Published by Associazione Teriologica Italiana
Hystrix, the Italian Journal of Mammalogy
Volume 27 (2): 150–157, 2016
OPEN ACCESS
Available online at:
http://www.italian-journal-of-mammalogy.it
doi:10.4404/hystrix-11835

Research Article

What can we learn from faeces? Assessing genotyping success and genetic variability in three mouse-eared bat species from non-invasive genetic sampling

Andrea Viglino[1,2,*], Romolo Caniglia[1], Aritz Ruiz-Gonzalez[1,3,4], Danilo Russo[11], Marco Galaverini[1], Roberto Toffoli[5], Paola Culasso[5], Paolo De Bernardi[6], Elena Patriarca[6], Paolo Agnelli[7], Felice Farina[8], Giovanni Mastrobuoni[9], Adriano Martinoli[10], Ettore Randi[1,12]

[1]*Laboratorio di Genetica, Istituto Superiore per la Protezione e la Ricerca Ambientale, Via Ca' Fornacetta 9, 40064 Ozzano dell'Emilia (BO), Italy*
[2]*Institute of Zootechnics, Università Cattolica del S. Cuore, 29122 Piacenza, Italy*
[3]*Department of Zoology and Animal Cell Biology, University of the Basque Country UPV/EHU, C/ Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*
[4]*Systematics, Biogeography and Population Dynamics Research Group, Lascaray Research Center, University of the Basque Country,UPV/EHU, Avda. Miguel de Unamuno 3, 01006 Vitoria-Gasteiz, Spain*
[5]*Chirosphera Associazione per lo studio e la tutela dei chirotteri e l'ambiente, Via Morghen 34, 10143 Torino, Italy*
[6]*Stazione Teriologica Piemontese S.Te.P., c/o Museo Civico di Storia Naturale, c.p. 89, 10022 Carmagnola (TO), Italy*
[7]*Museo di Storia Naturale la Specola, Via Romana 17, 50125 Firenze, Italy*
[8]*FaunaViva, Viale Sarca 78, 20125 Milano, Italy*
[9]*GIRC, Dipartimento di Biologia Animale, Via Taramelli 24, 27100 Pavia, Italy*
[10]*Department of Theoretical and Applied Sciences, Environment Analysis and Management Unit – Guido Tosi Research Group, University of Insubria, Via J. H. Dunant 3, 21100 Varese, Italy*
[11]*Wildlife Research Unit, Laboratorio di Ecologia Applicata, Dipartimento di Agraria, Università degli Studi di Napoli Federico II, via Università 100, 80055 Portici (Napoli), Italy*
[12]*Department 18/Section of Environmental Engineering, Aalborg University, Aalborg, Denmark*

Keywords:
dropping DNA
species genetic detection
genotyping success
individual identification
microsatellites
mouse-eared bats
mtDNA
non-invasive genetic sampling

Article history:
Received: 19 April 2016
Accepted: 24 May 2016

Acknowledgements
A. Viglino and R. Caniglia contributed equally to this work.
Performed the sampling: AV, RC, RT, PC, PD, EP, AM, PA, FF, GM. Conceived and designed the experiments: RC, AV, ER. Performed the experiments: RC, AV. Analyzed the data: RC, AV, ARG. Contributed reagents/materials/analysis tools: ER, RC. Wrote the paper: AV, RC, DR, ARG, MG. Revised the manuscript: AV, RC, ARG, MG, ER, RT, PC, PD, EP, AM, PA, FF, GM, DR.
A. Viglino was supported by a PhD fellowship awarded by the University of Bologna. This study was supported by the Italian Ministry of Environment. A. Ruiz-Gonzalez holds a Post-doc fellowship awarded by the Dept. of Education Universities and Research of the Basque Government (Ref. DKR-2012-64). Many thanks to Martina Spada (University of Insubria), Giacomo Maltagliati (Florence Natural History Museum) and Roberto Facoetti (Lecco Province) for sample collection, Elena Fabbri (ISPRA) for laboratory assistance and John Parsch (Ludwig Maximilians Universität) for English revision.

Abstract

In the last century bat populations significantly declined mainly due to habitat fragmentation and degradation. As management-dependent species, bats need appropriate monitoring programs for the implementation of sound conservation strategies. However, bats' small size, high mobility, elusiveness and nocturnal lifestyle make them difficult to survey. Non-Invasive Genetic Sampling (NIGS) may offer safe and cost-effective solutions, but requires well-planned sampling strategies, informative molecular markers and reliable laboratory protocols. Here we developed a NIGS protocol for species and individual identification of three mouse-eared bats, the Geoffroy's bat (*Myotis emarginatus*), the long-fingered bat (*Myotis capaccinii*) and the Daubenton's bat (*Myotis daubentonii*). Species identification was accomplished by mitochondrial (mt) DNA sequencing of reference tissue (n=49) and droppings (n=285) from Central-Northern Italy. In addition, we optimized a multiplex panel of seven microsatellites suitable for species and individual identification of the three species from droppings. We obtained a good success with mtDNA sequencing (245/285; 86%) and microsatellite genotyping (129/245; 53%). All microsatellites were successfully amplified with low error rates, and were polymorphic in the three *Myotis* species, with probabilities of identity $\leqslant 0.001$ and observed heterozygosities of H_o=0.48 in *M. emarginatus*, 0.62 in *M. capaccinii* and 0.71 in *M. daubentonii*. Our protocol represents a useful tool for population genetic studies on mouse-eared bats that could likely be extended to other bat species and provide useful information to implement effective conservation plans.

Introduction

Bats (order Chiroptera, Blumenbach 1779) constitute at least one-fifth of the extant mammals, with over 1300 known species (Russo and Jones, 2015). They play an important role in ecosystems, acting as pollinators and pest controllers, and occupy almost all existing habitats, exploiting a variety of trophic niches through different morphological and behavioural adaptations (Simmons, 2005).

Bat populations have considerably declined worldwide during the last century, mainly due to their high sensitivity to human disturbance, roost destruction, intentional killing, intensive agriculture and land use changes leading to fragmentation and degradation of their foraging habitats (Grzimek, 2004; Haysom et al., 2013). For this reason, all European bat species are protected under the European "Habitats" Directive 92/43/EEC and the UNEP "Eurobats" Agreement.

Understanding bat population structure and dynamics is thus essential to design sound preservation strategies (Haysom et al., 2013). However, the application of classical bat surveying methods, such as direct counting, camera trapping, telemetry and genetic analysis from captured individuals, may not be trivial because of bats' small size, high mobility and nocturnal lifestyle. Moreover, the application of techniques requiring the capture and handling of bats can represent an additional source of disturbance when frequently or incorrectly carried out, with possible detrimental consequences for their conservation.

*Corresponding author
Email address: andrea.viglino@gmail.com (Andrea Viglino)

doi:10.4404/hystrix-11835

Table 1 – Sampling details for ME, MC and MD. Sampling sites: progressive numbers (1–11). Sample type: droppings (D) and tissue (T) samples. Sample collectors: AM: Adriano Martinoli; AV: Andrea Viglino; EP: Elena Patriarca; FF: Felice Farina; GMA: Giacomo Maltagliati; GM: Giovanni Mastrobuoni; MS: Martina Spada; PA: Paolo Agnelli; PC: Paola Culasso; PD: Paolo Debernardi; RF: Roberto Facoetti; RT: Roberto Toffoli.

Species	Sampling site ID	Colony name	n	Sample type	Collector
ME	1	Trinita	43	D	RT & PC
	2	Tetti Pesio	20	D	RT & PC
	3	Venaria reale	20	D	PD & EP
	4	Bricherasio	15	D	PD & EP
	5	Passerano	15	D	PD & EP
	6	Revello	20	D	PD & EP
	7	Paperia	20	D	PD & EP
	8	Racconigi	12	D	PD & EP
	9	Campo dei Fiori	29	T	MS & AM
	10	Tenuta S. Rossore	25	D	PA & GMA
	11	Cormons	20	D	AV
MC	12	Isola Bella	50	D	PD & EP
	13	Lierna	15	T	FF, RF, & AV
MD	14	Fondi	25	D	GM & AV
	15	Villa di Domiziano	5	T	GM & AV

Non-invasive genetic sampling (NIGS) may offer safer, more efficient and cost-effective survey solutions, minimizing disturbance (Agnelli et al, 2004; Puechmaille et al., 2007) and is already widely applied to other elusive animal species (e.g. Beja-Pereira et al., 2009; Arandjelovic et al., 2010; Caniglia et al., 2013; Wultsch et al., 2014). DNA can be extracted from a broad range of biological samples, including faeces (Taberlet and Luikart, 1999; Caniglia et al., 2014), hair follicles (Woods et al., 1999), blood traces (Atterby et al., 2010), and even salivary swabs taken from prey carcasses (Caniglia et al., 2013). These approaches do not disturb the study subjects and make molecular identification of species and individuals possible, also providing DNA samples for further population genetic studies (Beja-Pereira et al., 2009; Wultsch et al., 2014). Although the analysis of DNA from bat droppings would be fully non-invasive and samples collectable by non-specialists — e.g. volunteers, park rangers or cavers — (Puechmaille and Petit, 2007; Boston et al., 2012; Puechmaille and Teeling, 2013), NIGS is still seldom applied to bats, with only a few exceptions (e.g. Puechmaille and Petit, 2007; Puechmaille et al., 2007; Boston et al., 2011, 2012).

The main drawbacks of this approach are that droppings might be affected by inter-individual contaminations, contain polymerase chain reaction (PCR) inhibitors and low-quality or quantity DNA that could potentially yield non-target mtDNA amplicons and microsatellite genotyping errors — i.e. false alleles (FA) and allelic dropout (ADO); Waits and Paetkau (2005); Broquet et al. (2007). Therefore, non-invasive genetic techniques should be tested in pilot studies before being applied to large-scale surveying projects (Taberlet and Luikart, 1999; Valière et al., 2007). In this study, using molecular identification of species from skin samples for validation, we developed a cost-effective method for typing mitochondrial and autosomal DNA extracted from the droppings of three species of the genus *Myotis* (family Vespertilionidae): the Geoffroy's bat (*M. emarginatus*; ME), the long-fingered bat (*M. capaccinii*; MC) and the Daubenton's bat (*M. daubentonii*; MD) sampled in Italy. We evaluated the effectiveness and reliability of this approach for species and individual identification. The three species showed different distribution and habitat. ME is mainly present in Southern, South-Eastern and central Europe (Červerný, 1999; Flaquer et al., 2004) and is also currently distributed from North Africa to Central Asia. MC was described with a distribution spanning Algeria and Tunisia in northern Africa, and with Spain, as its Western border in Europe, extends into Iran and South-Western Asia in the East (Koopman, 1994). MD showed a distribution from Portugal, Ireland and Norway through Europe and Northern Asia to the Far East (Korea and Japan) (Stubbe et al., 2008). Regarding the habitat ME is predominantly a forest insectivorous species (Goiti et al., 2011), while MC and MD are trawling insectivorous bats (Siemers et al., 2001; Simmons, 2005; Nardone et al., 2015). These three species often use the same roost types and dwell in riparian habitats, sometimes forming mixed colonies. In particular, MC and MD have similar foraging behaviour and ecological niches, and outside Italy they can co-occur with the only other European trawling bat *Myotis dasycneme* (Britton et al., 1997; Siemers et al., 2001). This NIGS method was thus applied to investigate the genetic variability of these three co-occurring species that have been only studied in a single phylogenetic case-study in Italy (Bogdanowicz et al., 2015), and for which no NIGS-based works have been carried out so far.

Materials and Methods

Sampling and DNA extraction

In 2010, between mid-June and September, a time that approximately corresponds to the pregnancy and lactation seasons of European bats (Racey and Entwistle, 2000), we collected reference tissue samples from the target species in three sampling localities: ME (n=29; sampling locality number 9), MC (n=15; sampling locality number 13) and MD (n=5; sampling locality number 15) (Fig. 1 and Tab. 1). Sampling in nursery sites occurred on one night for each colony. Bats were captured using mist nets and harp traps set up near roost entrances (Kunz, 1988). Using a sterile puncher, we collected a 3 mm biopsy of the wing membrane (Worthington Wilmer and Barratt, 1996) which was fixed in 95% ethanol (v/v) and stored at −20 °C until DNA was extracted.

Non-invasive sampling was conducted between 2010 and 2011 in 12 different bat roosts in Northern and Central Italy (caves, mines or attics; Fig. 1). To minimise stress to bats we conducted a non-invasive sampling scheme based on a single survey per session per roost during the post-reproductive season, always carried out at night. We removed old droppings from the floor, which was afterwards covered with paper sheets (Puechmaille et al., 2007). The sampling taken no more than six hours for each visited site. Thus, samples were collected paying attention to discard all the overlapping droppings. Allowing us to avoid any contact between faecal pellets, minimize contamination and limit resampling of the same individual. Droppings were immediately preserved in 95% (v/v) ethanol, stored at room temperature during the transfers and subsequently at −20 °C in the laboratory. We collected a total of 285 droppings from 10 colonies of ME (n=210), one of MC (n=50) and one of MD (n=25) (Tab. 1). To minimize DNA degradation of ethanol-preserved samples, DNA was extracted within two days

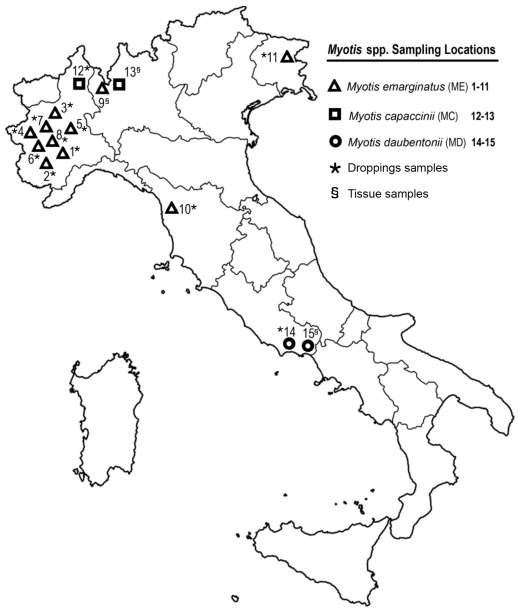

Figure 1 – Sampling localities in Northern and Central Italy (marked with progressive numbers 1–11; see Tab. 1 for further details) of the three analysed *Myotis* spp..

from sampling. Before processing, droppings were air-dried on filter paper to remove as much ethanol as possible. Biopsy tissue samples and droppings were sliced into tiny pieces, then DNA was automatically extracted using a MULTIPROBE IIEX Robotic Liquid Handling System (Perkin Elmer, Weiterstadt, Germany) and the Zymo research© Tissue or Stool DNA extraction kits (Zymo research, Corporation, Irvine, CA, USA), according to the manufacturer's instructions and following the modifications reported in Caniglia et al. (2013) for stool and salivary samples. DNA from droppings was extracted, amplified, and genotyped in separate rooms reserved to low template DNA, under sterile ultraviolet laminar flow hoods, using filter tips. Moreover, negative (no DNA in PCR) and positive (samples with known genotypes) controls were used during each laboratory step for both tissue and faecal samples.

DNA concentration was evaluated only for tissue samples using the Qubit v. 2.0 (Invitrogen, Carlsbad, CA, USA) and the appropriate quantification assay (dsDNA BR Assay kits). Since DNA concentration in faeces is certainly affected by bacterial or prey DNA, the DNA content was not quantified in non-invasive samples, but their quality was double screened by: 1) PCR-amplifying each faecal DNA sample at the mtDNA Cytochrome b (Cyt-b) gene; 2) subsequently, for samples that gave reliable results at Cyt-b, by four PCR replicates of three microsatellite loci (Multiplex 1: A13, H19 and H29 loci).

Species identification

DNA fragments of respectively 772 bp (ME), 653 bp (MC) and 743 bp (MD) of the Cyt-b gene, containing diagnostic mutations for the molecular identification of the three bat species (Ruedi and Mayer, 2001; Bilgin et al., 2008), were amplified using an ABI GeneAmp© PCR System 9700 with the following thermal profile: 94 °C/15 min, 94 °C/30 s, 57 °C/90 s, 72 °C/60 s (30 cycles), followed by five minutes of final extension at 72 °C. Amplifications were carried out in a total volume of 10 µl, including 1 µl (from wing membranes) or 2 µl (from pellets) of DNA solution, 0.3 pmol of the primers L15162 and H15915 Irwin et al. (1991); Ruedi and Mayer (2001), 1 µl of 10× PCR buffer with 2.5 mmol Mg^{2+}, 0.4 µmol dNTPs, 2 mg/ml of bovine serum albumin (BSA), 0.25 units of Taq polymerase (5 PRIME Inc., Gaithersburg, USA) and RNase-free water up to the final reaction volume. PCR products were purified using the exonuclease/shrimp alkaline phosphatase procedure (Exo-Sap; Amersham Life Sciences, UK) and sequenced in both directions using the ABI Big Dye Terminator kit v.3.1 with the following steps: 96 °C/10 s, 50 °C/5 s, 68 °C/4 min of final extension (25 cycles). Sequences were analysed in an ABI DNA sequencer 3130XL (Applied Biosystems, Foster City, CA), corrected with the software Seqscape v.2.5 and aligned using Clustal W (Thompson et al., 1997) in Bioedit (Hall, 1999). Identical haplotypes were matched in DNASP v.5 (Librado and Rozas, 2009). We then used the software Blast (Altschul et al., 1990) to determine the possible correspondence of mtDNA haplotypes discovered in this study with the eight ME, ten MC, and eleven MD haplotypes already published in GenBank (Tab. S1).

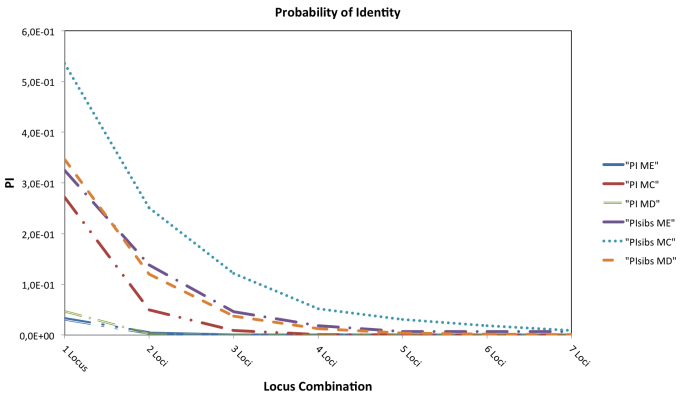

Figure 2 – Probability of identity values estimated from droppings in unrelated ($P_{(ID)}$) and sibling ($P_{(ID)sib}$)) individuals for the three multiplex reactions (seven microsatellites) in ME (n=104), MC (n=10) and MD (n=15).

Microsatellite selection and multiplex protocol development

Thirteen microsatellites (A13, B11, B22, C113, D09, D15, E24, F19, G09, G25, G30, H19, H29), originally identified and successfully amplified in DNA of *Myotis* spp. (*Myotis myotis*, Castella and Ruedi, 2000; *Myotis bechsteinii*, Kerth et al., 2003; *Myotis nattereri*, Kerth et al., 2003) or related species (*Plecotus auritus*, Furmankiewicz and Altringham, 2007; *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*, Kaňuch et al., 2007), were selected for their high variability and tested for cross-species amplification and polymorphism in tissue DNA of ME, MC and MD.

PCR conditions were firstly optimised for each primer pair in singleplex amplifications selecting the optimal annealing temperatures through a gradient PCR (from 50 °C to 60 °C with ΔT=1 °C) on a subset of tissue samples, using an ABI Veriti© Thermal Cycler. The reaction mix, with a total volume of 10 µl, included 1 µl of DNA solution, 0.3 pmol of each primer, 1 µL of 10× PCR buffer with 2.5 mmol Mg^{2+}, 0.4 µmol dNTPs, 2 mg/ml of bovine serum albumin (BSA), 0.25 units of Taq polymerase (5 PRIME Inc., Gaithersburg, USA) and RNase-free water up to the final reaction volume. Tissue samples were thus independently amplified twice at all loci using the optimal thermal profile in an ABI GeneAmp©PCR System 9700: 96 °C/30 s, 57 °C/90 s (for loci A13, H19, H29, D15 and E24) or 60 °C/90 s (for loci G25 and F19), 72 °C/60 s (40 cycles), followed by seven minutes of final extension at 72 °C. Microsatellite performance was evaluated through allelic variation, amplification success, and genotyping error rates; allowing us to select a suite of seven high-quality markers (see results). Multiplex Manager v.1.0 (Holleley and Geerts, 2009) was used to design three multiplexed PCRs: M1 (loci A13, H19 and H29), M2 (D15 and E24) and M3 (G25 and F19), avoiding any overlap between different amplicons of similar size. The reliability of multiplex protocols was tested by re-analysing tissue-derived DNA of all the three species (n=49) another two times per multiplex. Results were compared to those obtained from singleplex amplifications and checking for genotyping errors: ADO (the number of allelic dropouts over the number of successful amplifications of heterozygous genotypes at a given locus), and FA (the number of amplifications leading to one or more false alleles at a locus over the total number of successful amplifications at that locus). The newly designed multiplex protocols were then applied only to those non-invasive samples that were successfully typed at the Cyt-b gene. Multiplexed amplifications were carried out in 10 µl total volume, including 1 µl (from wing membranes) or 2 µl (from pellets) DNA solution,5 µL Qiagen Multiplex PCR mix (Qiagen Inc, Hilden, Germany), 1 µL Qiagen Q solution, from 0.1 µL to 0.4 µL of 10 µmol primer mix (forward and reverse, Tab. 3). and RNase-free water up to the final volume. Multiplexed amplifications were performed using an ABI GeneAmp©PCR System 9700, and the following thermal profiles: 94 °C/15 min, 94 °C/30 s, 57 °C/90 s, 72 °C/60 s (30 cycles), followed by five minutes of final extension at 72 °C for M1 and M2; 94 °C/15 min, 94 °C/30 s, 60 °C/90 s, 72 °C/60 s (30 cycles), followed by five minutes of final extension at 72 °C for M3.

PCR products were analyzed on an ABI 3130XL automated sequencer (Applied Biosystems, Foster City, CA). ROX 500 was used for M1 and ROX 350 for M2 and M3, while allele sizes were estimated using the ABI software GeneMapper v.4.0.

Multiple-tube approach, data reliability and individual identification

To minimize ADO and FA, we used a multiple-tube PCR protocol (Taberlet and Luikart, 1999) modified according to Caniglia et al. (2014) (for more details see Fig. S2). Faecal DNA quality was initially screened by amplifying each sample four times independently at multiplex M1, chosen for the high amplification success and low error rates of loci A13, H19 and H29, giving a good view of DNA quality with amplifications of both small, medium and large products. Only

Table 2 – List of Cyt-b haplotypes detected in tissue and droppings of ME, MC and MD.

Species	Haplotypes	Tissue	Droppings	Total	GenBank Acc. Number	Reference
ME	H1_ME	29	191	226	DQ120905	Ibañez et al. 2006
	H2_ME	0	1	1	KM396316	This study
	H3_ME	0	1	1	KM396317	This study
	H4_ME	0	1	1	KM396318	This study
	H5_ME	0	1	1	KM396319	This study
MC	H1_MC	10	0	10	KM406572	This study
	H2_MC	2	0	2	KM406573	This study
	H3_MC	3	25	28	KM406574	This study
MD	H1_MD	5	25	30	KP742480	This study

samples showing more than 50% positive PCRs were selected and further amplified independently four times at multiplexes M2 and M3. A reliability analysis was performed using Reliotype (Miller et al., 2002) based on the allele frequencies obtained from tissue sample genotypes. Unreliable genotypes (at threshold $R \leqslant 0.95$) were replicated other four times. Samples not reliably typed at all loci after eight PCR replicates were definitively discarded. Consensus genotypes were reconstructed using Gimlet v.1.3.3 (Valière, 2002), accepting heterozygotes only if both alleles were seen in at least two replicates, and homozygotes only if a single allele was seen in at least four replicates. Gimlet was also used to estimate PCR success rate (the number of successful PCRs divided by the total number of PCR runs across samples) and genotyping errors: ADO and FA following Pompanon et al. (2005). Statistical differences of PCR success and genotyping errors between groups were evaluated using nonparametric Kruskal-Wallis and post hoc Wilcoxon rank-sum tests in R v.3.0.1 (R Development Core Team, 2009). Any occurrence of null alleles was tested using Microchecker (Van Oosterhout et al., 2004). The probability of identity (P_{ID}) and the expected P_{ID} among full sibling dyads (P_{ID}sib; Mills et al., 2000; Waits et al., 2001) were computed by GenAlex v.6.5 (Peakall and Smouse, 2012).

Assignment tests and genetic variability

Patterns of differentiation among genotypes were visualized through a multivariate discriminant analysis of principal components (DAPC) computed with the Adegenet package (Jombart, 2008) implemented in R (R Development Core Team, 2009). Additionally, we used a K-means clustering algorithm to find groups of individuals that maximize between-group genetic variation (function `find.clusters`). Based on this test, multilocus genotypes were grouped into three different clusters, representing the three studied species (see Results). GenAlex was then used to estimate allele frequency by locus and group, observed (H_O) and expected unbiased (H_E) heterozygosity, mean (N_A) and expected (N_E) number of alleles per locus and number of private alleles (PA). Deviations from Hardy-Weinberg (HWE) was tested using GenePop v.4.0 (Rousset, 2008) with exact P values being estimated using the Markov chain algorithm with 10000 dememorization steps 100 batches and 1000 iterations. The Bayesian software Structure v.2.3.4 (Falush et al., 2003) was run for each species, in order to assign the multilocus genotypes to their cluster of origin (population), independently of any prior non-genetic information. Eleven independent runs for ME, five for MC and MD were conducted for increasing values of K (K=1–11 and K=1–5) using 1000000 Markov chain Monte Carlo (MCMC) iterations, after a burn-in of 100000 iterations and assuming independent allele frequencies and the admixture model (Falush et al., 2003). The most likely number of clusters K was identified based on the best value of LnP(K). At the optimal K, we assessed the average proportion of membership (Qi) to each different cluster and individual assignment was consequently based on the proportions of membership (qi) estimated for each individual. Principal component analysis (PCA) in GenAlex was used to visualize the subpopulations found in Structure.

Results

Species identification

We obtained reliable mtDNA Cyt-b sequences for all (n=49) tissue samples and for 86% of the 285 *Myotis* faecal samples we analysed: i) 29 tissues and 195 out of 210 droppings of ME (93%), ii) 15 tissues and 25/50 (50%) droppings of MC, and iii) 5 tissues and all the 25 droppings (100%) of MD. The remaining 15 ME and 25 MC droppings provided unreliable results and thus were discarded.

After matching all sequences in DNASP and blasting them against GenBank (pairwise identity >99.4%), we identified five haplotypes for ME (one corresponding to the haplotype H1 described by Ibáñez et al. (2006) and renamed as H1_ME in this study, four novel haplotypes named H2_ME, H3_ME, H4_ME and H5_ME), three new haplotypes for MC (H1_MC, H2_MC and H3_MC), and a single and novel MD haplotype (H1_MD). All the new haplotypes from this study were submitted to GenBank (Tab. 2; Tab. S1).

Microsatellite selection in tissue samples

After evaluating the performance and cross-species amplification success of the 13 microsatellites initially selected through singleplex reactions from the 49 tissue samples of the three species, we discarded six loci (C113, D09, B22, B11, G09, G30) due to low genetic variation (N_A=1 or H_E <0.5 for C113 and D09 in ME and MC; B22 in ME and G30 in MD), low PCR success (<0.30%) or high (>0.70%) unspecific amplification rates (B22 and G30 in MC; B11 and G09 in all species), high ADO (G09, ADO=0.57) and/or FA (C113, FA=0.44) rates. Conversely, the seven best-performing microsatellites had multiple alleles and did not show any signal of ADO or FA. Moreover, we obtained the same results when they were re-amplified in the three multiplexed reactions tested on the 49 tissue samples. This panel of multiplexed loci allowed us to identify multilocus genotypes for tissue samples of ME, MC and MD with an overall $P_{(ID)}=3.61\times10^{-6}$, 3.63×10^{-5} and 4.58×10^{-9} and a $P_{(ID)sib} = 8.25\times10^{-3}$, 9.21×10^{-3} and 1.07×10^{-3}, respectively.

Genotyping success and error rates from droppings

The 245 *Myotis* spp. droppings successfully identified at the species level by mtDNA sequencing were analyzed with the novel multiplex protocol of seven highly polymorphic microsatellites to further confirm species assignment through nuclear DNA (nDNA) analyses, as well as to provide reliable individual identification. Results of the multiplex panel of seven microsatellites loci for *Myotis* spp. are summarized in Tab. 3.

After the first four PCR replicates at multiplex M1 (2940 amplifications), the first screening step of multi-tube protocol was failed by 95 (39%) non-invasive samples that were immediately discarded (Tab. S3). The remaining 150 (61%) samples were further amplified four independent times at multiplex M2 and multiplex M3. After the first four replicates at all three multiplex PCRs, 121 out of the 150 (80%) droppings that successfully passed the quality screening showed a reliability score $R \geqslant 0.95$ and were accepted. DNA from the 29 droppings with R>0.95 were further amplified another four times at unreliable loci by simplex reactions. Eight of them (28%) reached $R \geqslant 0.95$ and were accepted, while the other 21 were definitively discarded.

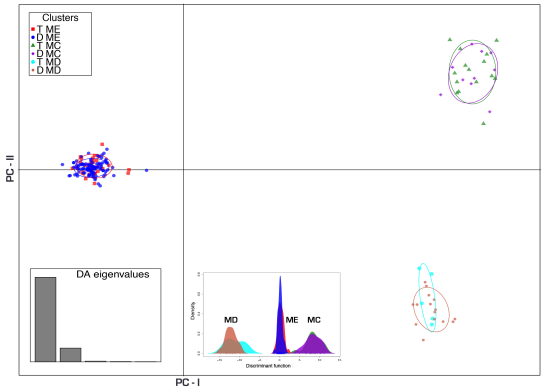

Figure 3 – Discriminant analysis of principal component scatter plot (DAPC, computed in `Adegenet`) of tissue (T) and dropping (D) samples of *Myotis* spp. showing the genetic distinction among ME (left side of PC-II), MC and MD (right side of PC-II). PC-I explains 56.85% of the total genetic variability (see also the DA eigenvalue histogram insert in the bottom left side). PC-II, explaining 9.27% of the total genetic variability, indicates the distinctions among three bats species (ME, MC and MD, see also the density plot insert in the lower centre side of the figure).

Overall, after 4–8 PCR replicates per dropping per locus, reliable and complete multilocus genotypes were obtained for 129 droppings (86% of the *Myotis* spp. samples that passed the M1 screening and 53% of the total analysed samples). After a regrouping procedure, no genetic re-captures were observed, thus the 129 genotypes from droppings corresponded to 129 different individuals (104 ME, 10 MC and 15 MD), also distinct from the 49 tissue-sampled individuals (Tab. S3).

The average proportions of positive PCRs on droppings were 87.1%, 90.3% and 97.7% for ME, MC and MD, respectively (Tab. 3) and varied among loci from 70% to 100% in the three species. The mean error rates ±SD across loci for all *Myotis* spp. were: ADO=0.04±0.06 and FA=0.01±0.01, Tab. 3. The meanADO rate for ME was 0.14±0.11 while ADO was absent in MC and MD (Tab. 3). FA rates averaged 0.01±0.02 for ME and 0.01±0.01 for MC but were absent in MD (Tab. 3). Amplification success did not differ significantly between species (H=2.95, p=0.2283; Kruskal–Wallis rank-sum test; p>0.005; Wilcoxon rank-sum tests for pairwise comparisons). Conversely, ADO significantly varied across species (H=12.21, p=0.002; Kruskal–Wallis rank-sum test), being significantly higher in ME than in MC (Wilcoxon rank-sum tests, W=7, p=0.011) and in MD (W=7, p=0.001). ME, MC and MD did not differ significantly from one another (H=3.36, p=0.186; Kruskal–Wallis rank-sum test) for FA (ME: 0.01±0.02 SD, MC: 0.01±0.01 SD, MC: 0.00±0.00 SD) (Tab. 3).

The seven loci selected allowed us to identify genotypes with $P_{(ID)}=1.26\times10^{-6}$, 3.64×10^{-5} and 6.60×10^{-10}, and $P_{(ID)sib}=6.89\times10^{-3}$, 9.02×10^{-3} and 7.18×10^{-4}, from droppings of ME, MC and MD, respectively (Fig. 2), that was enough to exclude any "shadow effect" (i.e. the probability of finding by chance more than one individual bearing the same genotype in a population) in all the three bats species.

Assignment tests

DAPC results showed that all ME, MC and MD samples plotted separately, with all the dropping genotypes included within their reference groups (Fig. 3). The 104 ME faecal genotypes were assigned to the ME cluster, while the ten MC faecal genotypes plot within the reference MC cluster and finally the 15 MD faecal samples were assigned to the MD cluster.

Thus, the assignment procedure concordantly led to identify 104 ME, 10 MC and 15 MD among the droppings DNA samples, concordantly with the CytB mtDNA identifications. Successively the `find.cluster` function (retaining 100 PC axes; Bayesian Information Criterion BIC=6) confirmed DAPC plot, showing the presence of three groups corresponded to the three species analysed in this study (Fig. S5).

Table 3 – Properties of the seven multiplexed microsatellites and summary of the genetic variability assessed per locus and species. M = Multiplex number; L = Locus name; D = Dye Label; V = primer pair volume; SR = size range (in bp); NA = Number of total alleles; H_O and H_E = observed and expected heterozygosity; PCR+ = positive PCR; ADO = allelic dropout rates; FA = false allele rates; SD = standard deviations .

M	L	D	V	SR		NA			H_E			H_O			PCR+			ADO			FA		
				ME	MC	MD	ME	MC	MD	ME	MC	MD	ME	MC	MD	ME	MC	MD	ME	MC	MD	ME	MC	MD
M1	A13	6-FAM	0.3	411–439	197–203	192–218	14	3	10	0.87	0.56	0.86	0.81	0.60	0.80	0.76	0.70	0.89	0.20	0.00	0.00	0.00	0.00	0.00
	H19	NED	0.2	82–104	87–89	89–107	11	5	9	0.75	0.73	0.85	0.41	0.72	0.85	0.90	0.94	0.97	0.16	0.00	0.00	0.00	0.00	0.00
	H29	6-FAM	0.2	164–184	168–174	158–186	9	2	12	0.76	0.50	0.90	0.58	0.44	0.95	0.79	1.00	1.00	0.23	0.00	0.00	0.00	0.00	0.00
M2	D15	6-FAM	0.2	82–96	97–105	95–131	6	4	10	0.71	0.65	0.86	0.65	0.76	0.60	0.95	1.00	1.00	0.10	0.00	0.00	0.00	0.05	0.00
	E24	NED	0.2	220–246	228–234	213–249	12	4	15	0.84	0.61	0.90	0.91	0.68	1.00	0.70	0.88	0.98	0.28	0.00	0.00	0.01	0.00	0.00
M3	G25	6-FAM	0.1	128–130	131–133	131–135	2	2	3	0.01	0.50	0.63	0.00	0.52	0.10	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.03	0.00
	F19	NED	0.4	198–200	197–205	197–213	2	4	9	0.06	0.70	0.78	0.00	0.64	0.70	1.00	0.80	1.00	0.00	0.00	0.00	0.02	0.02	0.00
						Mean	8.00	3.43	9.71	0.57	0.61	0.82	0.48	0.62	0.71	0.87	0.90	0.98	0.14	0.00	0.00	0.01	0.01	0.00
						SD	4.80	1.13	3.64	0.37	0.09	0.10	0.37	0.11	0.30	0.12	0.12	0.04	0.11	0.00	0.00	0.02	0.01	0.00

Genetic variability

Allelic ranges and species-specific alleles were described for the three species using both tissue and dropping genotypes (Tab. 3, Tab. S4). All the seven microsatellites were polymorphic in the three species, with a mean number of alleles per locus of 8.00±4.80 (range 2–14) in ME, 3.43±1.13 (2–5) in MC and 9.71±3.64 (3–15) in MD (Tab. 3). Only 2 out of 123 identified alleles (1.63%) were shared by all species, while six (4.88%) were shared exclusively between ME and MC, seven (5.69%) between ME and MD and 14 (11.38%) between MC and MD (Tab. S4). Conversely, among the 123 alleles described, 45±4.83 (36.58%) were species-specific for ME, 6±1.21 (4.89%) for MC and 49±4.51 (39.84%) for MD (Tab. S4).

The mean observed heterozygosity was 0.48±0.37 in ME, 0.62±0.11 in MC and 0.71±0.30 in MD, not significantly different (χ^2=0.03, p>0.001; χ^2 test) from their expected heterozygosity (0.57±0.37, 0.61±0.09 and 0.82±0.10, respectively).

Micro-Checker rejected the hypothesis of putative null alleles. We observed significant departures from HWE after Bonferroni correction (p<0.0023): two loci deviated significantly from HWE in ME (G25 and F19) and in MD (G25 and H24); these loci were thus excluded from further analysis with Structure. No significant departure form HWE was detected in MC.

Structure result showed that for ME the optimal number of genetic clusters was set at K=2 (Fig. 3), revealed by the best ln$p$(K)=-2300.80 (K=1, average ln$p$(K)=-2406.24; and K>3, average ln$p$(K)$\geqslant$ -2327.94). At K=2 all ME genotypes were assigned to two clusters with average Q_{ME1}=1.00 (90% CI: 0.99–1.00) and Q_{ME2}=1.00 (90% CI: 0.99–1.00) (Fig. 4A). Considering MC colonies, Structure revealed that the optimal K was 1, having the best ln$p$(K)=-376.48 (K>2, average ln$p$(K)$\geqslant$ -377.60). At K=1 all genotypes were assigned to one single cluster with Q_{MC}=1.00 (90% CI: 0.99–1.00). Finally MD Structure colonies analysis evinced that the most fitting K was 1, as demonstrated by the best ln$p$(K)=-461.60 (K>2, average ln$p$(K) $\geqslant$ -462.14). At K=1 all MD genotypes were assigned to single cluster with Q_{MD}=1.00 (90% CI: 0.99–1.00). Individual q_i were never <0.98 and ranged between 0.99<q_{ME}<1.00 in ME, 0.99<q_{MC}<1.00 in MC and 0.98<q_{MD}<1.00 in MD. Structure outputs for each species were confirm by PCA analysis performed using GenAlex. MD and MC showing no distinct genetic subpopulation structure but only one component common in all colonies, while ME pointed out two genetic components, resulting copresent in all colonies for this species (Fig. S6). Successively we added samples from others geographic range of ME to better investigated this result, 15 tissue samples from Spain and 4 tissue samples from Morocco. Structure was thus rerun with the same option used for the previous analysis, confirming that the best K was set at 2, but the 4 samples from Morocco showed only one of the two genetic components founded across all colonies, with an individual q_{MEm} ranging from 0.98 to 1.00 (Fig. 4B). The separation into two groups found in the population structure analysis was confirmed by PCA run in GenAlex(Fig. S6D).

Discussion

Given the threatened status of many bat species, in several circumstances NIGS may represent the most appropriate tool to identify species and assess the genetic variability of their populations in a non-invasive way, since such method provides a remarkable amount of information without any direct contact with the animals (Taberlet and Luikart, 1999). To overcome the difficulties in collecting samples containing sufficient amounts of good-quality DNA (Zielinski and Mazurek, 2007; Puechmaille et al., 2007; Puechmaille and Petit, 2007; Puechmaille and Teeling, 2013), optimizing primer design and selection (e.g. Housley et al., 2006) and implementing appropriate sampling and laboratory techniques (e.g. Beja-Pereira et al., 2009; Soto-Calderón et al., 2009; Stenglein et al., 2010), in this study we developed a sampling protocol maximising the freshness of droppings under analysis (Santini et al., 2007) while limiting cross-contaminations, and designed laboratory protocols (Fig. S2) to increase amplification success and reduce working time and costs Taberlet and Luikart (1999); Caniglia et al. (2014).

Sampling strategy: maximizing DNA quality for DNA analysis

Sampling protocols represent a crucial step in non-invasive genetic studies. Indeed, not only should they be as accurate and efficient as possible, but also well planned and designed for the specific purposes of the project (Puechmaille and Petit, 2007; Beja-Pereira et al., 2009). Most of the existing non-invasive genetic studies on bats primarily aimed to estimate the size of the monitored populations (Puechmaille and Petit, 2007). Thus, they applied sampling schemes designed to collect as much material as possible per sampling session (Puechmaille and Petit, 2007; Puechmaille et al., 2007). As the main aims of this study were to develop a set of markers to allow cross-species amplifications, multi-species detection, individual identification, and to investigate the genetic variability of the bat species studied, we developed a non-invasive sampling method based on a single and short sampling session per colony (ca. six hours). In this way it was possible to reduce disturbance as well as the possibility of contamination, avoiding individual resampling and providing a good representation of the colony under investigation.

Sample choice and species identification by mtDNA

A correct and unambiguous molecular identification of material collected non-invasively is the second critical step to ensure that only target species' samples will be used for further population genetic analysis, especially when habitat or trophic niches can potentially overlap between species exhibiting similar ecological requirements (Siemers et al., 2001; Fenton and Bogdanowicz, 2002; Ruiz-Gonzalez et al., 2015).

Previous studies on bats showed that sequencing large fragments (>600 bp) of mitochondrial DNA from well-preserved faecal samples is achievable and can be performed routinely with high success rates (even >85%; e.g. Boston et al., 2011; Ruedi et al., 2012; Goodman et al., 2012; Boston et al., 2012). Concordantly, ca. 86% of the droppings analysed in this study were successfully sequenced and most of the Cyt-b haplotypes identified in faecal samples matched those obtained from reference tissue samples, confirming the correct identification of the three bats species from the non-invasive DNA analysis. A lower sequencing success rate (ca. 50%) was obtained for MC droppings, probably because the material was degraded by the high humidity of the roost — a cave on a protected island (Isola Bella, Lago Maggiore).

Selection and performance of microsatellites

Microsatellites represent powerful and presumably neutral genetic markers, commonly used to answer a variety of questions in population genetics and ecology (Allendorf et al., 2013; Wultsch et al., 2014). We succeeded in cross-amplifying 13 microsatellites, originally developed for *M. myotis* (Castella and Ruedi, 2000), in three other vespertilionids (i.e. ME, MC and MD). The seven best performing markers were organised in three multiplexed PCRs, since a lower number of multiplexes

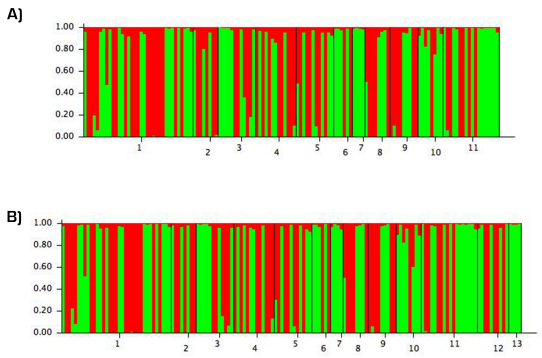

Figure 4 – Subpopulation assignment of *Myotis emarginatus* (ME) genotypes performed by Structure assuming K=2 (optimal number of genetic clusters) and using dropping and reference tissue sample genotypes. Each subpopulation cluster is represented by a colour (green and red). 4A: Structure plot for the Italian colonies of ME numbered from 1–11 (see Tab. 1 for further details); 4B: Structure plot including Italian colonies (1–11), Spanish (12) and Moroccan sample.

was not achievable because of the different annealing temperatures of primers, and the need to avoid overlap between amplicons. However, the three best performing loci were included in multiplex M1, which was used for a further quality screening step, while multiplex M2 and M3 were developed to reach a finer resolution for species and individual identification, as well as genetic structure (Fig. S2). The high genotyping success rate we obtained (86% of the samples that passed the M1 screening showed a complete genotyping profile) is comparable with that of another NIGS study on bats (91%; Puechmaille and Petit, 2007). Genotyping error rates were relatively low compared to other non-invasive genetic studies on bats (*Rhinolophus hipposideros*, Puechmaille and Petit, 2007; *Myotis nattereri*, Boston et al., 2012.

Moreover, the high probabilities of identity we obtained confirmed that the panel of loci we optimized can be used to distinguish *Myotis* bats individually with $>$ 99% likelihood of correct classification (Mills et al., 2000; Waits et al., 2001).

Genetic variability

As only a few previous studies have focused on the population genetic variability of mouse-eared bat species (MC and MD; Bilgin et al., 2008; Ngamprasertwong et al., 2008) and none had been conducted on Italian populations, our study adds novel information on their gene pool and represents the first genetic survey of ME, for which no previous genetic data were available (Dekeukeleire et al., 2013).

All species were polymorphic at the selected loci and showed species-specific alleles. DAPC multivariate assignment tests clearly separated the three species as distinct genetic units. Moreover, both reference tissue and non-invasively collected samples were assigned to the expected *Myotis* taxon, further confirming mtDNA species identification. Considering separately each species in MC and MD both Structure and PCA analysis revealed that there was not a clear genetic structure; in fact only one genetic component was found in all colonies both in MC and MD. This situation was probably due to fact that in both cases colonies are quite close to each other, with a distance of about 50 km and because both the MC and MD move between roosting sites (Papadatou et al., 2008; Ngamprasertwong et al., 2008); moreover, the limited numbers of sampled colonies (2, both for MC and MD) could have affected the final result. Differently in ME the analysis with Structure and GenAlex showed that there were two distinct genetic components. Extra samples from Spain and Morocco were added to verify if the situation was the same in other colonies outside Italy. The subsequently analysis confirm the previous result, but the Moroccan samples showed only one of the two genetic component, while in the other European colonies both components were present. These results allowed to describe for ME subpopulation the presence of one genetic component as characteristic of Morocco, considering the fact that the four samples from that area showed all the same component, which was found in others colonies; while the second component might be typical of populations in Europe, because it was found in all Italian and Spain colonies, but not in the samples from Morocco. This hypothesis may be supported by the fact that ME could have an African ancestor as suggested by its phylogenetic position in a specific Ethiopian clade (Stadelmann et al., 2004).

MC and MD showed high levels of genetic variability. In MC, heterozygosity was comparable (H_o=0.62; Tab. 3) to that reported for populations of Greece and Turkey (mean H_o=0.60 ranging from 0.10 to 0.75; Bilgin et al., 2008) and MD showed similar values (H_o=0.71) to those found in Scottish populations (H_o=0.64–0.84; Ngamprasertwong et al., 2008).

Conversely, ME showed the lowest heterozygosity values among the three species analysed. This might witness the occurrence of a recent bottleneck, as seen for Belgian and Dutch populations (Dekeukeleire et al., 2013), or be due to an ascertainment bias, since ME is phylogenetically more distant from *Myotis myotis* (the species from which the microsatellites we used were originally identified) than MD and MC (Stadelmann et al., 2004), thus leading to an underestimation of the true variability.

Conclusions

The different molecular markers we used (mtDNA and nDNA) provided reliable species and individual identification of three different mouse-eared bat species. Our sampling method is efficient and easily applicable, showing that the freshness of the samples, together with the optimized multiple-tube approach, allowed us to achieve high genotyping success and low error rates.

This study demonstrates that NIGS, followed by a strict step-by-step laboratory workflow, represents a powerful tool to study bats, and highlights how non-invasive genetic methods can provide a reliable and cost-effective multi-species surveying approach for bat populations that are otherwise difficult to investigate, as long as the allele frequencies of the markers employed are variable enough to discriminate the target species. Moreover, the availability of entire genomes for a growing number of taxa can foster the development of species-informative markers (Allendorf et al., 2010). Thus, this approach could likely be used for other bat species, and also for several social vertebrates resting in the forest canopy, on cliffs or other sites difficult to access, such as tropical birds (e.g. the blue-winged macaws *Primolius maracana* or the toco toucan *Ramphastos toco*), arboreal primates (e.g. the Northern rufous mouse lemur *Microcebus tavaratra*) and nocturnal raptors (e.g. the European eagle owl *Bubo bubo*,or the Eurasian pygmy owl *Glaucidium passerinum*), providing a widely applicable and valuable tool for conservation genetic studies.

References

Agnelli P., Martinoli A., Patriarca E., Russo D., Scaravelli D., Genovesi P., 2004. Linee guida per il monitoraggio dei Chirotteri: indicazioni metodologiche per lo studio e la conservazione dei pipistrelli in Italia. Quaderni di Conservazione della Natura. Ministero dell'Ambiente e della Tutela del Territorio - Istituto Nazionale per la Fauna Selvatica "A. Ghigi", Roma.

Allendorf F.W., Hohenlohe P.A., Luikart G., 2010. Genomics and the future of conservation genetics. Nature Genetics Reviews 11: 697–709.

Allendorf F.W., Luikart G., Aitken S.N., 2013. Conservation and the genetics of populations Blackwell Pub., Malden, MA, Oxford.

Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403–410.

Arandjelovic M., Head J., Kühl H., Boesch C., Robbins M.M., Maisels F., Vigilant L., 2010. Effective non-invasive genetic monitoring of multiple wild western gorilla groups. Biological Conservation 143(7): 1780–1791.

Atterby H., Aegeter J.N., Smith G.C., Conyers C.M., Allnutt T.R., Ruedi M., Mac Nicoll A.D., 2010. Population genetic structure of the Daubenton's bat (*Myotis daubentonii*) in western Europe and associated occurrence of rabies. European Journal of Wildlife Research 56: 67–81.

Beja-Pereira A., Oliveira R., Alves P.C., Schwartz M.K., Luikart G., 2009. Advancing ecological understandings through technological transformations in non-invasive genetics. Molecular Ecology Resources 9: 1279–1301.

Bilgin R., Karataş A., Çoraman E., Morales J.C., 2008. The mitochondrial and nuclear genetic structure of *Myotis capaccinii* Chiroptera: Vespertilionidae in the Eurasian transition, and its taxonomic implications. Zoologica Scripta 37: 253–262.

Blumenbach J.F., 1779. Handbuch der naturgeschichte, Volume 1. Jonathan Christian Dieterich. Göttingen.

Bogdanowicz W., Hulva P., Černá Bolfíková B., Buś M.M., Rychlicka E., Sztencel-Jabłonka A., Cistrone A., Russo D., 2015. Cryptic diversity of Italian bats and the role of the Apennine refugium in the phylogeography of the Western Palaearctic. Zoological Journal of the Linnean Society 174: 635–648.

Boston E.S.M., Hanrahan N., Puechmaille S.J., Ruedi M., Buckley D.J., Lundy M.G., Scott D.D., Prodöhl P.A., Montgomery W.I., Teeling E.C., 2011. A rapid PCR-based assay for identification of cryptic *Myotis* spp. (*M. mystacinus*, *M. brandtii* and *M. alcathoe*). Conservation Genetics Resources 3: 557–563.

Boston E.S.M., Puechmaille S.J., Buckley D., Lundy M., Scott D.D., Prodohl P.A., Montgomery I.W., Teeling E.C., 2012. Empirical assessment of non-invasive population genetics in bats: comparison of DNA quality from faecal and tissue samples. Acta Chiropterologica 14: 45–52.

Britton A.R.C., Jones G., Rayner J.M.V., Boonman A.M., Verboom B., 1997. Flight performance, echolocation and foraging behaviour in pond bats, *Myotis dasycneme* (Chiroptera: Vespertilionidae). Journal of Zoology (London) 241: 503–522.

Broquet T., Menard N., Petit E., 2007. Non-invasive population genetics: a review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates. Conservation Genetics 8: 249–260.

Caniglia R., Fabbri E., Mastrogiuseppe L., Randi E., 2013. Who is who? Identification of livestock predators using forensic genetic approaches. Forensic Science International: Genetics 7: 397–404.

Caniglia R., Fabbri E., Galaverni M., Milanesi P., Randi E., 2014. Non-invasive sampling and genetic variability, pack structure, and dynamics in an expanding wolf population. Journal of Mammalogy 95: 41–59.

Castella V., Ruedi M., 2000. Characterization of highly variable microsatellite loci in the bat *Myotis myotis* (Chiroptera: Vespertilionidae). Molecular Ecology 9: 1000–1002.

Červerný J., 1999. *Myotis emarginatus* (E. Geoffroy, 1806). In: Mitchell-Jones A.J., Bogdanowicz W., Krystufek B., Reijnders P.J.H., Spitzenberger F., Stubbe C., Thissen J.B.M., Vohralík V., Zima J. (Eds.) The atlas of European mammals. Academic Press, London. 112–113.

Dekeukeleire D., Janssen R.E.A., Van Schaik J., 2013. Frequent melanism in Geoffroy's bat (*Myotis emarginatus*, Geoffroy 1806). Hystrix 24(2): 197–198. doi:10.4404/hystrix-24.2-8770

Falush D., Stephens M., Pritchard J.K., 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164: 1567–1587.

Fenton M.B., Bogdanowicz W., 2002. Relationships between external morphology and foraging behaviour: bats in the genus *Myotis*. Canadian Journal of Zoology 80: 1004–1013.

Flaquer C., Ruìz-Jarillo R., Arrizabalaga A., 2004. Contribución al conocimiento de la distribución de la fauna quiropterológica de Cataluña. Galemys 16: 39–55. [in Spanish]

Furmankiewicz J., Altringham J.D., 2007. Genetic structure in a swarming brown long-eared bat (*Plecotus auritus*) population: evidence for mating at swarming sites. Conservation Genetics 8: 913–923.

Goodman S.M., Puechmaille S.J., Friedli-Weyeneth N., Gerlach J., Ruedi M., Schoeman M.C., Stanley W.T., Teeling E.C., 2012. Phylogeny of the Emballonurini (Emballonuridae) with descriptions of a new genus and species from Madagascar. Journal of Mammalogy 93: 1440–1455.

Goiti U., Aihartza J., Guiu M., Salsamendi E., Almenar D., Napal M., Garin I., 2011. Geoffroy's bat, *Myotis emarginatus*, preys preferentially on spiders in multistratified dense habitats: a study of foraging bats in the Mediterranean. Folia Zoologica 60: 17–24.

Grzimek B., 2004. Grzimek's animal life encyclopedia. Volume 13: Mammals II. Second Edition. The Gale Group, location Farmington Hills, Michigan.

Hall T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Haysom K., Dekker J., Russ J., van der Meij T., van Strien A., 2013. Technical report No 19/2013 — European bat population trends. European Environment Agency.

Holleley C.E., Geerts P.G., 2009. MULTIPLEX MANAGER 1.0: a crossplatform computer program that plans and optimizes multiplex PCR. Biotechniques 46: 511–517.

Housley D.J.E., Zalewski Z.A., Beckett S.E., Venta P.J., 2006. Design factors that influence PCR amplification success of cross-species primers among 1147 mammalian primer pairs. BMC Genomics 7: 253.

Ibáñez C., García-Mudarra J.L., Ruedi M., Stadelmann B., Juste J., 2006. The Iberian contribution to cryptic diversity in European bats. Acta Chiropterologica 8: 277–297.

Irwin D.M., Kocher T.D., Wilson A.C., 1991. Evolution of the cytochrome b gene of mammals. Journal of Molecular Ecology 32: 128 –144.

Jombart T., 2008. `Adegenet`: a R package for the multivariate analysis of genetic markers. Bioinformatics 24: 1403–1405.

Kaňuch P., Hájková P., Řehák Z., Bryja J., 2007. A rapid PCR-based test for species identification of two cryptic bats *Pipistrellus pipistrellus* and *P. pygmaeus* and its application on museum and dropping samples. Acta Chiropterologica 9: 277–282.

Kerth G., Kiefer A., Trappmann C., Weishaar M., 2003. High gene diversity at swarming sites suggest hot spots for gene flow in the endangered Bechstein's bat. Conservation Genetics 4: 491–499.

Koopman K.F., 1994. Chiroptera: systematics. In: Niethammer J., Schliemann H., Starck D. (Eds.) Handbuch der Zoologie. De Gruyter, Berlin, Germany. [in German]

Kunz T.H., 1988. Ecological and behavioural methods for the study of bats. Smithsonian Institution Press, Washington.

Librado P., Rozas J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452.

Miller C., Joyce P., Waits L.P., 2002. Assessing allelic dropout and genotype reliability using maximum likelihood. Genetics 160: 357–366.

Mills L.S., Citta J.J., Lair K.P., Schwartz M.K., Tallmon D.A., 2000. Estimating animal abundance using non-invasive DNA sampling: promise and pitfalls. Ecological Applications 10: 283–294.

Nardone V., Cistrone L., Di Salvo I., Ariano A., Migliozzi A., Allegrini C., Ancillotto L., Fulco A., Russo D., 2015. How to Be a Male at Different Elevations: Ecology of Intra-Sexual Segregation in the Trawling Bat *Myotis daubentonii*. PLOS ONE 10: e0134573. doi:10.1371/journal.pone.0134573

Ngamprasertwong T., Mackie I.J., Racey P.A., Piertney S.B., 2008. Spatial distribution of mitochondrial and microsatellite DNA variation in Daubenton's bat within Scotland. Molecular Ecology 17: 3243–3258.

Papadatou E, Butlin R.K., Altringham J.D., 2008. Seasonal roosting habits and population structure of the long-fingered bat *Myotis capaccinii* in Greece. Journal of Mammalogy 89: 503–512.

Peakall R., Smouse P., 2012. GenAlex 6.5: genetic analysis in Excel. Population genetic software for teaching and research — an update. Bioinformatics 28L 2537–2539.

Pompanon F., Bonin A., Bellemain E., Taberlet P., 2005. Genotyping errors: causes, consequences and solutions. Nature Reviews Genetics 6: 847–856.

Puechmaille S.J., Mathy G., Petit E.J., 2007. Good DNA from bat droppings. Acta Chiropterologica 9: 269–276.

Puechmaille S.J., Petit E.J., 2007. Empirical evaluation of non-invasive capture-mark-recapture estimation of population size based on a single sampling session. Journal of Applied Ecology 44: 843–852.

Puechmaille S.J., Teeling E.C., 2013. Non-invasive genetics can help find rare species: a case study with *Rhinolophus mehelyi* and *R. euryale* (Rhinolophidae: Chiroptera) in Western Europe. Mammalia 78: 251–255.

R Development Core Team, 2009. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Racey P.A., Entwistle A.C., 2000. Life-history and reproductive strategies of bats. In: Crichton E.G., Krutzsch P.H. (Eds.) Reproductive biology of bats. Academic Press, London. 314–364.

Rousset F., 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Molecular Ecology Resources 8: 103–106.

Ruedi M., Mayer F., 2001. Molecular systematics of bats of the genus *Myotis* (Vespertilionidae) suggests deterministic ecomorphological convergences. Molecular Phylogenetics and Evolution 21: 436–448.

Ruedi M., Friedli-Weyeneth N., Teeling E.C., Puechmaille S.J., Goodman S.M., 2012. Biogeography of Old World emballonurine bats (Chiroptera: Emballonuridae) inferred with mitochondrial and nuclear DNA. Molecular Phylogenetics and Evolution 64: 204–211.

Ruiz-Gonzalez A., Cushman S.A., Madeira M.J., Randi E., Gómez-Moliner B.J., 2015. Isolation by distance, resistance and/or clusters? Lessons learned from a forest-dwelling carnivore inhabiting a heterogeneous landscape. Molecular Ecology 24: 5110–5129.

Russo D., Jones G. 2015. Bats as bioindicators: an introduction. Mammalian Biology, 80, 157–246.

Santini A., Lucchini V., Fabbri E., Randi E., 2007. Ageing and environmental factors affect PCR success in wolf (*Canis lupus*) excremental DNA samples. Molecular Ecology Notes 7: 955–961.

Siemers B.M., Stilz P., Schnitzler H.U., 2001. The acoustic advantage of hunting at low heights above water: behavioural experiments on the European "trawling" bats *Myotis capaccinii*, *M. dasycneme* and *M. daubentonii*. The Journal of Experimental Biology 204: 3843–3854.

Siemers B.M., Dietz C., Nill D., Schnitzler H.U. 2001. *Myotis daubentonii* is able to catch small fish. Acta Chiropterologica 3: 71–75.

Simmons N.B., 2005. Order Chiroptera. In: Wilson D.E., Reeder D.M. (Eds.) Mammal Species of the World: A Taxonomic and Geographic Reference. Johns Hopkins University Press, Baltimore.

Soto-Calderón I.D., Ntie S., Mickala P., Maisels F., Wickings E.J., Anthony N.M., 2009. Effects of storage type and time on DNA amplification success in tropical ungulate faeces. Molecular Ecology Resources 9: 471–479.

Stadelmann B., Jacobs D.S., Shoeman C., Ruedi M., 2004. Phylogeny of African *Myotis* bats (Chiroptera, Vespertilionidae) inferred from cytochrome b sequences. Acta Chiropterologica 6: 177–19

Stenglein J.L., Debarba M., Ausband D.E., Waits L.P., 2010. Impacts of sampling location within a faeces on DNA quality in two carnivore species. Molecular Ecology Resources 10: 109–114.

Stubbe M., Ariunbold J., Buuveibaatar V., Dorjderem S., Monkhzul Ts., Otgonbaatar M., Tsogbadrakh M., Hutson A.M., Spitzenberger F., Aulagnier S., Juste J., Coroiu I., Paunovic M., Karataş A., 2008. *Myotis daubentonii*. The IUCN Red List of Threatened Species 2008.

Taberlet P., Luikart G., 1999. Non-invasive genetic sampling and individual identification. Biological Journal of the Linnean Society 68: 41–55.

Taberlet P., Waits L., Luikart G., 1999. Non-invasive genetic sampling: look before you leap. Trends in Ecology and Evolution 14: 323–327.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876–4882.

Valière N., 2002. Gimlet: a computer program for analysing genetic individual identification data. Molecular Ecology Notes 10: 1046–1046.

Valière N., Bonenfant C., Toigo C., Luikart G., Gaillard J.M., Klein F., 2007. Importance of a pilot study for non-invasive genetic sampling: genotyping errors and population size estimation in red deer. Conservation Genetics 8: 69–78.

Van Oosterhout C., Hutchinson W.F., Wills D.P.M., Shipley P., 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Resources 4: 535–538.

Waits L.P., Luikart G., Taberlet P., 2001. Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. Molecular Ecology 10: 249–256.

Waits L.P., Paetkau D., 2005. Non-invasive genetic sampling tools for wildlife biologists: A review of applications and recommendations for accurate data collection. The Journal of Wildlife Management 69: 1419–1433.

Woods J.G., Paetkau D., Lewis D., McLellan B.N., Proctor M., Strobeck C., 1999. Genetic tagging of free-ranging black and brown bears. Wildlife Society Bulletin 27: 616–627.

Worthington Wilmer J., Barratt E., 1996. A non-lethal method of tissue sampling for genetic studies of chiropterans. Bat Research News 37: 1–3.

Wultsch C., Waits L.P., Kelly M.J., 2014. Non-invasive individual and species identification of jaguars (*Panthera onca*), pumas (*Puma concolor*) and ocelots (*Leopardus pardalis*) in Belize, Central America using cross-species microsatellites and fecal DNA. Moleculer Ecology Resources 14: 1171–1182.

Zielinski W.J., Mazurek M.J., 2007. Identifying the species of bats roosting in redwood basal hollows using genetic methods. Northwest Science 81: 155–162.

Associate Editor: P. Colangelo

Supplemental information

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

Table S1 Total list of haplotypes in *Myotis* spp.

Figure S2 Flow chart illustrating the multiple-tube approach developed in this study.

Table S3 Summary of the mtDNA sequencing and microsatellite genotyping.

Table S4 Allele size ranges in base pairs (bp) for tissue and faecal DNA samples.

Figure S5 Discriminant analysis of principal components.

Figure S6 PCA for each bats species using GenAlex.

Data accessibility

The raw microsatellite data of the 178 individuals of the three *Myotis* spp. will be submitted to DRYAD (http://datadryad.org/) upon acceptance of the manuscript.