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Research Article

Genetic diversity of Italian greater horseshoe bats (*Rhinolophus ferrumequinum*) and distinction of the Sardinian colonies

Alessandra Palladini[1,2,6], Mauro Mucedda[3], Ermanno Pidinchedda[3], Adriano Martinoli[4], Danilo Russo[5], Giuseppe Bogliani[1], Nadia Mucci[2,*]

[1] *Department of Earth and Environmental Sciences, University of Pavia, via A. Ferrata 1, 27100 Pavia, Italy*
[2] *Italian Institute for Environmental Protection and Research (ISPRA)*
[3] *Centro Pipistrelli Sardegna, Via G. Leopardi 1, 07100 Sassari, Italy*
[4] *Environment Analysis and Management Unit – Guido Tosi Research Group, Department of Theoretical and Applied Sciences, University of Insubria, via J.-H. Dunant 3, 21100 Varese, Italy*
[5] *Wildlife Research Unit, Sezione di Biologia e Protezione dei Sistemi Agrari e Forestali, Dipartimento di Agraria, Università degli Studi di Napoli Federico II, via Università 100, 80055 Portici, Italy*
[6] *Paul Langerhans Institut Dresden, Tatzberg 47/49, 01307 Dresden, Germany*

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Abstract

Characterizing the genetic outfit of a species is fundamental to evaluate its status and devise optimal conservation plans to maintain or enhance the viability of its populations. The greater horseshoe bat (*Rhinolophus ferrumequinum*) underwent declines across Europe over the 20th century and was thus listed in the EU Habitat Directive and the species protection legislation of EU countries. To understand what is the state of the species in Italy, we described the genetic variability and population structure derived from 327 bats sampled in twenty-two Italian colonies. The analysis of the first part of the mitochondrial DNA control region (525 bp) and a panel of 12 microsatellite loci revealed high genetic variability throughout the study area and the presence of two genetically distinct groups: the Sardinian and the peninsular pools. We could also identify indications of a genetic substructure within the peninsular population, with a significant divergence concerning colonies located at the northern borders. Notably, the relevant genetic differentiation between Italian and Sardinian colonies should be carefully taken into account for conservation planning.

Introduction

The Convention on Biological Diversity (CBD) and the International Union for the Conservation of Nature (IUCN) state that genetic diversity is one of the three primary forms of biodiversity deserving conservation. Precautionary principles and experimental data indicate that both short- and long-term persistence of natural populations are conditioned by their adaptability to climate and biotic changes. In particular, the persistence of small and isolated populations is threatened by genetic diversity loss. In the short term, the deleterious consequences of inbreeding may depress some reproductive fitness traits. In the long-term, small effective population size is expected to increase the stochastic fixation of a genetic variant by drift, thus weakening the adaptability of populations (Felsenstein, 1971; Hedrick and Kalinowski, 2000; Reed and Frankham, 2003).

The number of genetic studies on bats has increased in the last decades, but substantial research efforts are still required to develop sound conservation plans. Some conservation studies on bats have investigated the genetic structure in temperate and tropical species (e.g. Rossiter et al., 2000; Carstens et al., 2004; Rivers et al., 2005; Rossiter et al., 2005; Moussy et al., 2015) to gain insight into their ecology or to explore the impact of human activities (wind farms, deforestation, intensive agriculture, urbanisation, etc.) on population viability. Nevertheless, the recent discovery of new cryptic species by using molecular tools (Mayer and von Helversen, 2001; Ibáñez et al., 2006; Racey et al., 2007; Furman et al., 2010), indicates how bat systematics and ecology are still poorly known. The greater horseshoe bat, *Rhinolophus ferrumequinum*, is the biggest of the five horseshoe bats occurring in Europe. It is a thermophilous species, but nurseries can also be found in northern Italy in situations offering warmer temperatures such as abandoned buildings, monuments or caves, karst massifs and gypsum or ophiolite mines. In the south and all through the coastal regions, maternity roosts are frequently located in caves that offer milder microclimatic conditions. The species hibernates in caves and mines across all Italy, at times in old cellars or icehouses, and in basements of monumental buildings.

The greater horseshoe bat is listed by the IUCN as a species of Least Concern but with a decreasing population trend (Piraccini, 2016). In Europe, the species is extinct in Belgium, Gibraltar and the Netherlands, possibly extinct in Malta, while marked declines were observed in Germany, Austria, and the UK (Piraccini, 2016). In Italy, the demographic trend is not the same across the whole peninsula, and recent surveys showed that it is declining in some regions where historical colonies became extinct (Agnelli et al., 2004). For this reason, the species is classified as Vulnerable in the Italian Red List of Threatened Species (Rondinini et al., 2013). Besides natural events, like severe winters followed by cold springs, *R. ferrumequinum* populations are affected by human activities causing destruction or alteration of roosts in caves and buildings, and reduction of insect prey due to habitat ob-

*Corresponding author
Email address: nadia.mucci@isprambiente.it (Nadia Mucci)

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literation, or spread of pesticides and chemicals for parasite control. For instance, the anthelmintic avermectin compounds used in animal farming are known to disrupt the life cycles of dung beetles by inhibiting their development (Strong et al., 1996). Consequently, the decline of Scarabaeidae populations which constitute a key prey item for *R. ferrumequinum*, especially for young bats learning to hunt (Ransome, 1996), will interfere with the availability of insects in cowpats and may impact on bat activity and survival (Duvergé and Jones, 2003).

At a broader spatial scale, the replacement of deciduous forests with conifer plantations is narrowing the extent of optimal bat habitat patches, since bat diversity is higher in broad-leaved woods (Boye and Dietz, 2005). The loss of hedgerows and treelines linked with agricultural intensification (Ransome and Hutson, 2000) is affecting species' movements, eventually limiting connection and gene flow among colonies. Over time, these anthropogenic impacts are expected to accelerate population size declines and progressive isolation of the small remnant colonies. This scenario is especially severe for *R. ferrumequinum* due to its low productivity (one pup per year), late maturity (around two to three years of age) and high mortality rate in first-time breeders (Ransome and Hutson, 2000).

The major goal of this study was to assess for the first time the status of the Italian *R. ferrumequinum* by defining the extent and patterns of its genetic diversity and structure using a suite of population genetic tools, thus providing the knowledge needed to design tailored management plans and lay the foundation for further genetic monitoring. DNA samples from bats located in northern, central and southern Italy were genotyped using 12 autosomal microsatellite loci and the hypervariable region of the mitochondrial DNA, with the following specific aims: 1) measure the extent of genetic divergence among colonies and infer population structure to estimate connectivity and genetic flow across Italy; 2) reconstruct population history and investigate recent and past demographic changes. Using genotypes and haplotypes from the sampled bats, we inferred population structure via principal component analysis and model-based clustering, we calculated haplotype and nucleotide diversity, allele frequencies, private alleles and pairwise genetic distances to assess genetic diversity and polymorphism of the colonies, and used haplotype network, mismatch distribution and Bayesian skyline plot to investigate the history of greater horseshoe bat populations in Italy.

Materials and methods

Study area

Italy offers a variety of climates ranging from temperate to arid going from north to south, with colder summers on the Alps and the Apennines. Urbanization and industrialization are high in the northern area known as the Po Valley, and overall there is a decrease in population density going from the coastal areas towards the Apennine Range, which constitutes the central backbone of the Italian territory.

Sampling

Our goal was to cover the largest possible area, according to the availability of roosts known and monitored by local bat researchers and activists at the time of the study (2009). A total of 327 samples (287 tissue samples and 40 fecal pellets) was collected from 22 sites spread over 11 Italian regions (Tab. 1). Bats were sampled under licenses from the Italian Ministry of the Environment (MATTM), the Institute for Environmental Protection and Research (ISPRA), Natural Reserves and local authorities, and following the national guidelines for bat monitoring (Agnelli et al., 2004). Bats were caught at the exit of their roost with a harp trap, placed individually in cloth bags, processed promptly and released immediately afterwards. One or two skin biopsies were taken from each individual using a 3 mm punch, preserved in 96° ethanol and stored at −20 °C. Droppings were collected on plastic sheets put inside the roost underneath the hanging animals from sunset until the next morning. Only isolated droppings were collected to avoid cross contamination between samples belonging to different individuals, alcohol-preserved and stored at −20 °C. Sampling and subsequent DNA analyses were performed in the same year (2009).

DNA extraction sequencing, and genotyping

DNA was extracted using the Zymo Research 96 Quick-gDNA™ kit, The biological samples were first treated with an overnight digestion with Proteinase K, then the solution was managed as described in the manufacturer's protocol.

DNA sequencing

The first part of the mtDNA control region was PCR-amplified using the universal primers VI-L15774 (`GTAAAACGACGGCCAGTACATGAATTGGAGGACAACCAGT`) and VII-H16498 (`CCTGAACTAGGAACCAGATG`) (Shields and Kocher, 1991) with the following protocol: 94 °C × 2 min + 40 cycles × (94 °C × 40 s + 50 °C × 40 s + 72 °C × 40 s) + 72 °C × 10 min + 4 °C × 10 min + hold 15 °C. PCR fragments were sequenced using the forward primer (VI-L15774) and the BigDye Terminator kit v.1.1 (Life Technologies), using the manufacturer protocols: 25 cycles × (95 °C × 5 s + 50 °C × 40 s + 72 °C x 4 min) + 72 °C × 10 min + 4 °C × 10 min + hold 15 °C.

The sequence products were then separated on an ABI 3130XL capillary sequencer and corrected using Genemapper 3.0.

DNA genotyping

Twelve microsatellite loci were chosen from the literature: Rferr03, Rferr08, Rferr09, Rferr11, Rferr12 (Rossiter et al., 1999) and Rferr16, Rferr19, Rferr22, Rferr25, Rferr27, Rferr28 and Rferr29 (Dawson et al., 2004). DNA samples were amplified in a volume of 10 µl using 2 mM $MgCl_2$, 0.04 mM of Bovine Serum Albumin Fraction V, 0.10 mM of dNTPs , 0.15 µM of each primer, 0.05 U of Taq (5 Prime). Cycling conditions were optimized for each primer pair and for tissue or scat samples. The number of cycles during the annealing varied from 35 (in tissue) to 40 (in faeces), starting from the following general PCR program: 94 °C × 2 min + 40 cycles × (94 °C × 40 min + primer annealing temperature × 40 s + 72 °C × 40 s) + 72 °C × 10 min + 4 °C × 10 min + hold 15 °C. DNA fragments were separated on an automated 16 capillary sequencer 3130 XL Genetic Analyzer (Life Technologies). Due to the high percentage of low DNA amount and quality, non-invasive DNA samples underwent from four to eight PCR amplifications to rule out any PCR error (multitube approach according to Taberlet et al., 1999) and obtain reliable unique genotypes. Consensus genotypes among replicates were obtained using GIMLET 1.3.3 (Valière, 2002).

Data analysis

Sequencing data analysis

Haplotype diversity (h), nucleotide diversity (π) and average number of nucleotide differences (k) were calculated with DnaSP 5 (Librado and Rozas, 2009). F_{ST} and standard analysis of the molecular variance (AMOVA) were computed in Arlequin 3.5 (Excoffier and Lischer, 2010). Phylogenetic reconstructions were done in Mega 5.10 (Tamura et al., 2011), using the maximum likelihood, neighbour-joining algorithm and maximum parsimony procedures (Saitou and Nei, 1987; Tamura et al., 2011). The optimal nucleotide substitution model (HKY) was identified with JModelTest 2.1.3 (Darriba et al., 2012; Guindon and Gascuel, 2003). A network was drawn using the median joining procedure in Network 4.6.1.2 (Bandelt et al., 1999). Past population dynamics of Italian colonies were obtained from the mismatch distribution graph from Arlequin and in the skyline plots computed by Beast 1.7.5 (Drummond et al., 2007). We assumed a mutation rate of 20% per million years (Petit et al., 1999) which is based on the control region mutation rate of the noctule bat *Nyctalus noctula* (Bilgin et al., 2009).

Genotyping data analysis

We used GenAlEx 6.5 to estimate allele frequencies, observed (H_O) and expected (H_E) heterozygosity, mean number of alleles per locus (N_A), the number of private alleles (N_P), pairwise F_{ST}, G_{ST} and Nei's G_{ST}. Deviations from the Hardy-Weinberg Equilibrium were quantified using the inbreeding coefficient F_{IS}, calculated according to Weir and Cockerham with 1000 permutations, in Genetix 4.2 (Belkhir et

Table 1 – Origin and number of the bat samples used in this study.

	Region	Province	Colony id	Type of roost	No. of samples	Sampled bats/ estimated colony size	Type of sample
NORTHERN ITALY	Valle d'Aosta	Aosta	AO	Nursery	40	25%	fecal pellets
	Piemonte	Cuneo	CN_A	Hibernaculum	19	25%	tissue
		Cuneo	CN_B	Hibernaculum	15	25%	tissue
	Liguria	Savona	SV	Hibernaculum	17	20%	tissue
	Lombardia	Como	CO	Nursery	6	5%	tissue
	Friuli Venezia Giulia	Gorizia	GO	Nursery	15	10%	tissue
	Emilia Romagna	Forlì-Cesena	FC	Nursery	20	20%	tissue
		Modena	MO_SC	Nursery	24	80%	tissue
		Modena	MO_T	Nursery	7	25%	tissue
		Piacenza	PC_A	Hibernaculum	23	30%	tissue
		Piacenza	PC_T	Hibernaculum	19	10%	tissue
CENTRAL ITALY	Marche	Macerata	MC	Nursery	11	10%	tissue
	Umbria	Perugia	PG	Hibernaculum?	8	unknown	tissue
SOUTHERN ITALY	Basilicata	Matera	MT_A	Nursery	17	15%	tissue
		Matera	MT_J	Nursery	10	5%	tissue
	Puglia	Foggia	FG_C	Summer male roost	13	20%	tissue
		Foggia	FG_F	Summer male roost	11	35%	tissue
		Foggia	FG_I	Nursery	15	10%	tissue
SARDINIA	Sardinia	Cagliari	CA	Nursery	10	10%	tissue
		Ogliastra	OG	Nursery	8	5%	tissue
		Oristano	OR	Nursery	7	10%	tissue
		Sassari	SS	Nursery	12	5%	tissue

al., 2001). Data were compared with those obtained from the exact test in Genepop on the web (Raymond and Rousset, 1995 http://genepop.curtin.edu.au/). MicroChecker 2.2.3 (Van Oosterhout et al., 2004) was run to verify the occurrence of null alleles and allelic drop out. We used Arlequin 3.5 (Excoffier and Lischer, 2010) to perform the analysis of the molecular variance (AMOVA) and assess the level of global and pairwise population differentiation. Population structure was inferred using Structure v. 2.3.4 (Pritchard et al., 2006; Falush et al., 2003) with the admixture model and correlated frequencies (F-model by Falush et al., 2003) with the USEPOPINFO selection flag column = 0. First, we performed 500000 repetitions after 50000 burn-in from 1 to 22 K and 1 repetition to check for the trend of the likelihood values, then we repeated the analysis considering K from 1 to 7 and 5 iterations with 750000 repetitions after 250000 burn-in. This second analysis was repeated with the same parameters also including the LOCPRIOR model developed by Hubisz et al., 2009 which requires sampling locations as prior information and can detect poor genetic structure. The subdivision of the sampling provided 11 groups according to the geographic distribution of the colonies. Further analyses were run separately on the peninsular and the Sardinian sampling with and without the LOCPRIOR model and applying the same parameters used for the analysis of the whole sampling. The results were processed with Structure Harvester (Dent and von Holdt, 2012) to identify the number of clusters best representing the dataset. To avoid a wrong interpretation of the number of K, both mean log-likelihood of the data and Evanno's ΔK (Janes et al., 2017) were used to choose the right number of subopulations. Clumpp (Jakobsson and Rosenberg, 2007) and Distruct (Rosenberg, 2004) were used to merge the data from the five repetitions and to draw a plot for the best cluster obtained from the analysis. As suggested by Janes et al., 2017, the result for each K was considered in section Results. Genetic variation and structure within all sampling and peninsular samples only was further investigated by means of Principal Component Analysis (PCA), Discriminant Analysis of Principal Components (DAPC) test with the adegenet package (Jombart, 2008; Jombart and Ahmed, 2011) in R 5.1 (R Core Team, 2018). PCA and DAPC were performed also on peninsular colonies after the removal of the Sardinian samples. A further attempt to discover a substructure in the peninsular sampling was done through the removal of the colonies that were plotted as outliers in the PCA graph. A Mantel test was also performed with the adegenet package for the R software to highlight a possible correlation between genetic and geographic distances. Probability of identity was calculated with GenAlEx 6.5 (Peakall and Smouse, 2006, 2012) using the whole data set to verify whether the combination of markers could reliably determine individual genetic profiles.

Results

Genetic diversity and divergence inferred from mitochondrial DNA

We obtained 263 mtDNA control region sequences (525 base pairs long) that included 32 distinct haplotypes (Genbank accession number MN037759 – MN037790) defined by 30 polymorphic sites (8 singletons and 22 parsimony informative sites). The mean haplotype diversity was Hd= 0.858 ± 0.017.

Bats sampled in the Sardinian island showed lower haplotype diversity (Hd=0.614 ± 0.096; six haplotypes) than those in peninsular Italy (Hd=0.832 ± 0.020; 27 haplotypes) but this difference was not found to be significant. Haplotype number and diversity (Tab. 2) were variable among colonies sampled in peninsular Italy, with the highest values in PC_A (hibernaculum in north-western Italy, district of Piacenza) (Hd=0.865; four haplotypes) and the lowest in AO (nursery in Aosta, Italian northwestern border) and MO_T (nursery in the district of Modena, Apennines, northern Italy) (Hd=0; one haplotype). Nucleotide diversity was π=$0.003\,58 \pm 0.000\,21$ in peninsular Italy and π=$0.001\,65 \pm 0.000\,34$ in Sardinia. The average number of nucleotide differences was k=1.9 in peninsular colonies and 0.9 in Sardinia. Detailed values for Hd, π and k are shown in Tab. 2. The most abundant haplotype, IT1, was spread across peninsular Italy and in two Sardinian bats (colony S_OG, located in the district of Ogliastra; Supplementary Figure S1).

Genetic diversity and divergence inferred from microsatellite DNA

We successfully genotyped 287 bat DNA samples extracted from skin biopsies at 12 microsatellite loci and obtained reliable unique genotypes from 27 out of the 40 droppings collected in AO (detailed data are shown in Tab. 2). The number of alleles per locus across all samples ranged from 5.0 (OR, Sardinia) to 8.3 (FC, Northern

Table 2 – Summary of mean gene diversity at mtDNA control region and 12 microsatellite loci in each sampled colony. Haplotype diversity (*Hd*), nucleotide diversity (π), number of successfully sequenced samples (*Nmt*), number of genotyped samples (*N*), average number of alleles per locus (*Na*), number of effective alleles, (*Ne*), 1 / sum of the squared population allele frequencies, observed heterozygosity (*Ho*), expected heterozygosity (*He*). F_{IS} values: significance is ***p<0.001; **p<0.05.

	Mitochondrial DNA				Microsatellite loci					
Population	**Nmt**	**Hd**	π	**k**	**N**	**Na**	**Ne**	**Ho (±SD)**	**He (±SD)**	**F_{is}**
AO	**9**	0	0	0	**27**	7.4	4.9	0.756 ± 0.053	0.742 ± 0.037	0.0002
CN_A	**11**	0.473	0.000 96	0.5	**19**	8.2	5.6	0.780 ± 0.050	0.766 ± 0.035	0.0078
CN_B	**11**	0.709	0.002 77	1.4	**15**	7.8	5.3	0.800 ± 0.029	0.777 ± 0.025	0.0054
SV	**16**	0.833	0.003 16	1.7	**17**	7.8	5.2	0.725 ± 0.053	0.763 ± 0.032	0.0806**
CO	**5**	0.4	0.003 81	2	**6**	5.1	4	0.764 ± 0.052	0.718 ± 0.030	0.0266
GO	**4**	0.473	0.002 22	1.2	**15**	7.2	4.7	0.767 ± 0.041	0.748 ± 0.032	0.0108
FC	**29**	0.837	0.004 18	2.2	**20**	8.3	5.6	0.767 ± 0.039	0.768 ± 0.034	0.0279
MO_SC	**23**	0.644	0.002 76	1.5	**24**	8.2	5.3	0.750 ± 0.042	0.762 ± 0.037	0.038
MO_T	**7**	0	0	0	**7**	5.4	3.9	0.762 ± 0.054	0.692 ± 0.042	–0.0221
PC_A	**19**	0.833	0.003 54	1.9	**23**	8	5.4	0.718 ± 0.043	0.746 ± 0.046	0.0599**
PC_T	**24**	0.865	0.003 05	1.6	**19**	8	5.3	0.772 ± 0.042	0.765 ± 0.033	0.0181
MC	**11**	0.618	0.001 66	0.9	**11**	6.3	4.5	0.773 ± 0.049	0.729 ± 0.040	–0.0117
PG	**8**	0.643	0.001 43	0.7	**8**	5.6	4	0.717 ± 0.070	0.681 ± 0.054	0.014
MT_A	**16**	0.125	0.000 24	0.1	**17**	7.9	5.1	0.773 ± 0.046	0.737 ± 0.049	–0.0185
MT_J	**9**	0.2	0.001 14	0.6	**10**	6.1	4.3	0.665 ± 0.076	0.707 ± 0.051	0.1130**
FG_C	**11**	0.345	0.000 69	0.4	**13**	7.1	4.9	0.729 ± 0.053	0.715 ± 0.044	0.0208
FG_F	**11**	0.509	0.000 97	0.5	**11**	6.6	4.8	0.778 ± 0.041	0.753 ± 0.028	0.0151
FG_I	**11**	0.709	0.002 35	1.2	**15**	7.4	4.6	0.750 ± 0.053	0.721 ± 0.046	–0.0064
Peninsular Average	**0.832**	**0.00358**	**1.9**		**7.1**	**4.8**	**0.752 ± 0.012**	**0.738 ± 0.009**	**0.0304*** **
CA	**7**	0.7	0.001 52	0.8	**10**	6	4	0.692 ± 0.078	0.647 ± 0.062	–0.0163
OG	**6**	0.857	0.003 27	1.7	**8**	5.3	3.8	0.637 ± 0.072	0.615 ± 0.071	0.0335
OR	**10**	0.333	0.000 63	0.3	**7**	5	3.7	0.667 ± 0.079	0.628 ± 0.067	0.0161
SS	**5**	0.2	0.000 38	0.2	**12**	5.9	4.1	0.715 ± 0.066	0.670 ± 0.051	–0.0226
Sardinian Average	**0.614**	**0.00165**	**0.9**		**5.6**	**3.9**	**0.678 ± 0.256**	**0.640 ± 0.218**	**–0.0041**
Total Average	**0.858**	**0.00373**	**1.9**		**6.8**	**4.7**	**0.739 ± 0.012**	**0.739 ± 0.012**	**0.0407*** **
Total N	**263**				**314**					

Italy) while the mean number of alleles across all colonies was 6.8. All microsatellite loci were found to be polymorphic in all sampling sites, except for OR colony in Sardinia (91.7%) in which Rferr28 was monomorphic. Observed heterozygosity ranged from intermediate (MT_J: H_O=0.665 ± 0.076; H_E=0.707 ± 0.051) to moderately high values (CN_B: H_O=0.800 ± 0.029; H_E=0.777 ± 0.025, with a mean value 0.752 ± 0.012 for the observed heterozygosity (H_O) and 0.738 ± 0.009 for the expected (H_E). Sardinian colonies displayed slightly lower values of H_E (from 0.615 to 0.670) when compared to peninsular colonies (from 0.681 to 0.777).

Two hibernacula (SV, PC_A) and a maternity roost (MT_J) showed a significant deficit of heterozygotes (*Fis*, Tab. 2), which disappears when a Bonferroni correction is applied. All other colonies were in Hardy-Weinberg Equilibrium (HWE). No equilibrium was reached when calculating *Fis* over all colonies. Data from Genepop found a departure from HWE in the central Italian nursery MC (p<0.00001) and a significant positive value for Rferr25. MicroChecker showed nor allelic drop out, neither scoring error due to stuttering in all the loci, but null allele occurrence was found at the locus Rferr12. As the two software listed above showed no congruency in the detection of the departure from HWE at these two loci, we preferred to not remove them from the analysis. Private alleles were found both in peninsular and in Sardinian samples, 46 and three respectively. The PIsibs value was 8.3×10^{-6} for a combination of 12 microsatellites. Genetic distance between pairs of colonies (pairwise F_{ST} and G_{ST} Gerlach et al., 2010 – Supplementary Figure S2) revealed that the three nurseries sampled at the northern borders of Italy, i.e. Como (CO), Aosta (AO) and Gorizia (GO), exhibit a significant divergence to most of the other colonies. In particular, considering a significance threshold of 0.0042 after Bonferroni correction, F_{ST} was significant for Aosta and Gorizia (p=0.002) and for Aosta and the male roost FG_C, located in the south (p=0.001). Como was significantly differentiated from the nursery MO_SC (located in the district of Modena, northern Italy; p=0.003), and from all southern Italian colonies (p-values ranging from 0.001 to 0.004), except the nursery MT_J. Also Gorizia is significantly differentiated from the nursery MO_SC (p=0.004), and from the southern Italian colonies MT_A (p=0.004), FG_C (p=0.003) and FG_I (p=0.001). Further, each Sardinian colony differed significantly (p=0.001) from any peninsular colony, but not from the other Sardinian colonies.

Population structure inferred from mitochondrial DNA

The optimal substitution model found by JModel Test was HKY+I for all Italian samples. D-loop analysis revealed a lack of a clear genetic structure. The topology of trees based on maximum likelihood, neighbour-joining algorithm and maximum parsimony lacked statistical support since bootstrap values ranged between 0% and 45%, with a few nodes reaching 65% (data not shown). The median-joining haplotype network showed a star-like pattern, which is usually indicative of a high level of gene flow (Fig. 1A). Nevertheless, some haplotypes appeared to be restricted to some geographical areas: bats in Sardinia exhibited five private haplotypes, and the colonies of north-western Italy (PC, CN, SV), north-eastern Italy (GO), northern Italy (CO), central Italy (PG, MC) and southern Italy (FG, MT) also featured private haplotypes (Fig. 1B and Supplementary Figure S1).

Haplotype AMOVA scores (Supplementary Table S3) were higher within (64.9%) than among (35.1%) colonies. When repeating the analysis after regrouping peninsular versus Sardinian haplotypes, the resulting variation among populations dropped down to 23%.

Population structure inferred from microsatellite DNA

Scores of molecular variance (AMOVA) (Supplementary Table S4) were high within (95%) and low among colonies (2%). Significant

values (p<0.00001) were retrieved between Sardinian and peninsular colonies (6.28%). Variance among peninsular colonies alone was almost negligible (0.84%). Mean log-likelihood of the data and Evanno's ΔK in Structure Harvester identified the best grouping at K=2 when all samples were analyzed (data not shown). At K=2, Sardinian individuals were unambiguously assigned to a single population with a mean Q value=0.97. All peninsular samples were assigned to the second cluster. Analysis performed with the LOCPRIOR model permit to identify the best cluster at K=5 (Fig. 2A). At K=2, Sardinian individuals were split into a separate cluster, as in the previous analysis. At K=3, Sardinian bats remained assigned unambiguously to a single cluster, while peninsular samples from South Italy sharply separated from the other peninsular samples and constituted a second distinct cluster. Individuals from CO an GO individuals were also associated to a third cluster, while all the other samples from centre and Northern Italy showed an admixed composition between the second and the third cluster (Fig. 2B).

STRUCTURE yielded the same information when it was performed only on peninsular samples, either with or without LOCPRIOR option (data not shown). Analysis of Sardinian samples did not provide a substructure nor with neither without the use of the LOCPRIOR model.

Unsupervised analysis of the whole dataset by means of PCA also identified two main groups (Fig. 3A), segregating peninsular from Sardinian bats. Repeating PCA on peninsular colonies alone showed that northern roosts (Fig. 3B, greendots) overlap with northwestern and southern ones, while AO (Aosta, northwestern border) segregates from the rest of the colonies. PCA performed after the removal of northern colonies (Fig. 3C) detected a moderate differentiation of the southern Italian colonies from the others, while AO and Gorizia are outliers.

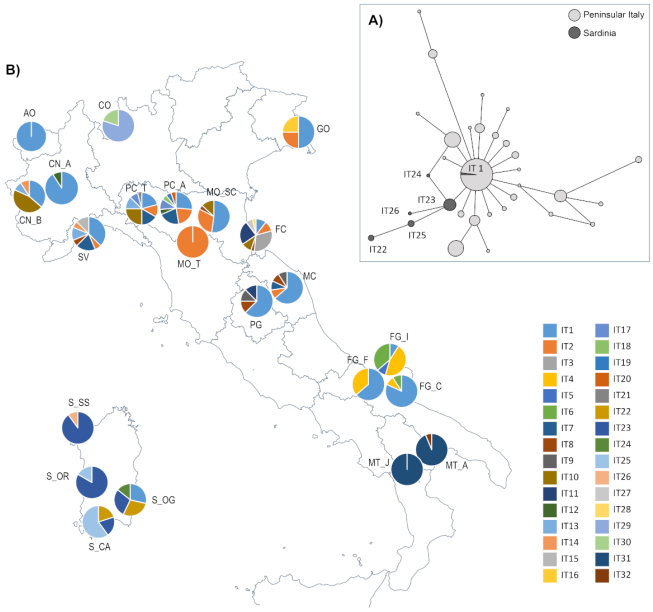

Figure 1 – A) Median-joining haplotype network of the D-loop sequences (mitochondrial DNA) obtained from greater horseshoe bats sampled across Italy. The network is colored such as to highlight the relationship between Italian peninsular (light gray circles) and Sardinian colonies (dark gray circles). Each circle represents a single haplotype and its diameter is proportional to its frequency in the sampled population. The star-like shape of the network indicates the absence of a clear genetic substructure. Black lines represent the connection between different haplotypes. Gray shades indicate geographical origin. Circles with different colors, but with the same origin identify sympatric haplotypes. IT1 is the haplotype with the highest frequency and it was found everywhere. Dark gray circles identify haplotypes found uniquely in Sardinia. B) Map of the haplotype frequencies found in the sampling sites. A total of 32 haplotypes are present in the 22 colonies. Each pie chart represents a colony, and the name of the colony indicates the city district: if more than one colony was sampled within the same district, a letter was added. The districts' codes are the following: AO=Aosta; CO=Como; GO=Gorizia; CN=Cuneo; SV=Savona; PC=Piacenza; MO=Modena; FC=Forlì-Cesena; PG=Perugia; MC=Macerata; FG=Foggia; MT=Matera. Sardinia: SS=Sassari; OR=Oristano; OG=Ogliastra; CA=Cagliari. The size of the slices depends on the relative frequency of the haplotype in the colony. Pie charts are color-coded according to the haplotypes present in the successfully sequenced bats.

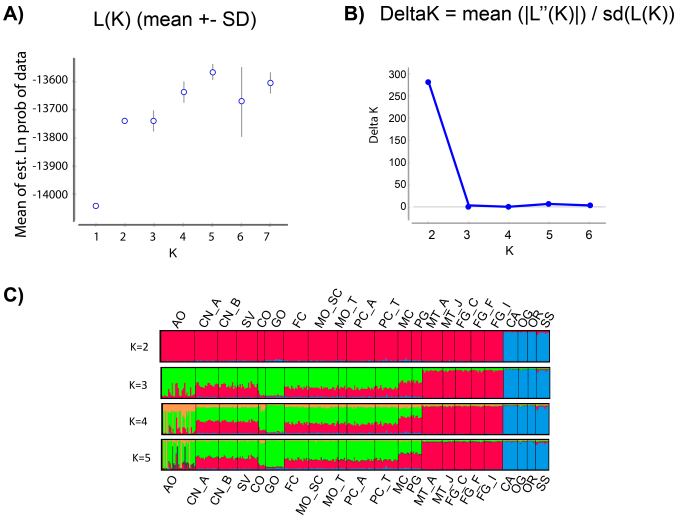

Figure 2 – Inference of the genetic population structure via model-based clustering using Structure: A) Mean log-likelihood of the data; B) Delta K; C) graphic plot from K=2 to K=5. Sardinia samples are the first to split in a separate cluster. At K=3 also Southern Italian samples and individuals from Northern colonies (GO and CO) separate each other. K=4 and K=5 identify individuals belonging to populations not sampled.

Further, the Discriminant Analysis of the Principal Components on the peninsular samples (Fig. 3C), also highlighted a slight differentiation of the Southern Italian bats (MT_A, MT_J, FG_C, FG_F, FG_I are all located on the right side of the DAPC plot) and of the individuals from the colonies located at the western and northern borders (Aosta and Como respectively, lie in the top part of the plot) when calculated on the peninsular colonies alone. Mantel test did not evidence any correlation between the genetic and geographic distances either with (r=0.055; p=0.768), or without the Sardinian sampling (r=0.0355, p=0.984).

Demographic history

As previously described, the haplotype network was star-shaped (Fig. 1A), with both peninsular and Sardinian haplotypes deriving from the central haplotype IT1. The number of nucleotide differences between haplotypes was k=1.7 within the peninsula and k=0.9 in Sardinia. The mismatch distributions of the Italian and the Sardinian haplotypes revealed non-significant p-values for the sum of squared deviations and Harpending's Raggedness index, under the sudden and the spatial expansion models. Both distributions were unimodal, which is consistent with an expanding population (Fig. 4A-B). The Bayesian Skyline Plot, calculated on all samples, confirmed the expansion model detected by the mismatch (Fig. 3C).

Discussion

Genetic diversity, divergence and population structure

The Italian populations of *R. ferrumequinum* displayed a high genetic variability. Mean haplotype diversity was 0.857, all microsatellite loci were found to be polymorphic. Allelic diversity and heterozygosity values (N_a=6.8; H_o=0.739) recorded in the study were comparable to the ones found by Rossiter et al. (2007) for France, Spain and two Italian colonies. The sample set departed from Hardy-Weinberg Equilibrium when it was considered as a unique population, while only three colonies out of twenty-two displayed the absence of random mating when analysed singly. The reduced heterozygosity that we found in one maternity roost (MT_J) and two hibernating sites (PC_A and SV) could be attributed to a sampling bias since MT_J was a large nursery where only a small number of bats could be sampled (Tab. 1), while hibernacula are aggregations of bats originating from different sites and distinct colonies and can therefore host bats carrying different allelic frequencies. Microsatellite and haplotype analyses revealed the presence of two distinct geographic populations, the Sardinian and the peninsular ones. Unique haplotypes and alleles were reported for Sardinia,

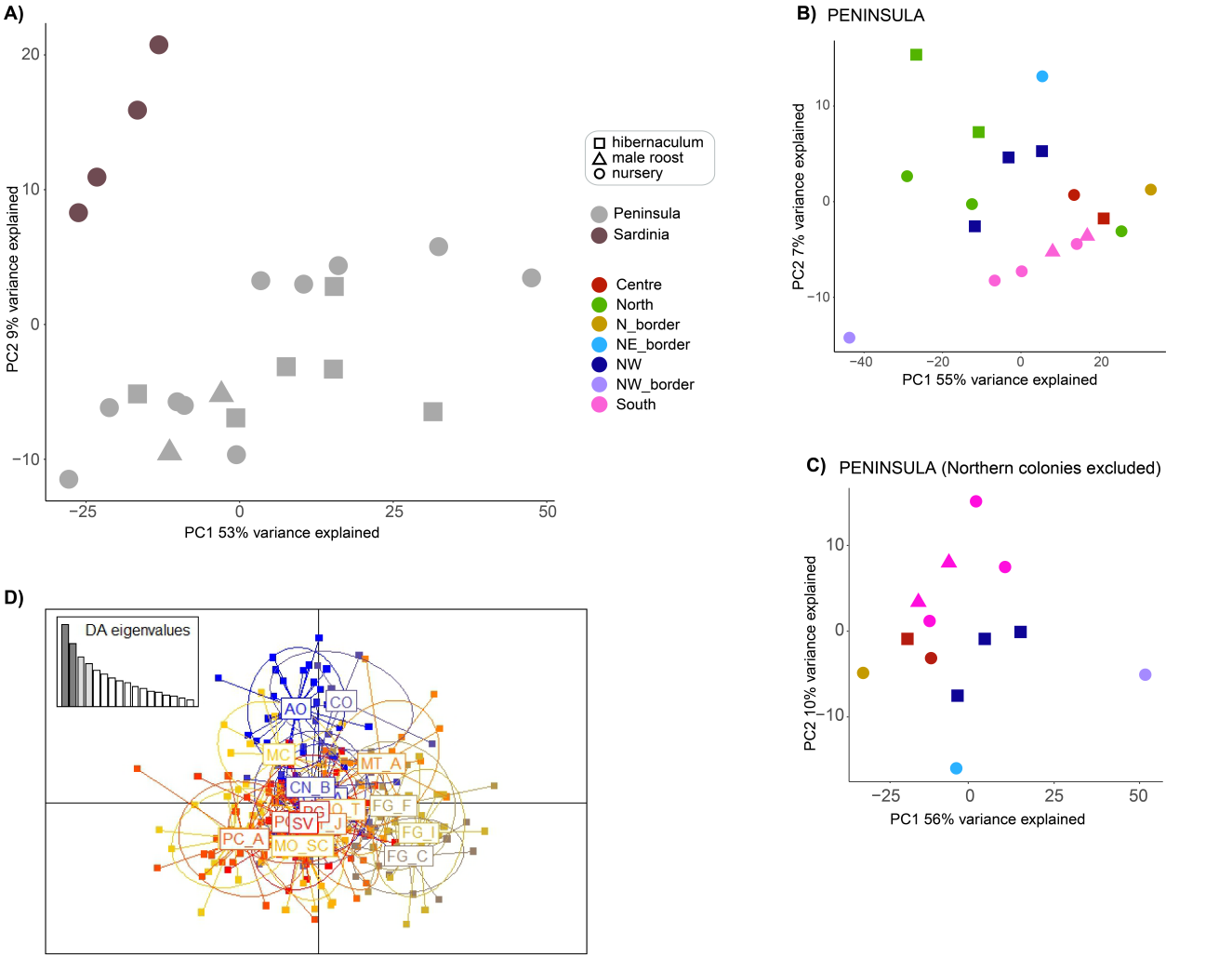

Figure 3 – Data exploration by means of unsupervised and supervised multivariate techniques, i.e. Principal component analysis (PCA) and Discriminat Analysis of Principal Components (DAPC), respectively. PCA was calculated for A) all colonies; B) peninsular colonies only; C) peninsular colonies after removal of northern Italian colonies. Plots are generated using the first and the second principal component, which together contribute to explain about 60% of the variance present in the dataset. Each dot is a colony and its phenotype is coded by the shape: squares are hibernacula, triangles are male roosts and circles indicate maternity colonies (nurseries). Colors indicate in which part of Italy the colonies are located. In A) peninsular sites are in gray, Sardinian ones are brown. In B) and C): red=centre; green=north; light brown=northern border; light blue=norteastern border; blue=north-west; violet=northwestern border; pink=south.

whose genetic diversity is lower than in the peninsula. Moreover, a total of 46 peninsular alleles was not found in the Sardinian colonies, thus indicating the absence of current gene flow between the two regions. Haplotype and allele divergence of Sardinian nurseries from peninsular colonies and the absence of migrants, as suggested by the evidence of no departures from the Hardy-Weinberg Equilibrium, led us to consider the Sardinian population as a Management Unit sensu Moritz (1994), namely a functionally independent unit with respect to gene flow and conservation requirements. These findings highlight the importance of the bats of Sardinia, which already include unique lineages, like the endemic *Plecotus sardus* and a Sardinian lineage of *Plecotus auritus* (Mucedda et al., 2002), haplotypes of *Pipistrellus* genealogically connected to haplotypes from Morocco and Sicily, and two lineages of *Hypsugo*: *H. savii* s. str. and *H.* cf. *darwinii* (Veith et al., 2011).

We could not resolve any further population structure within the peninsula, although a slight differentiation was detected by Bayesian and Principal Component Analysis in the Southern (Matera and Foggia) and in two Northern colonies (Como, Gorizia). Variation among populations decreased from 35% to 23% when AMOVA was performed on colonies regrouped in peninsular versus Sardinian haplotypes, indicating that part of the variation was still incorporated in the peninsular group. Pairwise F_{ST} and G_{ST} statistics also revealed significant divergences of most sampled colonies with respect to Como (far north), Aosta (north-west) and Gorizia (north-east). This could be attributed to the geographical position of these nurseries, which are located on the northern border of the species distribution in Italy and may therefore host genetic components from Switzerland, France and Slovenia. In fact, some samples from Aosta (northwestern Italy, close to the Swiss and French borders) stand out for high Q values, possibly a cross-border genetic component. Significant differentiation was also present in comparison with only one of the two hibernacula located in Cuneo, CN_B, although both were located in the northwest. *R. ferrumequinum* females are known to be loyal to either specific males or mating sites (Rossiter et al., 2005); thus, the discovery and sampling of reproductive and mating sites from the same area should help explain such divergence and show how colonies are related. Interestingly, the AO nursery also showed significant haplotype differences from the southern nursery FG_I, but not from the two male roosts located in the same area (FG_C and FG_F), while its F_{ST} and G_{ST} values were significantly different from all three roosts. Overall, there are peninsular haplotypes that are unique to the south (MT and FG), the far north-east (GO), the north-west (PC, CN, SV), and the centre (MC, PG).

Demographic history

The unimodal mismatch distributions found under the sudden and the spatial expansion models, as well as the skyline plot indicate that greater horseshoe bat populations did not suffer any recent decline in Italy. Unfortunately, we have no demographic records to support such findings. This trend seems to contrast with the ones described for the species in several European countries (Piraccini, 2016), where *R. ferrumequinum* populations have been declining (Austria, Germany,

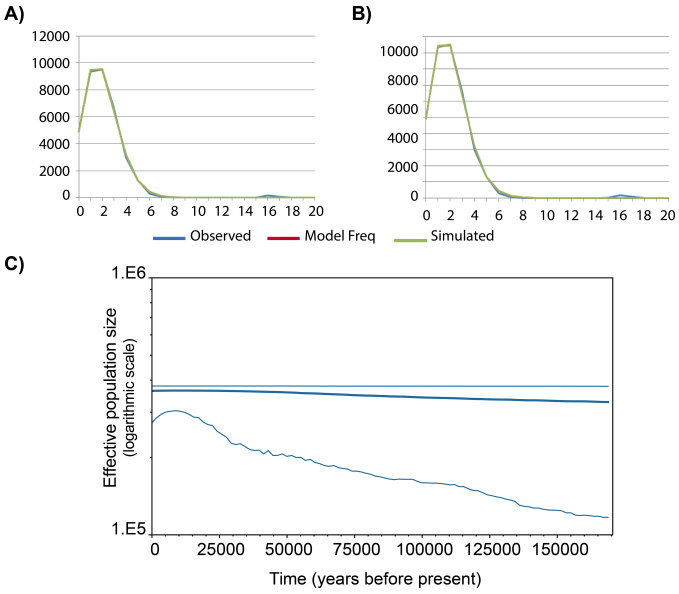

Figure 4 – Demographic changes in the Italian population. Mismatch distributions A) under the sudden expansion model and B) under the spatial expansion model. These graphs represent the frequency of pairwise differences between alleles (x axis). In both graphs, observed and simulated values overlap. C) In the Bayesian skyline plot the thick blue line represents the demographic trend defined inside a minimum and maximum vale (thin blue lines). A mean value higher than 5.5×10^5 is expected from the graph trend. All the models are consistent with an expansion of either the range of the population or the population.

Spain) or have gone extinct (Belgium, Netherlands, Malta). In any case, the data reflect what is happening in a minority of countries where populations are stable after a decreasing period (UK, Switzerland, Croatia), or have been slowly increasing thanks to a return to traditional agriculture (Romania).

The haplotype network is star-shaped indicating that the Italian *R. ferrumequinum* has a recent origin, as confirmed by the low number of nucleotide differences between haplotypes (k=1 within the peninsula and 0.7 in Sardinia). Former phylogeographical studies performed on European colonies (Rossiter et al., 2007; Flanders et al., 2009) showed that the present distribution of *R. ferrumequinum* alleles and haplotypes is the result of postglacial population expansions from refugia such as Iberia and/or Italy and the Balkans/Greece, where the species retreated during the LGM. We hypothesize the significant genetic differences between southern and northern colonies may depend on the isolation occurred during the LGM: haplotype IT1, which is spread all over Italy and also found in Sardinia, may represent the ancestral haplotype that colonised or re-colonised Italy after the LGM, when temperatures started increasing.

Management implications

Results from this study should encourage the planning of conservation strategies based on molecular and field data. Molecular data suggest that colonies are not suffering a population decline. Nevertheless this genetic survey did not cover the whole Italian territory and it should be expanded to carry out continuous sampling from north to south. This will allow to complete the genetic characterization of the species and provide additional information to evaluate ecological corridors or critical barriers to genetic flow. Further, a systematic genetic monitoring of the colonies would serve as an effective tool to evaluate the response of bat populations to environmental changes, and also to the effectiveness of conservation practices. Genetic monitoring over multiple years as a means to investigate demographic parameters in wildlife populations (Schwartz et al., 2007) has been used especially on larger terrestrial mammals, like the brown bear, to evaluate the reintroduction of a brown bear population in northern Italy (De Barba et al., 2010), and to track population expansion and pack structure of the Italian wolf (Caniglia et al., 2012, 2014). In many bat studies genetic sampling is mostly used to gain further insight into their ecology and history (e.g. Rossiter et al., 2000; Bogdanowicz et al., 2012; Wright et al., 2018; Andersen et al., 2019). Yet, there are a few examples of genetic mark-recapture as a successful approach to enhance knowledge on the dynamics of bat populations, as the estimation of colony size and survival rates for the Indiana bat (Oyler-McCance et al., 2018), and the estimation of population size, sex ratio and sex-related behaviour in colonies of lesser horseshoe bats in Northern France (Zarzoso-Lacoste et al., 2017).

The interpretation of the results obtained from genetic monitoring requires, indeed, the availability of a wealth of ecological data, such as the extension and distribution of suitable roosting and foraging habitats around the colonies, and the threats linked with human activities. The results of this study indicate that special attention should be given to the nurseries located at the northern border, which showed significant genetic differences from the other colonies, i.e. Como (far north), Aosta (north-west) and Gorizia (north-east). These maternity roosts are located at the northern extreme of the species national distribution range and are therefore more likely subject to isolation. They can also be concurrently in contact with populations from across the border and this should be investigated by sampling nearby cross-border colonies, to understand whether their peculiarity is due to a limited gene flow, to the presence of genetic components from abroad, or both.

Even the southern colonies deserve further studies to investigate the origin of their differentiation, particularly to assess if it can be due to natural isolation or to anthropogenic barriers hampering the gene flow with other Italian colonies. Like the isolated colonies at the northern border, Sardinian colonies too require special attention, as they constitute a separate cluster where gene flow with the peninsula is no longer ongoing. Island populations are generally closely watched because, over time, geographic isolation brings to the loss of allelic diversity and fixation of genetic variants by stochastic processes, thus depleting the potential of a species to adapt. In such a context, any perturbation that causes a population to shrink may be the trigger to an extinction vortex if the population remains isolated and unable to numerically recover. British greater horseshoe bats have been thoroughly studied as part of a species recovery project aiming at enhancing its population levels by applying favourable land management practices in the foraging areas surrounding maternity and hibernation colonies (Longley, 2003). Genetic sampling showed that the British population has a lower genetic diversity than continental European colonies, indicating that genetic flow is limited or interrupted (Rossiter et al., 2000). Sardinia too, showed lower genetic diversity compared to the peninsula, with lower expected and observed heterozygosity and average number of alleles. As an island, it grants a limited area to its wildlife, and already hosts a number of other endemic bat lineages. On top of this geographical constraint there are threats specifically concerning the four colonies of *R. ferrumequinum* that were analysed for this study. Only SS is within a protected area, yet the maternity roost is located in a dangerous building. The maternity roost in OG is also inside a building in danger of collapse, while the colony in OR lies in an area that is highly impacted by agricultural activities and the nursery has recently disappeared (Mucedda *pers. comm.*). Lastly, the maternity roost in CA lies in an abandoned mine that may be closed for safety reasons.

Genetic monitoring of hibernacula, nurseries and other roosts should be performed to gather information on the population genetics and parental relationships existing within the island. This would enable conservation authorities to properly intervene in case of negative population trends and to provide further information about the ecology of greater horseshoe bats in Italy.

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Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Table S1 Haplotipe distribution among colonies.

Table S2 Pairwise analysis for F_{ST} (A), and G_{ST} (B).

Figure S3 Haplotype Analysis of the molecular variance (AMOVA).

Figure S4 Microsatellite analysis of the molecular variance (AMOVA).