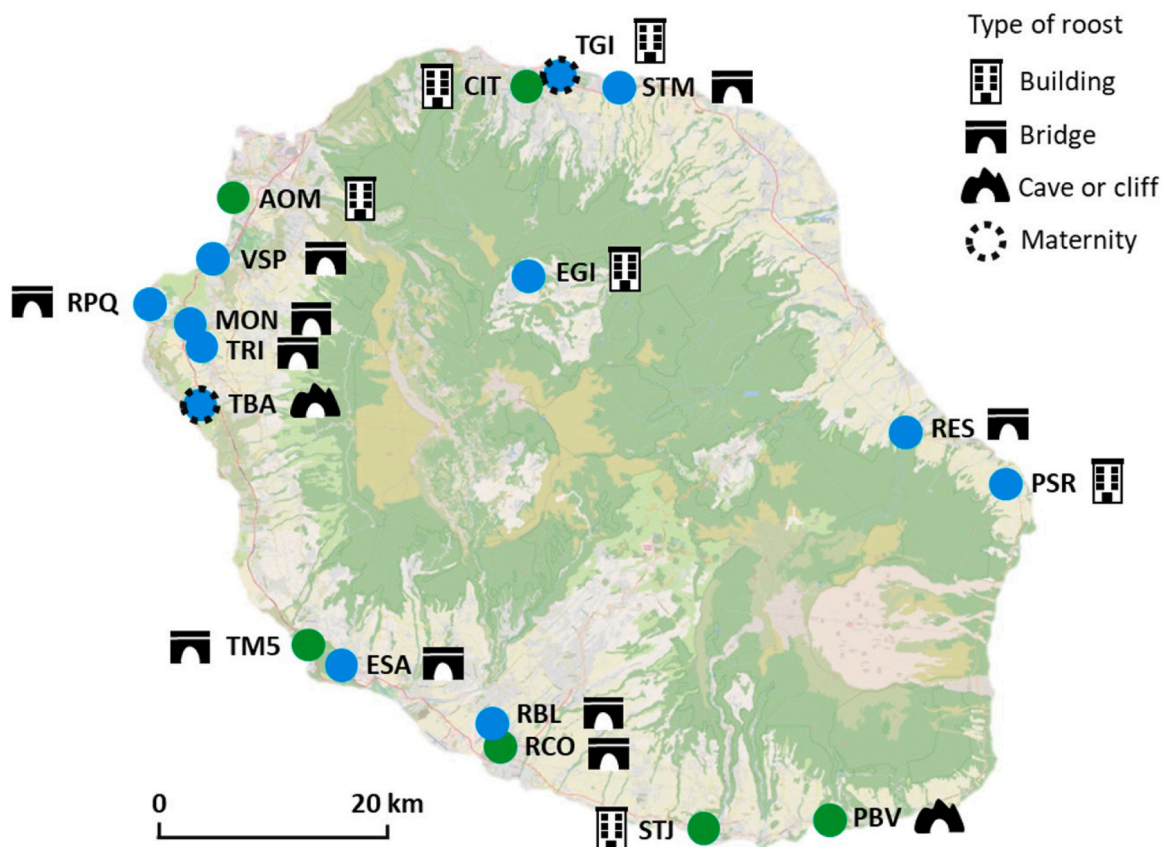


Fig. 1. Sampling sites of *Mormopterus francoismoutoui* on Reunion Island. Details on roost sites are presented in Table 1. The 12 roosts in blue were monitored regularly over two years while the six in green were sampled only once. The green colour indicating forested areas and the pale one indicating urbanized areas. Modified from Aguillon et al. (2023).

**Table 1**

Summary of mitochondrial and microsatellite data used in this study for *Mormopterus francoismoutoui*. For each roost, the first number indicates the total number of mitochondrial sequences, while the number of samples with microsatellites is indicated below, and details of females and males are in parentheses. The roost codes refer to those in Fig. 1. Maternity roosts are indicated with an asterisk (\*).

Roost	Habitat	Pregnancy 2018 (summer)	Non-reproductive 2019 (winter)	Mating 2019 – 2020 (March)	Opportunistic sampling		Total
					February 2019	October 2018	
AOM	Building			14 (8/6)			14
				30 (15/15)			30
CIT	Building	15 (0/15)					15
		29 (0/29)					29
EGI	Building	26 (8/18)		15 (8/7)			41
		34 (11/23)		30 (15/15)			64
ESA	Bridge	15 (9/6)	16 (9/7)	15 (8/7)			46
		36 (19/16)	34 (18/16)	26 (19/7)			96
MON	Bridge	18 (3/15)	15 (7/8)	15 (8/7)			48
		33 (3/30)	30 (15/15)	25 (16/9)			88
PBV	Cliff				11 (4/7)		11
PSR	Building	17 (6/11)	15 (2/13)	15 (7/8)	11 (4/7)		11
		34 (12/22)	34 (2/32)	28 (7/21)			47
RAC	Bridge					16 (4/12)	16
						14 (4/10)	14
RBL	Bridge	16 (8/8)	15 (7/8)	15 (7/8)			46
		31 (13/18)	34 (11/23)	28 (20/8)			93
RES	Bridge	15 (7/8)		15 (7/8)			30
		32 (15/17)		30 (7/23)			62
RPQ	Bridge	15 (6/9)	15 (8/7)	15 (8/7)			45
		37 (19/18)	33 (11/22)	30 (15/15)			100
STJ	Building		15 (8/7)				15
			30 (13/17)				30
STM	Bridge	15 (7/8)	13 (3/10)	15 (3/12)			43
		34 (17/17)	13 (3/10)	23 (3/20)			70
TBA*	Cave	31 (29/2)		15 (7/8)			46
		34 (31/3)		29 (22/7)			63
TGI*	Building	19 (12/7)	15 (7/8)	15 (7/8)			49
		38 (20/18)	36 (13/23)	29 (15/14)			103
TM5	Bridge				15 (7/8)		15
					28 (13/15)		28
TRI	Bridge	15 (1/14)	15 (8/7)				30
		29 (1/28)	29 (9/20)				58
VSP	Bridge	16 (7/9)	15 (7/8)	15 (7/8)			46
		38 (18/20)	35 (7/28)	28 (15/13)			101
Total		233	149	179	26	16	603
		439	308	336	39	14	1136

Wing punch samples (~ 2 mm) were taken on each wing, and stored dried in a cool box in the field before being transferred at -80°C at the laboratory. Finally, each bat was tattooed on the right propatagium with an individual alphanumeric code (Markotter et al., 2023): we first disinfected the propatagium with a cotton swab soaked with diluted ethanol and then applied a special tissue oil (AIMSTM) so that the dermatograph can glide over the propatagium and prevent any tearing. After completing the tattooing (a few seconds), the excess ink was absorbed and a second disinfection step was performed with a new cotton swab. After all manipulations, bats were released at the capture site.

Handling of bats was performed using personal protective equipment and gloves were disinfected between each individual bat and changed regularly, and all the equipment was disinfected between sites (see protocol in Aguillon et al., 2023 for more details). Bat capture and manipulation techniques were evaluated by the ethic committee of Reunion Island, approved by the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation (APAFIS#10140–2017030119531267), and conducted under a permit (DEAL/SEB/UBIO/2018–09) delivered by the Direction de l'Environnement, de l'Aménagement et du Logement (DEAL) of Reunion Island.

Samples (organ pool: spleen, lung, kidney) of *M. acetabulosus* from Mauritius (2012) and *M. jugularis* from Madagascar (2012–2013) were obtained from vouchered individual bats collected associated with zoonotic disease studies (Gomard et al., 2016; Joffrin et al., 2020; Mélade et al., 2016), and from three different roost sites on each island. Mauritius samples were collected under a memorandum of agreement for the supply of biological material by Government of Mauritius (delivered by the National Park and Conservation Service for authorization of Mauritius), signed on 17 December, 2010. Madagascar samples were collected under the permits delivered by the Direction du Système des Aires Protégées and Direction Générale de l'Environnement et des Forêts: no. 350/10/MEF/SG/DGF/DCB.SAP/SCB, no. 032/12/MEF/SG/DGF/DCB.SAP/SCBSE, no. 067/12/MEF/SG/DGF/DCB.SAP/SCBSE, no. 194/12/MEF/SG/DGF/DCB.SAP/SCB, no. 283/11/MEF/SG/DGF/DCB.SAP/SCB, no. 077/12/MEF/SG/DGF/DCB.SAP/SCBSE, no.

238/14/MEEF/SG/DGF/DCB.SAP/SCB, and no. 268/14/MEEF/SG/DGF/DCB.SAP/SCB.

## 2.2. DNA extraction, PCR, sequencing, and genotyping

Wing punch samples of Reunion free-tailed bats were processed with the Cador Pathogen 96 Qiacube HT kit (Qiagen, Hilden, Germany). Samples were lysed before DNA extraction, in 180  $\mu$ L of ATL buffer and 20  $\mu$ L of Proteinase K at 56°C during 1h30. Then, the buffer VXL mixture was prepared replacing Proteinase K by sterile water. Total nucleic acids were extracted in an automated extractor Qiacube with slight modifications of the Q Protocol, including 350  $\mu$ L of ACB, 100  $\mu$ L of AVE, and 30  $\mu$ L of TopElute. Nucleid acids from Mauritius and Madagascar samples were already available (protocols of extraction described in [Gomard et al., 2016](#); [Joffrin et al., 2020](#); [Mélade et al., 2016](#)).

Subsequently, a fragment of the hypervariable domains I and II of the D-loop region (with no repeats) was amplified by PCR (expected: 896 bp) in a 20  $\mu$ L reaction mixture containing 2  $\mu$ L of DNA, 10  $\mu$ L of GoTaq® Green Master Mix 2X (Promega, Madison, Wisconsin, United States), 1  $\mu$ L of each newly designed primers at 10  $\mu$ M D-loop-F (5'-CAAGACTTCAGGAAGAAGCTAACCA-3') and D-loop-R-Lg (5'-TATTCGTATGTATGTCCTGTAACCA-3'). PCR program included an initial denaturation step (95°C for 2 min), followed by 35 cycles of denaturation (95°C for 30 sec), annealing (50°C for 30 sec), elongation (72°C for 1 min 30 sec), and a final elongation step (72°C for 7 min). PCR products were Sanger-sequenced by the GENOSCREEN platform (Lille, France). The D-loop chromatograms were visually checked using Geneious 9.1.8 (Biomatters Ltd, Auckland, New Zealand) and sequences were aligned using CLC Sequence Viewer 7.6.1 (Qiagen Aarhus A/S, Aarhus, Denmark). DNA extracted were genotyped by GENOSCREEN, using a panel of 12 previously described microsatellite markers, according to the protocol and primers of [Dietrich et al. \(2019\)](#).

## 2.3. Genetic relationships among regional *Mormopterus*

In order to investigate genetic relationships among the three regional *Mormopterus* species, we reconstructed a Bayesian tree based on Yule model ([Yule, 1925](#)), based on a dataset including 30 D-loop sequences (alignment of 811 bp) for each island, and using the island as a trait to resolve the spatial origin of the nodes using BEAST v.2.6.4 ([Bouckaert et al., 2019](#)). We used a HKY model ([Hasegawa et al., 1985](#)) with invariant and gamma distribution according to the best substitution model on AIC criterion using JModelTest ([Darriba and Posada, 2016](#)). We used an uncorrelated lognormal relaxed molecular clock ([Drummond et al., 2006](#)) of 0.2 substitutions/site/million years ([Petit et al., 1999](#)), with a 100 million chain length and sampling every  $10^4$  steps, and a burning of 10 %. We ran three analyses and combined log outputs (removing 10 % of burning for each output) using LogCombiner v2.6.4 ([Rambaut and Drummond, 2015](#)). Traces of Markov Chain Monte Carlo (MCMC) were checked for convergence of the posterior estimates of the effective sample size (ESS) to the likelihood using Tracer v1.7.1 ([Rambaut et al., 2018](#)). We combined tree outputs (removing 10 % of burning for each output) to obtain a consensus tree using LogCombiner v2.6.4 ([Rambaut and Drummond, 2015](#)) and then TreeAnnotator v2.6.4 ([Rambaut and Drummond, 2019](#)). We also investigated the haplotype number and genetic distances between sequences from each island using DnaSP v6.12.03 ([Rozas et al., 2017](#)).

Microsatellite analysis of regional samples was performed using STRUCTURE v2.3 ([Pritchard et al., 2010](#)), with LocPrior ([Hubisz et al., 2009](#)) and no admixture model, using uncorrelated allele frequencies among group. We ran 10 replicates with a burn-in of  $10^6$  steps,  $10^6$  recorded steps for the MCMC, and K from 1 to 5 groups (corresponding to the number of species, plus two). We apply the Evanno method ([Evanno et al., 2005](#)) to estimate the best K. We also calculated the global number of genotypes for each island using GenAlex 6.503 ([Smouse and Peakall, 2012](#)).

## 2.4. Analyses of *M. francoismoutoui*

Using mitochondrial D-loop data, genetic diversity indices, including haplotype number, haplotype diversity (Hd), and nucleotide diversity ( $\pi$ ), were measured at the roost-level using DnaSP v6.12.03 ([Rozas et al., 2017](#)), for the entire Reunion Island dataset and then separately for each sex and season. Differences among roosts, sexes, and seasons were tested using analyses of variance (ANOVA) in RStudio 1.4.1106 ([Rstudio Team, 2021](#)). Spatial structure was assessed by calculating genetic distances ( $\Phi_{st}$ ) among roosts for the entire Reunion Island dataset and then separately for each sex and season using Arlequin 3.5.2.2 ([Excoffier and Lischer, 2015](#)). The significance of multiple tests was corrected with the Holm method ([Holm, 1979](#)) using RStudio. To test for temporal differences in the spatial structure,  $\Phi_{st}$  values were compared among seasons using ANOVAs and Tukey's post-hoc tests. Subsequently, to test for the presence of isolation by distance (IBD), we performed a Mantel test with 1000 permutations using Arlequin and calculated the correlation between genetic and geographic distances. IBD was first tested for the complete Reunion Island dataset, and then separately for each sex and season. Finally, we also used an AMOVA test (analysis of molecular variance) in Arlequin and defined "population" as individuals from a single roost to test for a roost-associated genetic structure in the Reunion population. The significance of this test was assessed by 1000 permutations of individuals among roosts.

We determined the most appropriate nucleotide substitution model of Reunion Island D-loop sequences based on AIC criterion ([Akaike, 1974](#)) using JModelTest v2.1.10 ([Darriba and Posada, 2016](#)). Then, using all Reunion D-loop sequences (alignment of 985 bp), we constructed a Bayesian tree using BEAST v.2.6.4 ([Bouckaert et al., 2019](#)) with TN93 site model with invariant and gamma distribution ([Tamura and Nei, 1993](#)) including roost location as a trait. We used the same parameters in BEAST as described above for the regional analysis.

To investigate the demographic history of *M. francoismoutoui*, we calculated the expected frequency distributions of pairwise differences between D-loop sequences (mismatch distribution) in DnaSP v6.12.03 ([Rozas et al., 2017](#)). We also used a neutrality test

with 1000 simulated samples using Arlequin v.3.5.2.2 (Excoffier and Lischer, 2015) based on Harpending's raggedness index ( $r$ , Harpending, 1994), Fu's  $F_s$  (Fu, 1997), and the sum of squared deviations (SSD) between observed and expected mismatch indices. We then calculated the expansion time using the formula  $t = \frac{\tau}{2\mu k}$  from Rogers and Harpending (1992) where  $\tau$  is the expansion date calculated with the mismatch distribution,  $\mu$  is the mutation rate, and  $k$  is the average number of nucleotide sites per haplotype. Global population size change through time was reconstructed using a coalescent Bayesian skyline model (CBS, Drummond et al., 2005) with the same parameters as described above in BEAST v.2.6.4 (Bouckaert et al., 2019). We performed BEAST analyses changing the dimension group parameter from 3 to 10 groups, according to the results of the Yule Bayesian tree. We ran three analyses for each group and followed the same method described for Yule Bayesian tree and choose the best  $k$  according to the higher ESS.

For the microsatellite data, genotype determination was performed using GeneMapper 6 (ThermoFisher). We tested the dataset for scoring errors, out of range allele, and null alleles for each roost using Microchecker v.2.2.3 (Van Oosterhout et al., 2004). Using GenAlex 6.503 (Smouse and Peakall, 2012), the global number of genotypes was calculated, and for each roost, deviations from Hardy-Weinberg equilibrium were tested for each locus. We then assessed the number of alleles per locus ( $N_a$ ), the observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosity, and the fixation index ( $F_{is}$ ) per locus using GenAlex 6.503 (Smouse and Peakall, 2012). A permutation test with 1000 permutations was used to perform linkage disequilibrium analysis between each pair of loci using Genetix v.4.05.2 (Belkhir et al., 2004). We used Fstat v.2.9.4 (Goudet, 2003) to calculate the inbreeding coefficient ( $F_{IS}$ ) within each roost (Weir and Cockerham, 1984) and tested the significance by randomizing alleles among individuals within roosts (5000 permutations). We estimated the observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosity in each roost, first using the whole dataset, and subsequently estimated these two parameters for each sex and season, testing for sex and temporal differences using an ANOVA in RStudio. Effective population size ( $N_e$ ) was estimated for the global population because of a lack of genetic structure (see results) with the linkage-disequilibrium model and assuming random mating using NeEstimator v2.1 (Do et al., 2013). For this, we used a minimum allele frequency of 0.05 and 0.02 to calculate upper and lower limits of  $N_e$ .

Genetic distances ( $F_{st}$ ) between roosts were calculated globally, and then separately for each sex and season, using Arlequin 3.5.2.2 (Excoffier and Lischer, 2015). The significance of multiple tests was corrected with Holm method using RStudio. To test for temporal differences in the spatial structure,  $F_{st}$  values were compared among seasons using ANOVAs and Tukey's post-hoc tests. To check for IBD, we performed Mantel tests (1000 permutations) using Arlequin 3.5.2.2 (Excoffier and Lischer, 2015), as for mitochondrial data.

To test for a roost-associated genetic structure in the population, we used an AMOVA in Arlequin, as described for mtDNA. Then, we employed STRUCTURE v2.3 (Pritchard et al., 2010), and performed two analyses with and without the LocPrior model, which uses location to test for a weak signal of population structure (Hubisz et al., 2009). We used the admixture model with correlated allele frequencies among groups, and 10 replicate runs were performed with a burn-in of  $10^6$  steps and  $10^6$  recorded steps for the Monte Carlo Markov Chain (MCMC). We ran  $K$  from 1 to 19 groups (corresponding to the number of studied roosts, plus one) and applied the Evanno method (Evanno et al., 2005) to estimate the best  $K$ . To determine the optimal number of genetic clusters, we performed a  $k$ -means clustering analysis, tested  $K$  from 2 to 19 over 26 indices according to the "majority rule", using the *NbClust* package (Charrad et al., 2014) in RStudio. We also performed a principal coordinate analysis (PCoA) using GenAlex 6.503 to visualize possible genetic clusters.

Finally, to compare mitochondrial and nuclear results, we overlaid the genetic clusters identified using microsatellite markers and clades depicted by the BEAST phylogeny obtained with the D-loop sequences. Based on the dimension group parameter estimated by the best skyline converging model in BEAST, we defined five mtDNA clusters according to posterior probabilities  $> 0.99$  (see results). We used a generalized linear model (GLM) in Rstudio, including the nuclear clusters as the numeric response variable (value of PC1 from PCoA) and the mtDNA genetic clusters as the explanatory response.

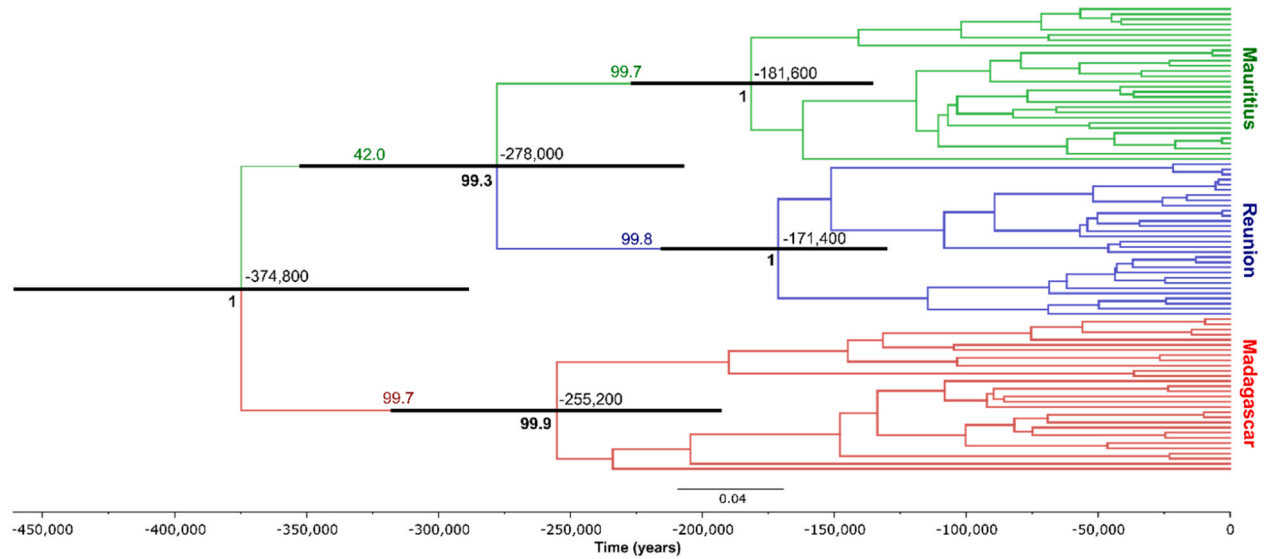
### 3. Results

#### 3.1. Data quality

Altogether, we obtained good quality D-loop sequences for 603 *M. francoismoutoui* and 30 sequences for each additional *Mormopterus* species (Mauritius and Madagascar). We genotyped 1136 individuals of *M. francoismoutoui* using the 12 microsatellite loci, and 30 individuals from Mauritius (*M. acetabulosus*) and Madagascar (*M. jugularis*). One locus (MF\_loc11) was removed because of a high percentage of uninterpretable weak signals in the Reunion Island dataset ( $\sim 41\%$  of individuals). Also, we removed individuals from Reunion for which at least six loci were not genotyped ( $n = 22$ ), leading to a final microsatellite data set containing 3.7% of missing alleles. In the Reunion dataset, a majority of loci significantly deviated from Hardy-Weinberg equilibrium, especially MF\_Loc03, MF\_Loc04, MF\_Loc05, and MF\_Loc15. Null alleles were detected in several loci, especially in MF\_Loc04 and MF\_Loc015. Three loci were implicated in several linkage disequilibria: MF\_Loc03, MF\_Loc05, and MF\_Loc28. Global  $F_{is}$  was low (mean: 0.022), and the expected heterozygosity ( $H_e$ ) per locus ranged from 0.481 to 0.881, and the observed heterozygosity ( $H_o$ ) ranged from 0.479 to 0.874 (Table S1). We also found important number of alleles, with an average of 14.8 per locus.

#### 3.2. Genetic relationship among regional *Mormopterus*

Mitochondrial DNA revealed a high number of haplotypes on each island: 30 for Madagascar, 29 for Mauritius, and 26 for Reunion Island (out of 30 sequences per island). Using the microsatellite markers, all genotypes were different. The time calibrated Bayesian phylogeny strongly supported three genetic clades corresponding to each bat species, thus confirming their monophyly (Fig. 2). The



**Fig. 2.** Bayesian chronogram inferred from mitochondrial data (D-loop) for three *Mormopterus* species occurring on southwestern Indian Ocean islands, using BEAST software. The Yule model was used with HKY (I+G) substitution model and relaxed molecular clock of 0.2 substitution/site/million years. Scale bar at the bottom represents divergence time in years from past (left) to recent (right) time. Colours of the branches depict sample locality: Mauritius (*M. acetabulosus*) in green, Reunion (*M. francoismoutoui*) in blue, and Madagascar (*M. jugularis*) in red. Posterior probabilities values of main nodes are indicated below branches in bold black. Black horizontal bars represent the 95 % HPD of node ages with main age values indicated above the bar. Posterior probabilities associated with island origin trait are indicated above branches and coloured accordingly.

species from Mauritius and Reunion were separated by a genetic distance of 5.0 %. Species from Mauritius and Madagascar, and then Reunion and Madagascar were separated by a divergence of 6.1 % and 6.4 %, respectively. Interestingly, STRUCTURE analyses identified two genetic clusters, separating the Malagasy species in one cluster, and the two species on Reunion and Mauritius in a second cluster (Fig. S1). Surprisingly, when  $K = 3$ , only 20 % of the runs assigned each bat species to a different cluster. Indeed, most (80 %) of the runs for  $K = 3$  grouped Reunion and Mauritius bats in a single genetic cluster, while Malagasy bats were composed of two clusters with a few individuals having mixed genotypes. The BEAST analysis showed that the inferred TMRCAs for the three *Mormopterus* species was 374,800 years ago (HPD: 287,500 – 467,000) and the divergence of *M. francoismoutoui* on Reunion from *M. acetabulosus* on Mauritius was dated at 278,000 years ago (HPD: 209,100 – 355,100). The diversification of *M. jugularis* on Madagascar was dated at 255,200 years ago (HPD: 192,800 – 319,200) and occurred later for the species on Mauritius (181,600; HPD: 135,800 – 232,500) and on Reunion (171,400; HPD: 129,600 – 218,000).

### 3.3. Genetic diversity of *Mormopterus francoismoutoui*

Mitochondrial DNA and microsatellite markers revealed a high genetic diversity within the population of *M. francoismoutoui*. For mtDNA, 410 haplotypes (out of 603 sequences) were identified with an average haplotype diversity (Hd) of 0.998 (Table 2) and a global nucleotide diversity ( $\pi$ ) of 0.0284. Using the microsatellite markers, we identified 1135 genotypes (in 1136 individuals), and only two individuals (both captured in the TGI roost) shared the same genotype. The average observed (Ho) and expected (He) heterozygosities were high (Ho = 0.778 ± 0.009, He = 0.797 ± 0.008) and there was no evidence of inbreeding between individuals occupying the same roosts, as none of the  $F_{IS}$  values were significantly different from zero (Table 2). For both the mtDNA (Hd and  $\pi$ ) and nuclear (Ho and He) data, there was no significant differences in the level of genetic diversity between roosts, nor between sexes and seasons (ANOVA, all  $p > 0.05$ , Table S2 and S3 for D-loop, Table S and S5 for microsatellites).

### 3.4. Genetic structure within the Reunion Island population

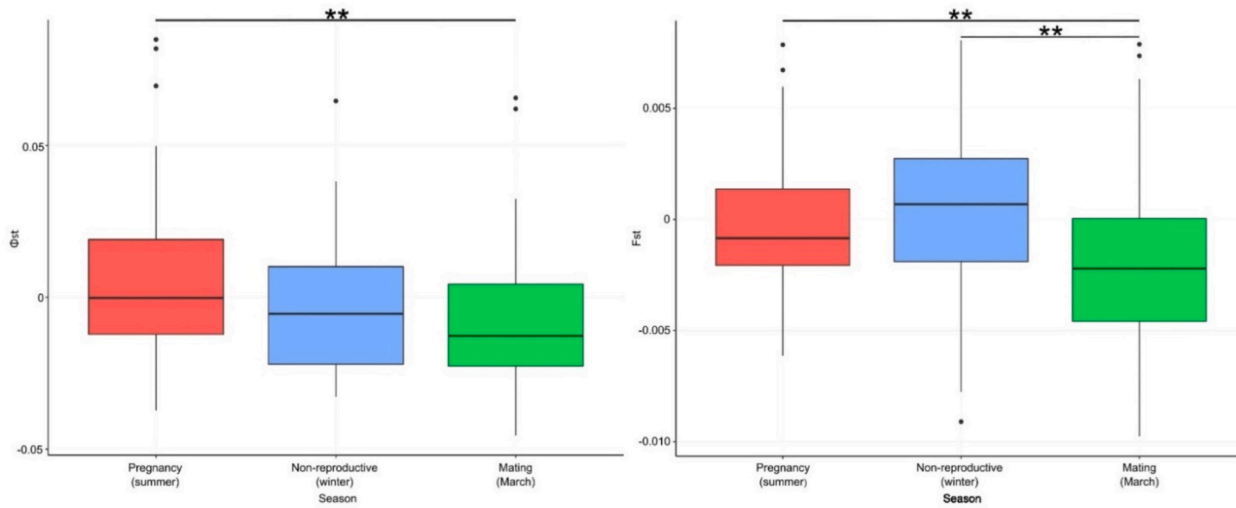
Globally, for both mitochondrial and nuclear markers, no isolation by distance ( $r_{mtDNA} = 0.006$ ,  $p = 0.46$ ;  $r_{nuclear} = 0.11$ ,  $p = 0.14$ ) was detected, nor significant pairwise differentiation among roosts (Fst and  $\Phi_{st}$ , Table S6). The same results were found when analyses were performed separately for each sex and season (Table S7 for IBD results). However, we identified differences in  $\Phi_{st}$  and Fst values among seasons (ANOVA,  $p = 0.003$  and  $p = 0.001$ , respectively). The mitochondrial marker showed higher  $\Phi_{st}$  values in the pregnancy period compared to those of the mating period ( $p = 0.002$ ), while for microsatellites, Fst values during both the pregnancy ( $p = 0.008$ ) and the non-reproductive period ( $p = 0.003$ ) were higher compared to those during the mating period (Fig. 3). AMOVA results with both markers showed that genetic variation (100 % for mtDNA and 99.99 % for microsatellites) was largely due to differences among individuals within roosts. Results from the STRUCTURE analyses revealed no genetic clustering (no conclusive Evanno result, Fig. S2), while the k-means clustering analysis evaluated the optimal number to three genetic clusters (Fig. S3). This result was corroborated by the PCoA but with only a small genetic variation explained by the two first axes (PC1: 4.73 % and PC2: 3.31 %, Fig. 4). Interestingly, these three clusters included a mixture of bats from the different roost sites.

The Bayesian tree built with the Yule model of speciation, revealed several well-supported genetic clusters (posterior probability > 0.95, Fig. 5). These clusters included bats from all roosts, but the best reconstruction of ancestral nodes failed to predict the roost origin

**Table 2**

Global genetic diversity indices of *Mormopterus francoismoutoui*, calculated with mitochondrial DNA (D-loop) and 11 microsatellite markers. For roosts details see Fig. 1 and Table 1. N: number of individuals, Hd: haplotype diversity,  $\pi$ : nucleotide diversity, Ho: observed heterozygosity ( $\pm$  standard error), He: expected heterozygosity ( $\pm$  standard error), and Fis: inbreeding coefficient (none are significantly different from zero).

Roost	Mitochondrial data			Microsatellite data			
	N	Hd	$\pi$	N	Ho	He	Fis
AOM	14	0.989	0.0264	30	0.762 ± 0.035	0.793 ± 0.037	0.058
CIT	15	1.000	0.0304	29	0.787 ± 0.035	0.791 ± 0.029	0.022
EGI	41	0.999	0.0287	64	0.766 ± 0.040	0.807 ± 0.030	0.060
ESA	46	0.998	0.0272	96	0.776 ± 0.033	0.806 ± 0.033	0.044
MON	48	1.000	0.0291	88	0.797 ± 0.031	0.800 ± 0.035	0.009
PBV	11	1.000	0.0342	11	0.759 ± 0.032	0.762 ± 0.030	0.053
PSR	47	0.997	0.0297	96	0.786 ± 0.037	0.797 ± 0.036	0.018
RAC	16	1.000	0.0309	14	0.836 ± 0.070	0.772 ± 0.036	-0.045
RBL	46	0.999	0.0282	93	0.763 ± 0.028	0.802 ± 0.035	0.055
RES	30	1.000	0.0284	62	0.744 ± 0.040	0.802 ± 0.033	0.081
RPQ	45	0.997	0.0297	100	0.788 ± 0.031	0.805 ± 0.034	0.026
STJ	15	1.000	0.0278	30	0.782 ± 0.029	0.796 ± 0.034	0.036
STM	43	1.000	0.0289	70	0.797 ± 0.037	0.809 ± 0.032	0.022
TBA	46	0.998	0.0290	63	0.783 ± 0.040	0.801 ± 0.034	0.032
TGI	49	0.997	0.0308	103	0.766 ± 0.044	0.806 ± 0.035	0.054
TMS	15	1.000	0.0305	28	0.752 ± 0.044	0.791 ± 0.035	0.070
TRI	30	1.000	0.0299	58	0.781 ± 0.038	0.801 ± 0.031	0.034
VSP	46	0.999	0.0290	101	0.779 ± 0.035	0.804 ± 0.034	0.036
TOTAL	603	0.998	0.0284	1136	0.778 ± 0.009	0.797 ± 0.008	0.034



**Fig. 3.** Temporal differences in  $\Phi_{st}$  (mtDNA) and  $F_{st}$  (microsatellites) values between seasons in *Mormopterus francoismoutoui*. \*\*  $p < 0.01$  (Tuckey's post-hoc tests).

of individuals. In addition, the best skyline converging model gave the same tree topology and indicated the occurrence of five genetic groups (highlighted in bold in Fig. 5), followed by models with six and 10 groups with a close likelihood ESS (Table S8). These five clusters were separated by a maximum of 3.3 % of divergence. When overlaying the three genetic clusters identified with microsatellite markers on the five clusters identified with the skyline BEAST phylogeny, no significant results were found revealing that genetic clusters from both markers are different (GLM,  $\chi^2_4 = 0.03$ ,  $p = 0.72$ ).

### 3.5. Population demographic history of *Mormopterus francoismoutoui*

Estimations of effective genetic population size with the lowest allele frequency at 0.05 and 0.02 led to infinite estimate of  $N_e$  (95 %  $CI_{0.05}$ : 7783.5 - Infinite and 95 %  $CI_{0.02}$ : 22347.5 - Infinite). Moreover, the mismatch distribution under the expansion model showed a clear signal of demographic expansion with a multimodal distribution with three peaks (Fig. 6a). Raggedness index and Sum of Square Deviation had non-significant values under the model of demographic expansion ( $r = 0.0005$ ,  $p = 1$ ;  $SSD = 0.002$ ,  $p = 0.71$ ). The result of Fu's  $F_s$  showed significant negative values indicating an excess of rare haplotypes compared to expected values under neutral model ( $F_s = -23.33$ ,  $p = 0.04$ ). All these results suggested an ancient demographic expansion in the population. Based on information calculated from the mismatch distribution test, we determined the expansion time  $t = \frac{\tau}{2\mu k}$  with  $\tau = 29.156$ ,  $\mu = 0.2$  substitutions/site/million years (Petit et al., 1999) and  $k = 811$  bp. We found an expansion time  $t = 89,876$  years. This was coherent with the Bayesian skyline plot showing a stable population size starting from 175,000 years and up to 90,000 years. Then, a slight increase in population size began and was followed by a drastic expansion around 55,000 years, lasting about 10,000 years. In the last 45,000 years, the population size still increased but at a slower rate. The end of the curve suggested a recent stabilization or a decrease in population size occurring about 500 years ago (Fig. 6b).

## 4. Discussion

Despite living on the small oceanic island of Reunion (2512 km<sup>2</sup>), our study revealed an extreme high genetic diversity in *Mormopterus francoismoutoui*, with 68 % of unique D-loop haplotypes and 99.9 % of unique microsatellite genotypes. Only one microsatellite genotype was found to be shared by two individuals, which were both captured in the same roost (TGI), suggesting a kinship link between them. Our results support those of Goodman et al. (2008) and are similar to previous studies reported in much bigger islands, such as in *M. jugularis* on Madagascar (587,041 km<sup>2</sup>, Rattrimomanarivo et al., 2009) and *Myotis punicus* on Corsica (8722 km<sup>2</sup>) and Sardinia (24,090 km<sup>2</sup>, Biollaz et al., 2010). High levels of genetic diversity in island endemic bats can be explained by large population size (Frankham, 1996), which is supported by our results providing infinite large effective population size estimates for *Mormopterus francoismoutoui*. Such results are consistent with the fact that Molossidae bats form large and dense colonies (e.g. *Tadarida brasiliensis* of the family Molossidae, McCracken and Wilkinson, 2000) and corroborate our field observations of numerous roosts across Reunion Island, with an estimated current population size probably far over 120,000 individuals (Aguillon et al., 2023).

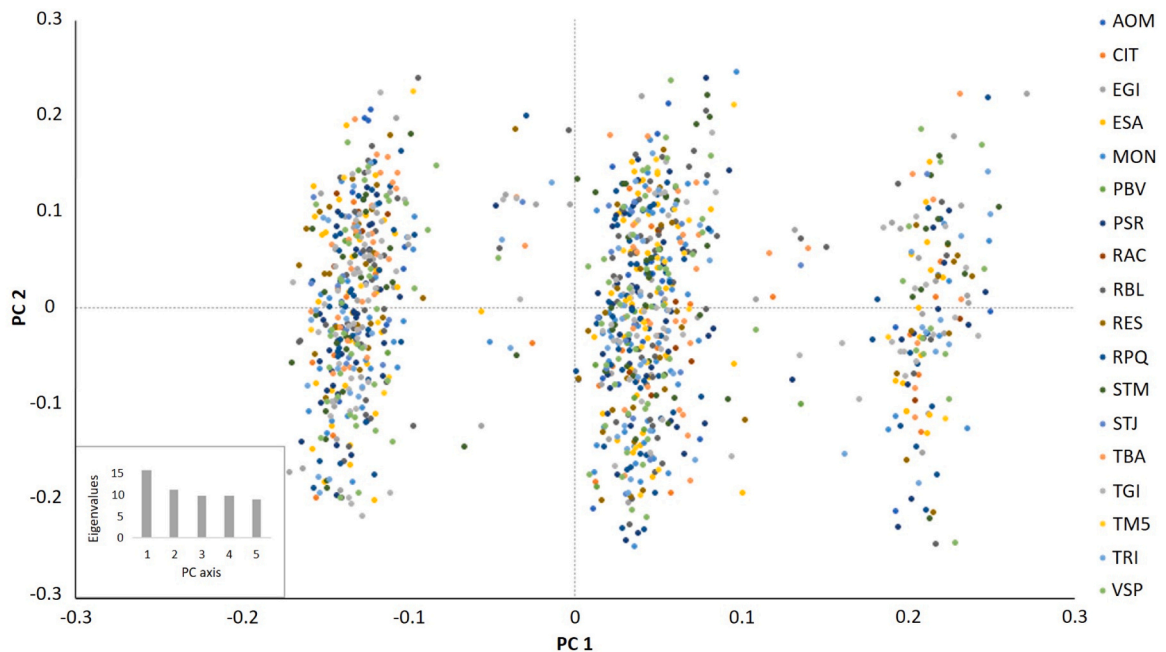
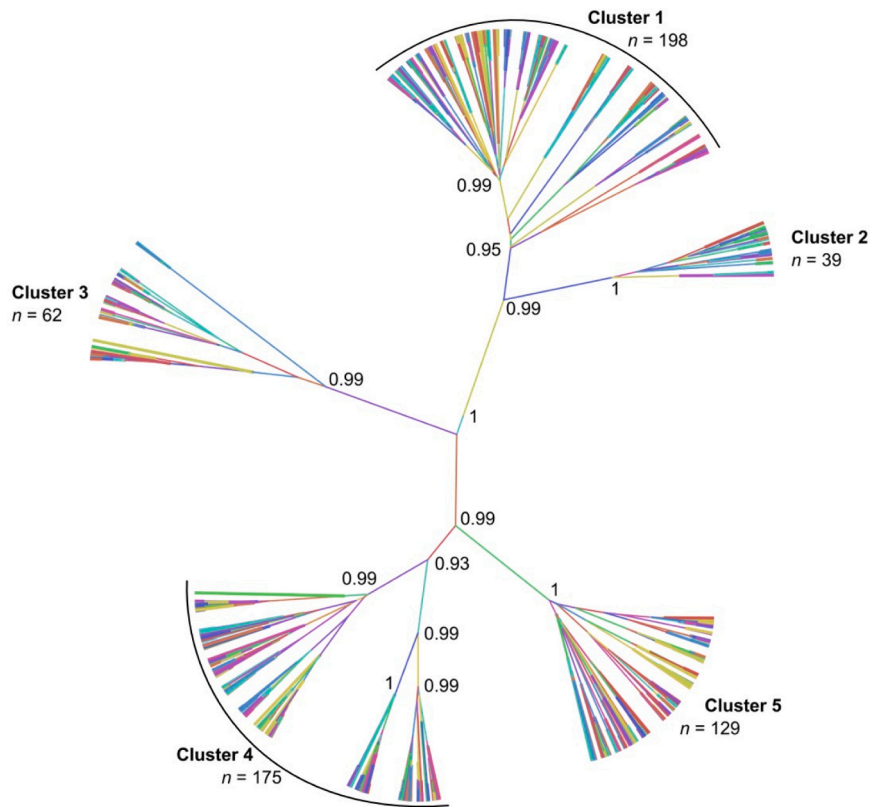


Fig. 4. Principal Coordinates Analysis (PCoA) of microsatellite markers for *Mormopterus francoismoutoui*. Roosts are indicated by different colours and the eigenvalues of the first five principal component (PC) are shown.

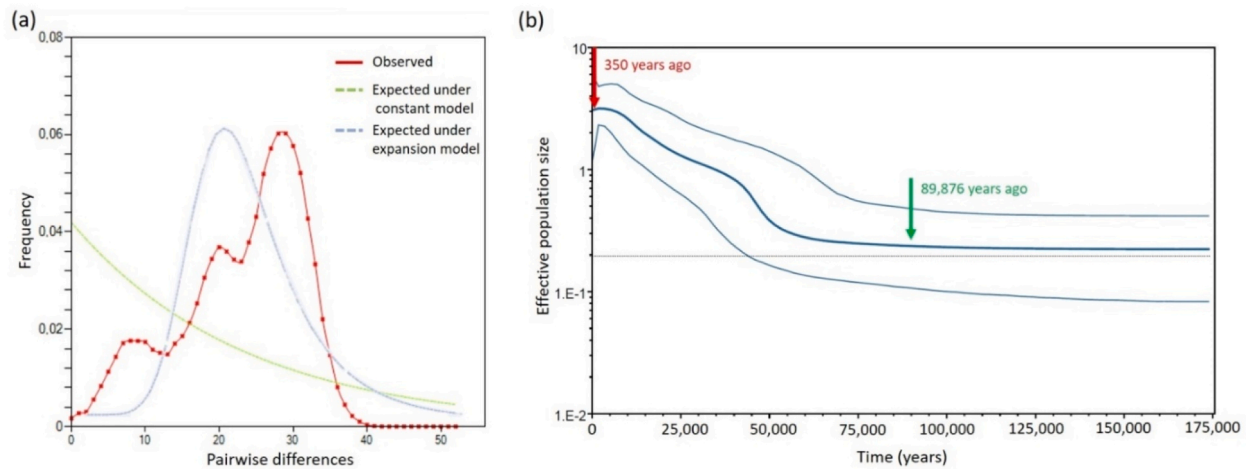


**Fig. 5.** Bayesian tree topology inferred from mitochondrial data (D-loop) for the 18 roosts of *Mormopterus francoismoutoui*. The Yule speciation model was used with TN93 (I+G) substitution model and a relaxed molecular clock of 0.2 substitution/site/million years. Colours correspond to different roost sites and line weigh represents the probability of roost location. Posterior probability values higher than 0.95 are indicated next to the node of main genetic clusters. The five clusters identified with the best skyline converging model are indicated in bold and correspond to posterior probabilities > 0.99.

Our Bayesian phylogenetic analysis indicates that *M. francoismoutoui* form a distinct monophyletic lineage that diverged about 278,000 years ago from *M. acetabulosus*. The monophyly of the Reunion species supports the hypothesis of a single colonization event by overwater dispersal, although the geographic origin of its ancestor could not be determined in our study. Previous taxonomic studies on *Mormopterus* bats from the Mascarene Islands showed morphological similarities with *M. norfolkensis* from Australia, while *M. jugularis* from Madagascar was reported closer to *M. doriae* of Sumatra (Goodman et al., 2008; Peterson, 1985). The different patterns of grouping among southwestern Indian Ocean island members of the genus obtained with STRUCTURE (Fig. S1) may indeed suggest different geographic origins for Mascarene and Madagascar *Mormopterus*, respectively. However, because of mixed genotypes in a few Malagasy bats, we cannot rule out migrant ancestry with occasional dispersal of bats from Mascarene Islands to Madagascar. Further, there is evidence of dispersal of the Mascarene populations to the African continent (Goodman et al., 2008). Detailed genetic studies are available for certain *Mormopterus* species, such as here for the southwestern Indian Ocean islands and Reardon et al. (2014) for the Australian species. No global analysis is available across the broad geographic range of members of the genus and this will be needed to better define the origin of the southwestern Indian Ocean island species.

Once established on Reunion Island, the population of *M. francoismoutoui* remained stable and started increasing slowly 90,000 years ago, with a notable expansion around 55,000 years ago (Upper Pleistocene). This timing coincides with the estimated period when both volcanos (Piton des Neiges and Piton de la Fournaise) were active: from 65,000 years to 20,000 years (Nehlig and Marie, 2005). Such volcanic activity could have created new suitable habitats (like cliff crevices and caves), and thus increased possible roost sites and enhanced range expansion of this species. Indeed, it has been previously suggested that certain bat species may benefit from volcano activity. For example, the New Zealand short tailed bat (*Mystacina tuberculata*, family Mystacinidae) underwent a range expansion possibly following rapid forest recolonization after a volcanic eruption (Lloyd, 2003).

Reunion Island was colonized by humans about 350 years ago, with a dramatic human population expansion during the 20th century (Sandron, 2007). Contrary to our predictions that recent urbanization of Reunion Island, specifically construction of permanent structures (*i. e.* buildings, bridges) might have enhanced bat population size, our results suggest that the population expansion of *M. francoismoutoui* ceased or slowed down about 500 years ago. This result could be due to model error and based on a single locus (Ho and Shapiro, 2011). However, such a pattern of human intervention and increased population size has already been described for several Amazonian bats of the family Phyllostomidae associated with 17th century deforestation (Silva et al., 2020). After human



**Fig. 6.** Demographic history of *Mormopterus francoismoutoui* inferred from mitochondrial data (D-loop). (a) Frequency of pairwise differences distribution (mismatch distribution). The frequency observed is represented by the red dotted line, the expected frequency under the hypothesis of population constant model is indicated by the green line, and population expansion model by the blue line. (b). Coalescent Bayesian skyline plot (with dimension group parameter = 5). Time is indicated in the x-axis from recent (left) to past (right) and the estimate effective population size ( $N_e$ ) is represented in the y-axis. The central blue line is the median surrounded by the upper and lower estimates of 95 % credibility interval. The Reunion free-tailed bat estimated time of expansion is indicated with the green arrow around 90,000 years before present and the red arrow represent the colonization of the island by humans since 350 years.

colonization of Reunion Island, the natural ecosystems were profoundly and rapidly modified associated with a growing urbanization of the island (Lagabrielle et al., 2009). *Mormopterus francoismoutoui*, which is not a forest-dwelling species, uses natural large caves and crevices along cliff faces as roosting sites, which can be disturbed by anthropogenic activities, such as human visitation and guano collection. This bat species also occupies day-roost sites in urban areas, but given the odour associated with such roosts, they are discouraged and a by-product might be higher rates of bat mortality (Augros et al., 2015). Moreover, extensive landscape modifications and human activities may have changed behaviour and physiology of this species, and may negatively affect the fitness of individuals and population size (suggested by the Bayesian skyline analyses), as has been shown for other bat taxa (Russo and Ancillotto, 2015). For example, previous work found that the molossid bat, *Tadarida brasiliensis*, roosting at human-constructed bridges in Texas, experienced reduced immune system functioning, which could be linked to physiological stress (Allen et al., 2008)), and which in turn could lead to negative impacts on bat populations. The evolution of *M. francoismoutoui* population size over recent decades has not been monitored, at least because the large and dense populations at roost sites make direct counts of individual bats difficult. Occupancy modelling based on acoustic survey data, together with data from recaptured bats and mark-recapture models, should provide an alternative method to precisely assess trends in population size in relation to human activities (Oyler-McCance et al., 2017; Rivers et al., 2006; Rodhouse et al., 2019).

Our results suggest that the large current population of *M. francoismoutoui* experiences important levels of gene flow, as no significant genetic differentiation among roosts was found, as well as, low levels of inbreeding and no isolation by distance across sampled populations. Further, the high genetic diversity linked to the large population size may counter-balance a weak signal of spatial genetic structure (Gauffre et al., 2008). Interestingly, we found stronger  $\Phi_{st}$  values during the summer, which might be associated with some degree of female philopatry during the pregnancy and parturition periods and supported with the massive aggregations of pregnant females observed during this period, specifically at the TBA roost (Table 1, Aguillon et al., 2023; Dietrich et al., 2015). Subsequently, in March, both  $\Phi_{st}$  and  $F_{st}$  values were the lowest, suggesting that bats dispersed and mixed within roosts, coherent with the mating period in a completely panmictic population (Moussy et al., 2013). Interestingly, the  $F_{st}$  values calculated with the microsatellites were still high during the non-reproductive winter months. This discordance between markers may be the consequence of behavioural aspects of males, which are probably more sedentary during non-reproductive period, and fits with observations that bats, particularly adult females, leave the studied roost sites during winter and disperse to unknown wintering sites (Aguillon et al., 2023). Our results thus suggest high levels of dispersal in this species across Reunion Island, and its capacity to disperse over the island's mountainous landscape is consistent with the existence of roosts at high altitude (Sanchez and Probst, 2013). However, it is important to note a similar lack of genetic structure in *M. jugularis* on Madagascar and not related to sex classes (Ratrimomanarivo et al., 2009). These results likely indicate a common evolutionary trend among *Mormopterus* species on southwestern Indian Ocean Islands, and more broadly in Molossidae species, such as the Mexican free-tailed bat *Tadarida brasiliensis* capable of long-distance migration and high-altitude displacements (Glass, 1982; McCracken et al., 2008), and yet showing genetic differentiation between islands in the Bahamas (Speer et al., 2017). Interestingly, roosts occupied by *M. francoismoutoui* are often located in urban areas which could have facilitated dispersal by increasing connectivity between populations as molossid bats in general seem less affected by human-induced land changes (Richardson et al., 2021; Russo and Ancillotto, 2015).

Despite the absence of a geographical pattern in the genetic structure of *M. francoismoutoui*, our phylogenetic analyses showed that it has diversified into at least five divergent mtDNA lineages that are not geographically separated, but rather found in sympatry in roosts. Sympatric mtDNA lineages are not commonly described in animals, and especially in bats (Andriollo et al., 2015; Sun et al., 2016) and their origin and maintenance are often difficult to resolve (Hogner et al., 2012; Makhov et al., 2021; Webb et al., 2011). This may be explained by stochastic lineage sorting processes that occur in panmictic populations with large effective population size (Hogner et al., 2012; Webb et al., 2011). Also, we cannot exclude the possibility of female philopatry that would increase population structure in the mtDNA marker (Moussy et al., 2013), and this remained the same even when genetic analyses were performed for each sex. The divergence of mtDNA lineages may also reflect long periods of geographical isolation of small bat populations after the colonization of Reunion Island. These initial bat populations could have limited dispersal because of the intense volcano activity leading topographic separation of portions of the island. Our Bayesian phylogeny dated the start of the *in-situ* diversification back to 175,000 years ago, and the sharp increase in population size (about 55,000 years ago) coincides with the apparition of multiples lineages within the population (Fig. 2). As well, we found a multimodal distribution under the expansion model of the mismatch distribution, which could be linked to the expansion of the different genetic clusters (Du et al., 2019). Interestingly, the mtDNA genetic structure was not observed in the microsatellite analyses, which can be explained by increased allelic homoplasy at microsatellite loci that may mask genetic differentiation over long periods of time in species with large populations (Estoup et al., 2002). Our results may also suggest the recent admixture of ancient lineages, that are no more reproductively isolated, which might have contributed to the high nuclear polymorphism detected (Andriollo et al., 2015; Sun et al., 2016). Indeed, such recent gene flow would erase genetic signatures at microsatellite loci more rapidly than mtDNA loci, explaining the absence of a strong signal of nuclear structure.

However, in the case of recent gene flow in *M. francoismoutoui*, our nuclear data suggest that it does not occur randomly within the population, as the clustering analysis and PCoA on microsatellite markers indicated the presence of three distinct clusters (also in sympatry within roosts), although the percentage of variance explained is low. More interestingly, these nuclear clusters did not overlay mtDNA clusters and were not detected with the STRUCTURE analysis. This discordance between both markers can be explained by different evolutionary time processes and inheritance (Harrison, 1989; Toews and Brelsford, 2012). Such opposite patterns between markers have previously been described in different bat species (Laine et al., 2023; Naidoo et al., 2016; Sun et al., 2016) and highlight the need to use several markers for reconstructing complex evolutionary histories, especially when population structure is weak (Kuo et al., 2015). The microsatellite structure may correspond to a few isolated mating roosts on the island or could reflect putative adaptations like morphological or acoustic differences, as described for the big-eared horseshoe bat (*Rhinolophus*

*macrotis*, family Rhinolophidae, Sun et al., 2016). Further bat tracking studies would provide better understanding of spatial and temporal movements of individuals on Reunion Island and potentially identify currently unknown mating sites (Conenna et al., 2019).

## 5. Conclusion

Our study illustrates how understanding the ecology and evolution of insular endemic bats can be challenging and thus the importance of integrating past evolutionary processes and contemporary gene flow. Here, we demonstrate that fine-scale sampling scheme and multi-marker comparisons at regional and local scales are necessary, but do not guarantee, to obtain a complete picture of the population structure and history of an island endemic bat species. Such genetic approaches in combination with understanding a range of ecological parameters are also crucial to reduce uncertainty in conservation decision making of vulnerable mammals due to their endemism status, such as *Mormopterus francoismoutoui*. For example, conservation actions for bats living in such a meta-population can focus on the identification of possible threats such as urbanization.

## Funding

This research was supported by the French National Research Agency (ANR JCJC SEXIBAT), by the European Regional Development Funds ERDF PO INTERREG V ECOSPIR number RE6875. Samantha Aguillon was supported by a “Contrat Doctoral de l’Université de La Réunion”.

## CRedit authorship contribution statement

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Mitochondrial sequences (D-loop) have been deposited in Genbank under ID’s from OR081945 to OR082607 and microsatellites genotypes and metadata are available at Zenodo (<https://doi.org/10.5281/zenodo.8069702>). D-loop sequences for the Reunion Island dataset are coded with the roost name, the field identification number and the sex (F = female and M = male). Regional sequences are coded with the island (MADA = Madagascar and MAU = Mauritius) and field identification number. A pre-print is available on bioRxiv (<https://doi.org/10.1101/2023.06.22.546033>).

## Acknowledgements

We are grateful to personnel of Eco-Med Océan Indien, Biotope, the Direction de l’Exploitation et de l’Entretien des Routes (DEER) of Région Réunion, the Direction des Routes et des Transports (DRT) of Département Réunion, and the Salazie city hall for their help in identifying and accessing bat roosts. We are thankful to David Wilkinson for fruitful discussions and help with analyses. We also thank Yann Gomard and Julien Mélade for previous laboratory work on Mauritius and Malagasy samples. We also thank Guillaume Verchère for his assistance in the field.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.gecco.2024.e03030](https://doi.org/10.1016/j.gecco.2024.e03030).

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