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METHODS PAPER

How to spot a black-footed cat? Successful application of cross-species markers to identify captive-bred individuals from non-invasive genetic sampling

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Abstract

The black-footed cat (*Felis nigripes*) is the smallest felid of Southern Africa, endemic to the arid steppe and savannah habitats. However, though threatened and characterized by decreasing sizes of its populations, a number of ecological, demographic, sanitary, and genetic aspects, essential for the long-term conservation of the species, still remain poorly known. Non-invasive genetic sampling may represent an appropriate and cost-effective tool to fill this lack of information. Thus, for the first time so far, we developed a protocol for species and individual identification of black-footed cats, starting from markers originally designed for the domestic cat and from 23 non-invasively collected samples of captive-bred individuals. We then tested its genotyping efficiency and reliability for future applications in non-invasive genetic monitoring programs of the wild populations. Most of the samples (65%), corresponding to 15 individuals, were successfully genotyped at 316 bp of the mtDNA ND5 and at nine autosomal microsatellites. We detected two black-footed cat mtDNA ND5 haplotypes that were clearly distinguishable from all the other wild and domestic felids. All microsatellites were polymorphic and showed low error rates, probabilities of identity < 0.001 and a mean observed heterozygosity $H_O = 0.68$. Subsequent approximate Bayesian computation simulations confirmed that black-footed cats and African and European wildcats likely experienced sequential population splittings that started during the Late Pliocene and continued through the Early Pleistocene. Our study provided the first reliable and cost-effective molecular multilocus characterization of non-invasively collected samples of black-footed cats. Though solely tested on captive-bred individuals, our method could be applied to design and implement effective long-term monitoring and conservation plans of poorly investigated black-footed cat wild populations.

Keywords Black-footed cats · Conservation genetics · Felids · Phylogenetics · Genetic variability · Microsatellites · mtDNA · Non-invasive genetic sampling

Introduction

The black-footed cat *Felis nigripes* (Burchell 1824) belongs to the order Carnivora, family Felidae, genus *Felis* (O'Brien and Johnson 2007) and, based on genomic analyses (Li et al. 2016), has been recently listed in the domestic cat lineage, which includes the African (*Felis s. lybica*; *Felis margarita*), Asian (*Felis s. bieti*; *Felis chaus*) and European (*Felis s. silvestris*) wildcats, together with the domesticated subspecies (*Felis s. catus*). Conversely to the African and European wildcats, for the black-footed cat to date, there are no evidences of inter-specific hybridization in the wild (Sliwa et al. 2016), whereas there are a few confirmed cases of black-footed cat *x* domestic cat hybrids documented in captive conditions (Leyhausen 1979). The black-footed cat is described as the smallest and rarest felid of Southern Africa (Nowell and Jackson 1996; Sliwa 2008; Sliwa et al.

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2016), being distributed only in South Africa, Namibia, and Botswana, with sporadic and marginal sightings in Zimbabwe and Southern Angola (Macdonald and Loveridge 2010; Sliwa et al. 2016). These factors, combined with the elusive and crepuscular behavior of this wild cat, have made it one of the least studied African carnivores (Sliwa 2004): only few research projects have been conducted so far and some of the available data are extrapolated solely from observations from captive individuals (Wells and Egli 2004; Sliwa 2008; Oh et al. 2017). Additionally, there is a still open debate about the possibility that two morphologically distinct subspecies exist (Meester et al. 1986): a northern subspecies, *Felis nigripes nigripes* (Shortridge 1931), native to the Kalahari, comprising South Africa, Namibia, and Botswana, and a southern one, *Felis nigripes thomasi* (Meester et al. 1986), described from the Eastern Cape of South Africa (Olbricht and Sliwa 1997). However, there are neither geographical nor ecological demarcations between their ranges (Olbricht and Sliwa 1997; Renard et al. 2015) and the differences in color, body size, and tail length used to distinguish them could represent individual variation detected in both captive and wild black-footed cats, rather than indexes of taxonomic differentiations (Olbricht and Sliwa 1997).

Despite these knowledge gaps, the species is currently described as vulnerable according to the International Union for Conservation of Nature (IUCN) red list and included in the Appendix I—threatened with extinction—by the Convention on the International Trade of Endangered Species (CITES), mainly due to the diffusion of overgrazing and extensive agriculture throughout its distribution range, which can cause fragmentation and degradation of its natural habitats and non-negligible declining trends of the abundance of wild populations (Sliwa et al. 2016).

Thus, reliable data about black-footed cat presence, distribution, and population structure are fundamental to design sound conservation strategies and effective management plans (Hedrick and Miller 1992; Bonin et al. 2007). However, monitoring a species so difficult to detect by classical surveying methods, such as direct counting, camera trapping, telemetry, and genetic analyses from captured individuals, is challenging because of the difficult individual identifications and the low trapping success (Caniglia et al. 2014; Kraus et al. 2015; Norman and Spong 2015; Viglino et al. 2016; Granroth-Wilding et al. 2017). Moreover, the application of techniques that require animal capture and handling can represent an additional source of disturbance when frequently or incorrectly carried out, with possible detrimental conservational consequences. Non-invasive genetic sampling (NIGS) approaches, though they require accurate field sampling strategies and are often based on low-quality or quantity DNA, can provide a safe, efficient, and cost-effective alternative tool (Lukacs et al. 2007; Beja-Pereira et al. 2009), as widely demonstrated for a number of other elusive species (Wultsch et al. 2014; Velli et al. 2015; Kubasiewicz et al. 2017; von Thaden et al. 2017; Verkuil et al. 2018). Well-planned and extended NIGS programs allow to obtain from biological samples such as hair follicles, feces, urine, saliva, and blood traces, collected without any direct human contact with the animals; reliable individual multilocus genotypes which can be successively used to estimate temporal trends of demographic (abundance and survival); and genetic (genetic variability, gene flow, inbreeding, relatedness, hybridization) parameters that would be almost impossible to achieve with traditional field methods (Waits and Paetkau 2005; Caniglia et al. 2014; Arandjelovic and Vigilant 2018). Moreover, non-invasive genetic data can be also used to clarify the systematic status of taxa and detect eventual cryptic species, subspecies, significantly differentiated populations, or priority management units (Oliveira et al. 2010; Ruiz-González et al. 2013; Adrados et al. 2018). Most non-invasive genetic monitoring studies on felids were based on DNA obtained from trapped hair samples (García-Alaníz et al. 2010; Steyer et al. 2013; Velli et al. 2015), which can be more easily collectable even by non-specialists (e.g., volunteers and park rangers) with the aid of olfactory attractants (Jerosch et al. 2010), are less prone than scat, urine, and saliva samples to DNA degradation caused by environmental factors (Ruell and Crooks 2007) and can represent a good quality DNA source also for genome-wide SNP genotyping (von Thaden et al. 2017; Steyer et al. 2018).

In this pilot study, for the first time to date, we developed a reduced panel of mitochondrial and nuclear molecular markers, originally characterized in the domestic cat (*Felis silvestris catus*; Menotti-Raymond and O'Brien 1995 and Menotti-Raymond et al. 1999) and successfully amplified across several felid taxa (Randi et al. 2001; Pierpaoli et al. 2003; Mattucci et al. 2013), and tested it on a small number of hair DNA samples available from captive-bred black-footed cats aiming at their species and individual identification.

We further evaluated the reliability and efficiency of such small multilocus marker panel in providing a preliminary description of the genetic variability and substructure of this poorly studied species. Since black-footed cats, European wildcats (*Felis s. silvestris*), African wildcats (*Felis s. lybica*), and domestic cats (*Felis s. catus*) are thought to have started to diverge during the last three million years with a progressive decrease of their effective population sizes (Johnson et al. 2006), we tried to reconstruct their phylogenetic relationships, past demographic scenarios, and divergence times, comparing the genetic profiles of the analyzed black-footed cats with published felid mtDNA sequences and microsatellite genotypes of a representative sampling of these three domestic cat lineage taxa (Li et al. 2016), previously typed at the same nuclear marker panel (Mattucci et al. 2013).

Materials and methods

Sample collection and DNA extraction

Twenty-three shed hair tufts containing a large number of follicles and found on the floor of their fences were individually

collected from 23 captive-bred black-footed cats of unknown geographical origins hosted at the Cat Conservation Trust (Cradock) and at the Hoedspruit Endangered Species Centre (Kapama Private Game Reserve) of the Republic of South-Africa, for which no morphological data were provided by collectors. No animals were hurt nor sacrificed for the purposes of this study. Samples were stored at room temperature in dry boxes containing regularly desiccated silica gel (Table 1). Total DNA was automatically extracted in a MULTIPROBE II^{EX} Robotic Liquid Handling System (Perkin Elmer, Heidelberg, Germany) using the *Qiagen DNeasy Blood & Tissue Kit* (Qiagen Inc., Hilden, Germany) and following the manufacturer's instructions but with an initial overnight digestion at 56 °C with Protease K and ATL lysis buffer and a final elution in 100 μL of the 10 μM AE buffer. To check for robustness in the extraction, sequencing, and genotyping procedures, two different hair samples were independently analyzed for two individuals.

Mitochondrial DNA sequencing

Each DNA sample was amplified by polymerase chain reaction (PCR) at a 316-bp fragment of the mtDNA NADH dehydrogenase subunit 5 (ND5) containing diagnostic mutations used by Johnson et al. (2006) to resolve the modern phylogeny of modern felid. Amplifications were performed in 10 μL total reactions containing 2 μL of DNA solution, 1× PCR buffer with 2.5 mM Mg2+, 0.3 μM of primers F2B (5′-TGCCGCCCTACAAGCAAT-3′) and R3B (5′-TAAG

Table 1 Genetic identification of the DNA extracted from the black-footed cat hair samples

ID ISRA[a]	ID sample[b]	Gender[c]	ND5 mtDNA[d]	Genotype[e]	*q*i (90% CI) at *K*2[f]	*q*i (90% CI) at *K*3[g]	*q*i (90% CI) at *K*4h[h]
1683	Sample1	M	H1	nd	nd	nd	nd
1684	Sample2	M	nd	nd	nd	nd	nd
1685	Sample3	M	H2	nd	nd	nd	nd
1686	Sample4	M	H1	Bfc1M	0.998 (0.994–1.000)	0.996 (0.979–1.000)	0.994 (0.967–1.000)
1687	Sample5	M	H2	nd	nd	nd	nd
1688	Sample6	F	H2	nd	nd	nd	nd
1689	Sample7	F	H1	Bfc2F	0.998 (0.993–1.000)	0.996 (0.977–1.000)	0.994 (0.964–1.000)
1690	Sample8	M	H2	Bfc3M	0.997 (0.989–1.000)	0.994 (0.963–1.000)	0.991 (0.947–1.000)
1691	Sample9	F	H1	Bfc4F	0.998 (0.994–1.000)	0.997 (0.980–1.000)	0.995 (0.969–1.000)
1692	Sample10	M	H1	Bfc5M	0.998 (0.994–1.000)	0.996 (0.979–1.000)	0.995 (0.967–1.000)
1693	Sample11	M	H2	Bfc6M	0.998 (0.993–1.000)	0.996 (0.978–1.000)	0.994 (0.966–1.000)
1693b	Sample11b	M	H2	Bfc6M	0.998 (0.993–1.000)	0.996 (0.978–1.000)	0.994 (0.966–1.000)
1694	Sample12	F	H1	Bfc7F	0.998 (0.993–1.000)	0.996 (0.975–1.000)	0.994 (0.962–1.000)
1695	Sample13	M	H1	Bfc8M	0.998 (0.993–1.000)	0.995 (0.974–1.000)	0.993 (0.958–1.000)
1696	Sample14	F	H1	Bfc9F	0.998 (0.994–1.000)	0.996 (0.979–1.000	0.995 (0.967–1.000)
1696b	Sample14b	F	H1	Bfc9F	0.998 (0.994–1.000)	0.996 (0.979–1.000	0.995 (0.967–1.000)
1697	Sample15	M	H1	nd	nd	nd	nd
1698	Sample16	F?	H1	Bfc10F	0.998 (0.994–1.000)	0.996 (0.980–1.000)	0.995 (0.968–1.000)
1699	Sample17	M?	H1	Bfc11M	0.998 (0.994–1.000)	0.997 (0.980–1.000)	0.995 (0.969–1.000)
1700	Sample18	F	H1	Bfc12F	0.998 (0.994–1.000)	0.996 (0.979–1.000	0.995 (0.968–1.000)
1701	Sample19	F	H1	nd	nd	nd	nd
1702	Sample20I	M	H1	nd	nd	nd	nd
1703	Sample21	M	H2	Bfc13M	0.998 (0.994–1.000)	0.997 (0.981–1.000)	0.995 (0.969–1.000)
1704	Sample22	M?	H1	Bfc14M	0.998 (0.992–1.000)	0.995 (0.972–1.000)	0.993 (0.954–1.000)
1705	Sample23	M	H1	Bfc15M	0.998 (0.993–1.000)	0.996 (0.977–1.000)	0.994 (0.963–1.000)

nd indicates information not detectable

[a] ID ISPRA indicates laboratory sample identification number

[b] ID Sample indicates field sample identification number

[c] Gender indicates the sex of each sampled individual: *F*, female; *M*, male

[d] H1 and H2 indicate the two haplotypes identified amplifying the 316-bp fragment of the mtDNA NADH dehydrogenase subunit 5 (ND5)

[e] Individual genotype acronyms (Bfc = black-footed cat, F = female; M = male) of each DNA sample determined at the nine unlinked microsatellite markers analyzed

[f, g, and h] Indicate the proportion of membership of individual genotypes (q_i) and their credibility intervals (90% CI) obtained from Bayesian clustering analyses performed in STRUCTURE with $K = 2$, $K = 3$, and $K = 4$, respectively

AGACGTTTAATGGAGTTGAT-3′; Driscoll et al. 2011) and 0.25 units of Taq polymerase (5 PRIME Inc., Gaithersburg, USA). Amplifications were performed with an initial DNA denaturation step at 94 °C for 2 min, followed by 45 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 15 s, extension at 72 °C for 30 s, and final extension at 72 °C for 10 min. 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 (Applied Biosystems, Foster City, CA) Big Dye Terminator kit v.3.1 with the following steps: 96 °C for 10 s, 50 °C for 5 s, 68 °C for 4 min of final extension (25 cycles). Sequences were analyzed in an ABI DNA Sequencer 3130XL, corrected with the ABI software SEQSCAPE v.2.5 and aligned with the complete black-footed cat mtDNA genome (*Felis nigripes*; NCBI reference sequence: NC028309; Li et al. 2016) using CLUSTAL W (Thompson et al. 1997) in BIOEDIT (Hall 1999). DNASP v.5.10.01 (Librado and Rozas 2009) was used to identify identical haplotypes and, taking into account for the presence of indels, to estimate haplotype (Hd) and nucleotide (π) diversity. Detected unique haplotypes were then compared for possible correspondences with homologue feline sequences available from GenBank using BLAST (Altschul et al. 1990).

Phylogenetic analyses

The detected mtDNA haplotypes were aligned with 18 homologue felid sequences downloaded from GenBank (Online Resource 1, Table S1) to evaluate the efficiency of the amplified short ND5 fragment to distinguish black-footed cats from closely related felid taxa. Such alignment was run in JMODELTEST2 (Darriba et al. 2012) to estimate the best nucleotide substitution model through the Akaike information criterion (AIC) and to construct in MEGA v.7.0 (Kumar et al. 2016) a Neighbor-Joining (NJ; Saitou and Nei 1987) phylogenetic tree, which was rooted using a servaline genet sequence (*Genetta servalina*, GenBank access number KJ624980.1; Hassanin 2014) as an outgroup and whose internode supports were obtained by 10,000 bootstrap replicates (Felsenstein 1985).

Microsatellite genotyping and error rate analyses

Nine feline-unlinked autosomal microsatellites (Fca126, Fca132, Fca23, Fca26, Fca43, Fca149, Fca58, Fca88, Fca08; Menotti-Raymond and O'Brien 1995; Menotti-Raymond et al. 1997), chosen for their high variability among different marker panels applied in some of the most recent studies on wildcat population genetics and hybridization in Europe (Pierpaoli et al. 2003; Oliveira et al. 2008; Anile et al. 2012; Mattucci et al. 2015), were used to test their cross-species amplification success and polymorphism in black-footed cats.

Microsatellites were amplified in three multiplexed reactions using the QIAGEN Multiplex PCR kit (Qiagen Inc., Hilden, Germany). Amplifications were carried out in 10 μL total volume, including 2 μL of DNA solution, 5 μL Qiagen Multiplex PCR mix (Qiagen Inc., Hilden, Germany), 1 μL Qiagen Q solution, 0.3 μL of 10 μmol primer mix for each primer pair (forward and reverse) 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 for 15 min, 94 °C for 30 s, 57 °C for 90 s, 72 °C for 60 s (45 cycles), followed by 5 min of final extension at 72 °C.

PCR products were analyzed on an ABI 3130XL automated sequencer and the allele sizes of the STR loci were estimated using the ABI ROX 350 size standard and the ABI software GENEMAPPER v.4.0. DNA extraction, amplification, and post-amplification procedures were carried out in separate rooms reserved to low-template DNA samples, adding a blank control (no biological material) during DNA extraction, and blank (no DNA) and positive (known European wildcat-DNA sample) controls during DNA amplification.

Following the multiple-tube approach described in Viglino et al. (2016), hair DNA samples were amplified at the autosomal STRs from four to eight independent times per locus. After the first four replicates at the nine autosomal STR loci, samples showing $\leq 50\%$ positive PCR (PCR+) were discarded. A reliability analysis was performed by the software RELIOTYPE (Miller et al. 2002) on samples showing > 50% PCR+, and unreliable loci (at threshold $R < 0.95$) were additionally replicated another four times. Only samples reliably typed at all loci ($R \geq 0.95$) were definitively accepted.

Consensus genotypes were reconstructed from the four–eight replicates 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, following Pompanon et al. (2005), to estimate PCR success rate (the number of successful PCRs divided by the total number of PCR runs across samples), allelic drop-out (ADO) and false allele (FA) and to match the detected genotypes to each other. The probability of identity (PID) and the expected PID among full sibling dyads (PIDsib; Mills et al. 2000; Waits et al. 2001) were computed by GENALEX v.6.5 (Peakall and Smouse 2012).

Assignment tests and variability analyses

In order to evaluate the resolution power of our small STR marker panel to distinguish closely related felid taxa belonging to the domestic cat lineage (Li et al. 2016) and to confirm the absence of any inter-specific introgression signal in the captive-bred black-footed cat genomes, assignment tests and variability analyses were performed together with the multilocus genotypes of 18 domestic cats (*Felis s. catus*), 19 European wildcats (*Felis s. silvestris*), 21 African wildcats (*Felis s. lybica*; including seven

Saharan and 14 Sardinian individuals), which were typed at the same nine STR marker panel (Mattucci et al. 2013).

Patterns of differentiation among individual genotypes were visualized through a multivariate analysis of principal (PCA) and discriminant (DAPC) components computed with the ADEGENET package (Jombart et al. 2008) implemented in R v.3.4.4 (www.r-project.org, last accessed March 1,2017).

Additionally, multilocus genotypes were assigned to their most likely taxon of origin using a Bayesian clustering model (minimizing departures from HWE and LE in the genetic clusters) implemented in STRUCTURE v.2.3.4 (Pritchard et al. 2000; Hubisz et al. 2009). Four independent runs were performed for increasing values of K (K from 1 to 10) using 1,000,000 Markov chain Monte Carlo (MCMC) iterations, after a burn-in of 100,000 iterations, assuming no prior information (option *usepopinfo* not activated), and choosing the *admixture* and *independent allele frequency* models, which are the most suitable ones to describe populations whose allele frequencies are expected to be reasonably different from each other (Pritchard et al. 2000; Hubisz et al. 2009). The highest rate of increase in the posterior probability LnP(K) between consecutive K was used to estimate most likely number of genetic groups K at which we assessed the average (Q_i) and individual (q_i) proportions of membership to each different cluster (Falush et al. 2003). The software CLUMPP v.1.1.1 (Jakobsson and Rosenberg 2007) was used to concatenate the data from the ten independent runs for each K value, and DISTRUCT v.1.1 (Rosenberg 2004) to graphically display the results. Based on the mtDNA results, to search any eventual substructure, clustering analyses were performed also running the black-footed cat genotypes alone with K from 1 to 5, 1,000,000 Markov chain Monte Carlo (MCMC) iterations, a burn-in of 100,000 iterations, the *admixture* and *independent allele frequency* models, and the option *usepopinfo* activated or not activated. In the former case, we assumed that individuals having mtDNA H1 and H2 (see "Results") were a priori correctly identified and assigned to their own clusters (popflag = 1).

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) were estimated using GENALEX. F statistics and deviations from Hardy-Weinberg (HWE) and Linkage (LE) equilibria were computed in GENETIX v.4.05 (Belkhir et al. 1996–2004) using 10,000 random permutations to assess significance levels.

Approximate Bayesian computation

The efficacy of our nuclear marker panel was further tested by performing approximate Bayesian computation (ABC) simulations (Beaumont et al. 2002) implemented in the software DIYABC 2.1.0 (Cornuet et al. 2014) using microsatellite multilocus genotypes and excluding domestic cats, to model plausible demographic scenarios and estimate divergence times (in generations) among the black-footed cats, the European and the African wildcat taxa used in the clustering analyses. According to Johnson et al. (2006), the tree taxa diverged, with a progressive decrease of their effective population sizes, approximately during the last three million years. Thus, we tested three demographic scenarios (Fig. 1), assuming that the three taxa split sequentially (i) without any bottleneck (scenarios 1), (ii) with simultaneous bottlenecks after the split (scenario 2), or (iii) with independent bottlenecks (scenario 3).

We ran 6×10^6 simulations for each scenario using uniform prior distributions of the effective population size and time parameters with default mutation settings. We selected the following summary statistics for all the microsatellites: (a) one sample: mean number of alleles, mean genetic diversity; (b) two samples: mean number of alleles, mean genetic diversity, F_{ST} (Online Resource 1, Table S2).

Scenarios were compared by estimating posterior probabilities with the logistic regression method in DIYABC using 1% of the simulated datasets. For the best models, posterior distributions of the parameters were estimated with a logit-transformed linear regression on the 1% simulated datasets closest to the observed data. Scenario confidence was evaluated by comparing observed and simulated summary statistics. Finally, the goodness-of-fit of the posterior parameters for the best performing scenario was tested via the model checking option with default settings, and significance was assessed after Bonferroni's correction for multiple testing (Rice 1989; Cornuet et al. 2014).

Results

Mitochondrial DNA analyses

From the 23 analyzed hair samples, we obtained 22 (96%) reliable mtDNA ND5 sequences of 316 bp that, after matching in DNASP, corresponded to two haplotypes (Hd = 0.416; $\pi = 0.013 \pm 0.0063$) differing for four polymorphic sites. Haplotype H1 was shared by 16 individuals and perfectly matched (pairwise identity 100%) to the black-footed cat haplotype AF006400 described by Johnson and O'Brien (1997), whereas the haplotype H2 was shared by seven individuals and perfectly matched (pairwise identity 100%) to the black-footed cat haplotype KP202277 described by Li et al. (2016).

The best fit evolutionary model for the alignment including the 18 felid fragments and the two black-footed cat haplotypes was the TrN + I + G with $I = 0.4950$, G category = 4, G shape = 2.0620, and Kappa = 47. Though the limited number of bp analyzed did not allow to achieve robust supports for most internodes, the NJ tree clearly identified three main clades roughly corresponding to the three main felid genus *Felis* (clade A), *Panthera* (clade B), and *Leopardus* (clade C), with the two black-footed cat haplotypes falling into the

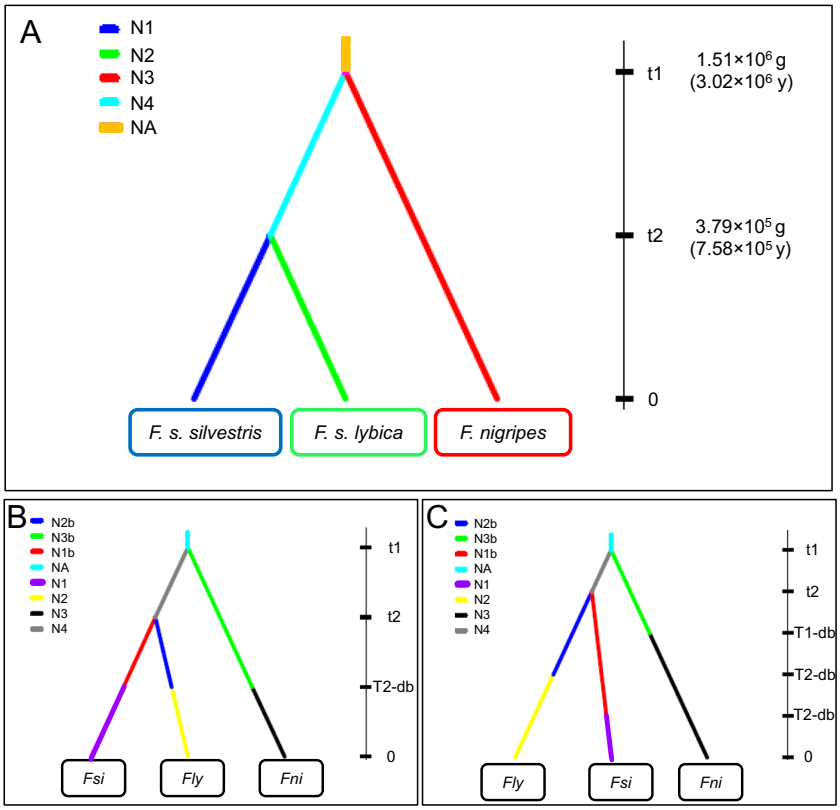

Fig. 1 Graphical representation of the three demographic scenarios in simulated in DIYABC. Scenarios assumed that the three taxa split sequentially (i) without any bottleneck (scenario 1; Fig. 1**a**), (ii) with simultaneous bottlenecks after the split (scenario 2; Fig. 1**b**) or (iii) with independent bottlenecks (scenario 3; Fig. 1**c**). For the best scenario (scenario 1; Fig. 1**a**), the resulting population sizes and divergence times (using a generation time $g = 2$ years) were reported. Fni *Felis nigripes*, Fly *Felis s. lybica*, Fsi *Felis s. silvestris*. N1, N2, N3, N4 black-footed cat-European wildcat-African wildcat post-bottleneck effective population sizes; NA effective population size of the started population, N1b, N2B, N3b, black-footed cat-European wildcat-African wildcat pre-bottleneck effective population sizes; t1 time of divergence from the common ancestor in thousand of generation, db duration of bottleneck

Felis subclade A2, but sharply distinguishable from all the other taxa (Fig. 2).

Microsatellite genotyping and error rate

At the first step of the multi-tube protocol, three samples (13%) were discarded showing PCR+ ≤ 0.50. Ten of the 20 samples (50%) showing PCR+ > 0.50 were directly accepted showing reliability scores $R \geq 0.95$, whereas the other 10 samples ($R < 0.95$) were further amplified four times at unreliable loci, finally allowing to obtain other five samples with $R > 0.95$. Thus, 15 samples (65%) were reliably genotyped at the nine autosomal STRs and after regrouping procedures and error rate analysis corresponded to 15 distinct genotypes (Table 1), showing an average number of positive amplifications per locus of 0.71 (ranging from 0.87 to 0.40) and average error rates ADO = 0.073 (SD = 0.011) and FA = 0.021 (SD = 0.010). The nine analyzed loci allowed to identify black-footed cat genotypes with PID = 3.13×10^{-7} and PIDsib = 1.47×10^{-3}, values low enough to exclude to find by chance more individuals bearing the same genotype in a small population. The pairwise similarity analysis between individuals showed that no pair differed for one or two allele mismatches, whereas at least two pairs showed three mismatches, four pairs showed four mismatches, and the other pairs more than 5 mismatches.

The genotypes of the two individuals that were reconstructed from two independent hair tuft samples regularly matched one another (100%).

Assignment tests and variability analyses

The DAPC (Fig. 3) plot identified three main groups with the black-footed cats clearly separated from the cluster of the European wildcats and from the cluster including the African wildcats and domestic cats, which were mostly overlapped (Fig. 3).

Multivariate analyses were clearly confirmed by the Bayesian clustering procedures implemented in STRUCTURE that showed progressive increase rates in the estimated posterior probability LnP(D) of the clusters until $K = 4$ (Online Resource 2, Fig. S1). At $K = 2$, corresponding to the optimal number of genetic clusters, the black-footed cats (mean estimated membership of population to the assigned cluster $Q_1 = 0.998$) were clearly separated from the other three taxa (Fig. 4a), which clustered together (mean

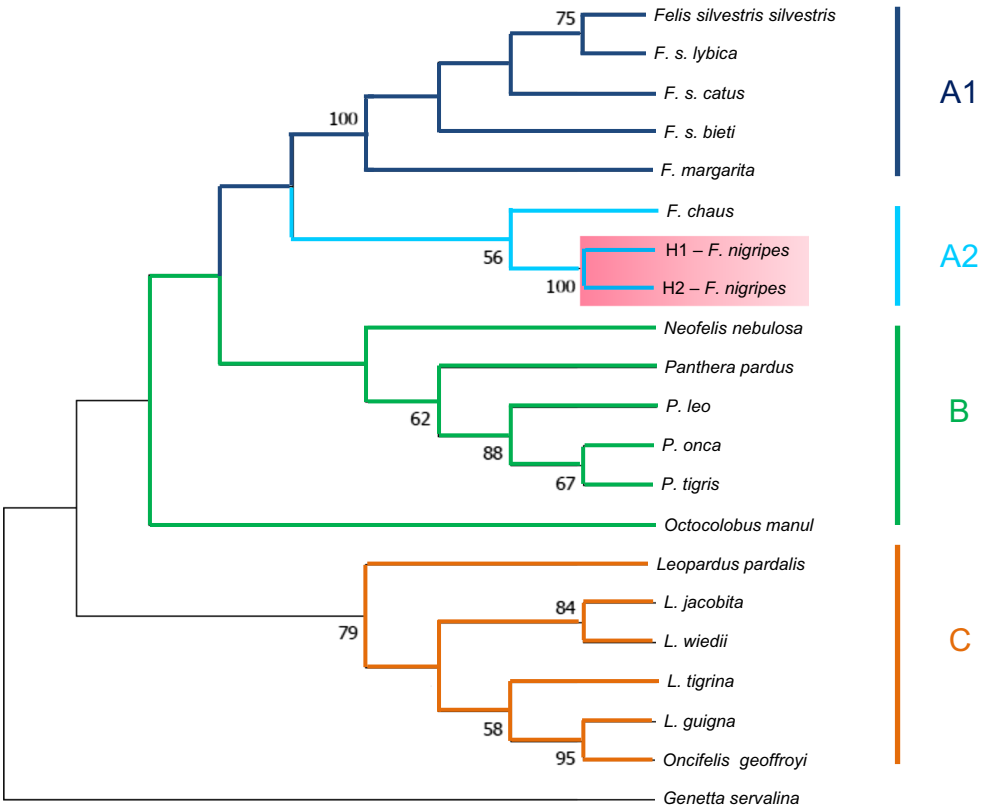

Fig. 2 Neighbor-joining (NJ) mtDNA ND5 phylogenetic tree computed in MEGA. The NJ tree was obtained aligning the two detected black-footed cat ND5 haplotypes with 18 homologue felid sequences and was rooted using a servaline genet sequence (*Genetta servalina)* as an outgroup. Bootstrap percentages ≥ 50% after 10,000 replicates are shown. The four main clades A1, A2, B, and C are indicated

$Q_2 = 0.997$). At $K = 3$ (Fig. 4b), the black-footed cats ($Q_1 = 0.996$) were clearly separated from both the European wildcats ($Q_2 = 0.993$) and from the African wildcats and domestic cats ($Q_3 = 0.979$), which were grouped in a same cluster. At $K = 4$ (Fig. 4c), the black-footed cats were assigned to cluster 1 ($Q_1 = 0.994$), the European wildcats to cluster 2 ($Q_2 = 0.988$), the Sardinian African

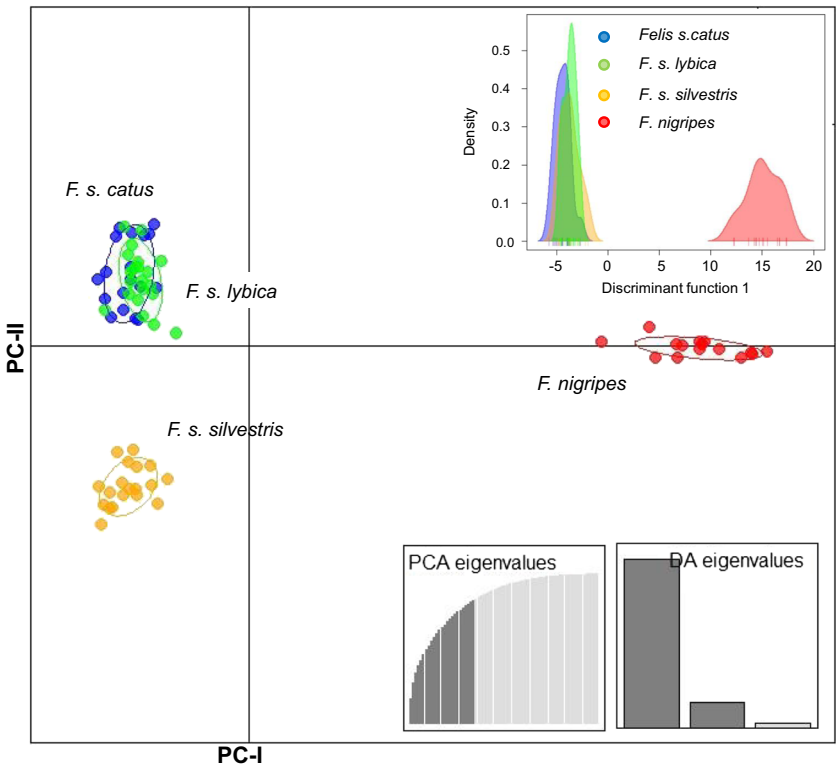

Fig. 3 Discriminant analysis of principal component scatter plot (DAPC, computed in ADEGENET) showing the genetic distinction among the black-footed cats (right side of PC-I), European wildcats (top-left side of PC-I), and the cluster including the African wildcats and domestic cats (bottom-left side of PC-I). PC-I explains 85.29% of the total genetic variability (see also the PCA and the DA eigenvalue histogram insert in the bottom right side). PC-II, explaining 12.56% of the total genetic variability, reinforces the main distinction between the black-footed cats and the other three felid taxa (see also the density plot insert in the in the top right side of the figure)

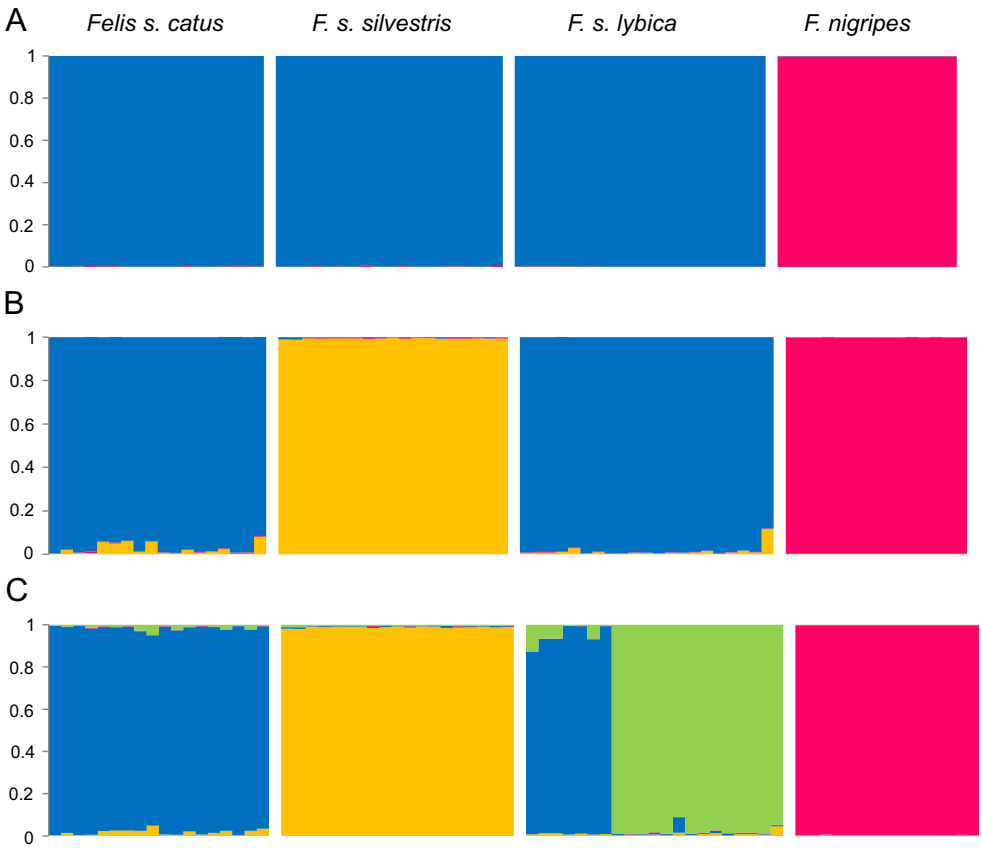

Fig. 4 Bayesian clustering of the black-footed cat, domestic cat, and European and African wildcat samples genotyped with nine autosomal microsatellite loci obtained by STRUCTURE assuming **a** $K = 2$, **b** $K = 3$, and **c** $K = 4$ genetic groups. Each individual is represented by a vertical line partitioned into K colored segments, whose length is proportional to the individual coefficients of membership in the assumed K clusters. At $K = 2$, corresponding to the optimal number of genetic groups, the black-footed cats and the other three felid taxa cluster separately. At $K = 3$, the three genetic groups are composed by the black-footed cats, the European wildcats, and the domestic and African wildcats. At $K = 4$, the Sardinian African wildcats separate in their own cluster, while the Saharan African wildcats continued to cluster together with domestic cats

wildcats to cluster 3 ($Q_3 = 0.941$), and the Saharan African wildcats together with domestic cats to cluster 4 ($Q_4 = 0.971$). For $K > 4$, the LnP(K) reached the plateau and no further interpretable substructure was observed in the data (Online Resource 2, Fig. S1). In any case, the analyzed black-footed cats showed no interspecific hybridization signal.

STRUCTURE results obtaining running the black-footed cat genotypes alone did not show any trace of substructure or correspondence with the two detected mtDNA haplotypes, neither with the option *usepopinfo* activated nor with the option not activated (data not shown).

All the nine microsatellites were polymorphic in the four sampled groups (Table 2) with a mean number of alleles per locus of 8.67 ± 0.83 (range 5–13) in the domestic cats, 5.78 ± 0.46 (4–8) in the European wildcats, 8.33 ± 0.41 (6–10) in the African wildcats, and 4.33 ± 0.37 (2–6) in the black-footed cats (Table 2). Only 4 out of 135 identified alleles (2.96%) were shared by all the four taxa, while the black-footed cats shared 7 (5.18%) alleles with the domestic cats, 6 (4.45%) with the European wildcats, and 4 (2.96%) with the African wildcats. Conversely, among the 135 alleles described, 12 (8.89%) were species-specific for the domestic cats, 8 (5.93%) for the European wildcats, 13 (9.63%) for the African wildcats, and 32 (23.70%) for the black-footed cats.

In a pairwise F_{ST} matrix of the genetic distances among groups the black-footed cats were significantly ($P < 0.001$) divergent from all the other felid taxa ($F_{ST} > 0.40$). The mean H_O was 0.68 ± 0.08 in the black-footed cats, ranging from 0.60 to 0.04 in the European wildcats to 0.73 ± 0.04 in the domestic cats and 0.74 ± 0.03 in the African wildcats (Table 2), all values not significantly (p values > 0.05; t test) different from their expected heterozygosity values. No significant departures from HWE were detected in the black-footed cats and African and European wildcats, whereas microsatellite loci were significantly out of Hardy-Weinberg only in the domestic cats, due to fewer expected than observed heterozygotes (significantly positive F_{IS}; Table 2).

Approximate Bayesian computation

Approximate Bayesian computation simulations provided the best support for scenario 1 (sequential population splitting without bottlenecks), which clearly better performed than the other two (Fig. 1; Online Resource 2, Fig. S2) and showed non-significant P values for all the posterior parameters after Bonferroni's corrections (Online Resource 1, Table S3). The posterior probability of the best scenario (Online Resource 2, Fig. S2) was 92.65% (95% CI, 90.16–91.15) and did not overlap with scenarios 2 and 3 (Online Resource 2, Fig. S2) that received equal, but considerably lower support (7.80, 95% CI, 7.34–8.25% and 1.55%, 95% CI, 1.41–1.69%). Under the best scenario, the median values of the divergence time showed that

Table 2 Genetic variability results obtained from the nine autosomal short tandem repeat (STR) loci analyzed in domestic cats, European wildcats, African wildcats, and black-footed cats

Group (N)	N_A[a]	N_E[b]	N_P[c]	H_O[d]	H_E[e]	uH_E[e]	F[f]	F_{IS}[g]	P[h]
Domestic cats (18)	8.67 (0.83)	4.94 (0.62)	12	0.73 (0.04)	0.77 (0.03)	0.79 (0.03)	0.05 (0.03)	0.087	0.036
European wildcats (19)	5.78 (0.46)	3.24 (0.36)	8	0.60 (0.04)	0.66 (0.04)	0.68 (0.04)	0.09 (0.03)	0.117	0.090
African wildcats (21)	8.33 (0.41)	4.71 (0.22)	13	0.74 (0.03)	0.78 (0.01)	0.80 (0.01)	0.05 (0.03)	0.081	0.054
Black-footed cats (15)	4.33 (0.37)	2.86 (0.25)	32	0.68 (0.08)	0.62 (0.04)	0.65 (0.04)	−0.06 (0.08)	0.048	1.899

Numbers of analyzed individuals for each group are in parenthesis (*N*)

[a] N_A = average observed number of alleles per locus

[b] N_E = expected number of alleles per locus

[c] N_P = number of private alleles

[d] H_O = observed heterozygosity

[e] H_E and uH_E = expected and unbiased expected heterozygosity

[f] F = fixation index

[g] F_{IS} = inbreeding coefficient

[h] P = probability to obtain F_{IS} values higher or lower than those observed after 10,000 random permutations of alleles in each population computed by GENETIX (corresponding standard deviation values are in parentheses)

the black-footed cats have been genetically isolated for the last 1.51×10^6 generations (5% quantile (q050) = 7.45×10^5 generations—95% quantile (q950) = 1.42×10^6 generations) and African and European wildcats for the last 3.79×10^5 generations (5% quantile (q050) = 3.01×10^5 generations—95% quantile (q950) = 6.41×10^5 generations; Online Resource 1, Table S4). Assuming a cat generation time of 2 years (Nowak and Walker 1999; Renard et al. 2015), the TMRCA of the black-footed cats, European, and African wildcats corresponds to 3.02×10^6 years ago, while the latter two taxa started their divergence 7.58×10^5 years ago (Fig. 1; Online Resource 1, Table S4), roughly consistent with divergence times of felid lineages estimated by Johnson et al. (2006) to molecularly clarify their Late Miocene radiation.

Discussion

During the last decades, habitat loss and other anthropogenic factors determined decreasing trends of wild populations of the black-footed cat (Sliwa et al. 2016; Oh et al. 2017). This species is the smallest African cat species (Sliwa et al. 2010) and also shows the most restricted range (Nowell and Jackson 1996), being endemic to the arid steppe and savannah habitats of the southern African sub-region (Sliwa 2004; Sliwa et al. 2016). However, though threatened, a number of ecological, morphological, demographic, sanitary, and genetic aspects, essential for its long-term conservation, still remain poorly known (Johnson et al. 2006; Sliwa 2008; Renard et al. 2015; Sliwa et al. 2016; Oh et al. 2017). In combination with other techniques, such as occasional observations and camera trapping, non-invasive genetic sampling (NIGS) may represent an appropriate tool to fill this lack of information. Thus, in this pilot study, we developed a multilocus detection method to molecularly characterize non-invasively collected hair samples of captive-bred black-footed cats at both mitochondrial and nuclear markers. Such approach can thus be applied in future monitoring projects of wild-living populations that could be based on the systematic analysis of hair tufts trapped by olfactory-treated sticks or snares along selected transects and integrated with other widely used low-quality DNA sources such as fecal, urine, and saliva samples that could be opportunistically collected (Steyer et al. 2013; Velli et al. 2015).

To test the cross-species amplification efficiency and reliability of our marker panel on different quality samples, we exploited the possibility to analyze hair samples of black-footed cats from zoo populations managed in captive breeding programs, which, to simulate natural conditions and situations, were randomly and non-invasively collected without a defined sampling strategy and without any direct contact with the animals.

The first critical step of our analysis protocol consisted in a preliminary quality screening of the non-invasively collected materials and their unambiguous molecular specific identification through the sequencing of a short fragment (< 400 bp) of the mitochondrial DNA. Such reduced mtDNA portion confirmed to be sufficiently diagnostic to distinguish strictly related felid taxa with potentially overlapping trophic niches or similar ecological contexts, thus could be successfully applied to more extensive non-invasive surveys. Almost all the analyzed hair samples (96%), even if randomly collected, were successfully sequenced at the selected mtDNA region, allowing to identify two already described black-footed cat ND5 haplotypes. Our rough phylogenetic reconstructions, although showing low support for the most basal nodes due to the short sequences they were based on, well reflected the evolutionary relationships described by Johnson et al. (2006), who tried to molecularly clarify the Late Miocene radiation of modern felids. However,

though in our preliminary molecular phylogeny the two detected black-footed cat haplotypes were clearly distinguishable from all the other wild and domestic felids included in the *Felis* lineage, we could not provide additional insights on the identification of the two hypothetical black-footed cat subspecies since neither geographical nor morphological information about the analyzed captive-bred animals were available. Despite their high homoplasy rates and low inter-laboratory comparability and interpretability, microsatellites are powerful genetic markers to answer a variety of questions in population genetics and ecology thanks to their high polymorphism (Allendorf et al. 2013; Wultsch et al. 2014). The successful cross-amplification of nine microsatellites, originally developed for the domestic cat (Menotti-Raymond and O'Brien 1995 and Menotti-Raymond et al. 1999), with three multiplexed PCRs of DNA extracted from non-invasively collected samples of black-footed cats, allowed a reliable and cost-effective resolution for both species and individual identification showing no evidence of inter-specific hybridization in the captive-bred individuals we analyzed. Although an accurate selection of fresh samples was not guaranteed due to the random sampling, and despite their supposed low-quality DNA content, our genotyping success rates were close to 70%, considerably higher than those usually obtained in most of the studies based on the analysis of non-invasive materials (Ruiz-González et al. 2013; Caniglia et al. 2014; Viglino et al. 2016; Fabbri et al. 2018), included those regarding felids (Lovari et al. 2009; Anile et al. 2012; Sugimoto et al. 2012; Velli et al. 2015; Steyer et al. 2018). Moreover, we did not find any signal of cross-sample contaminations and the mean genotyping error rates were much lower than in other non-invasive genetic studies performed on felids with a similar number of microsatellites (Bhagavatula and Singh 2006; Borthakur et al. 2011; Lovari et al. 2009; Sugimoto et al. 2012; Rozhnov et al. 2013; Wultsch et al. 2014; Steyer et al. 2018) and on other carnivores (De Barba et al. 2010; Caniglia et al. 2014; Stansbury et al. 2014; Granroth-Wilding et al. 2017; Fabbri et al. 2018). Additionally, the very low probabilities of identity, together with the high number of allele mismatches among genotypes, confirmed that the optimized panel of nine felid-diagnostic loci can be used to distinguish without ambiguity black-footed cat individuals, even when related, in future non-invasive long-term monitoring projects and to estimate minimum population sizes, being sure that matching genotypes can be considered recaptures of the same individual (Mills et al. 2000; Waits et al. 2001).

Concordantly with results obtained from the analysis of the mtDNA, our preliminary genetic screening at nuclear DNA, based on pairwise F_{ST} values, multivariate and assignment procedures, despite the limited sample size, showed that the analyzed black-footed cats were highly differentiated from all the other analyzed felid taxa, consistent with other studies, based on different types and number of markers, about the evolutionary histories of modern felids (Johnson et al. 2006). Overall, the analyzed black-footed cats were polymorphic at all the selected microsatellites, though they were mostly designed on the domestic cat variation, showing numerous species-specific alleles and high values of autosomal genome-wide heterozygosity, very similar to those observed in the other considered taxa. However, all these comparisons should be treated with caution because such estimates might have been inflated by the limited sample size, not fully representative of the real wild populations of the examined taxa. The multivariate and clustering procedures did not find any substructure in the analyzed black-footed cats; therefore, no correspondence between nuclear and mtDNA variation was possible, not allowing any further speculation on the supposed subspecies. However, once again, our results cannot be considered conclusive because a significant part of the genetic variability might have been unsampled and only future genotyping of a larger number of wild individuals sampled from the whole distribution range of the species could confirm or deny this apparent lack of genetic substructure.

Our demographic scenarios, even if they were based on a restricted number of presumably molecular markers and limited sample sizes, roughly confirmed the evolutionary history of genus *Felis* reconstructed by Johnson et al. (2006) analyzing autosomal, X-linked, Y-linked, and mitochondrial markers. Accordingly, our ABC simulations showed that black-footed cats and African and European wildcats likely experienced sequential population splittings that started during the Late Pliocene and continued through the Early Pleistocene during a period of relatively low sea levels before the onset of the Pleistocene glacial oscillations, with good statistical support despite the low number of samples and coherently with inferences from genome-wide data (Johnson et al. 2006).

Conclusions

This study provided the first application of a reliable and cost-effective protocol to molecularly identify the species and individuals of black-footed cats from non-invasively collected samples, as well as to preliminary characterize their genetic variation. Though solely tested on captive-bred individuals, our method revealed to ensure relatively high genotyping success and low error rates, while well differentiating the investigated species from the other closely related felid taxa.

The genotyping of black-footed cat non-invasive DNA at maternally inherited (mtDNA) and autosomal (STRs) loci highlighted relatively high levels of genome-wide heterozygosis and variability, although we did not detect any substructure in the analyzed samples.

Nonetheless, the easy applicability of our approach and the robustness of the optimized multiple-tube protocol make it a reliable tool for future non-invasive genetic monitoring projects to achieve ecological and genetic information directly from wild-

living populations within the whole distribution range of the species and to monitor its actual spatio-temporal trends in abundance and density. In this way, it will be also possible to verify, through landscape genetic analyses and habitat suitability models (Milanesi et al. 2016; Reddy et al. 2017), any possible correlation between the standing genetic variability of the extant black-footed cat populations and different geographic habitats; detect critical anthropogenic barriers to dispersal, which might fragment the species ranges or limit gene flow; and identify zones with high mortality risk due to direct or indirect human activities where to concentrate conservation efforts and resources. Additionally, the availability of entire genomes for a growing number of taxa, including the domestic cat (Pontius et al. 2007), can allow future investigations on the whole genome variation of the black-footed cat, providing essential information to better address a number of evolutionary, selective, and adaptive questions and definitively clarify the taxonomic uncertainties regarding the possible presence of two subspecies (Allendorf et al. 2010; Montague et al. 2014). Moreover, genomic platforms could soon allow the extensive and cost-effective screening of thousands of single nucleotide polymorphisms (SNPs), which represent the most widespread source of genome-wide variation and promote the development of specific ancestry informative markers (AIMs), overcoming the few limitations of microsatellites, to reliably and routinely apply also in conservation genetics of endangered taxa or priority management units.

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