

## Can scat-based species identification be a misleading sign of presence? More evidences from northeastern Portugal

¿Puede la identificación de especies basada en excrementos ser un signo engañoso de presencia? Más evidencias del noreste de Portugal

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### Abstract

Species identification of non-invasively collected samples using molecular genetics tools has become an important tool in ecological research. For decades, scat-based ecological studies were almost exclusively rooted in morphological identification of scats, within local context, in the field. However, this approach raised a controversial debate, due to species and context-specific probability of error and lack of validation. In this study, we aimed to test the accuracy of mesocarnivore scats identification, based on morphological criteria, using a carnivore guild in northeastern Portugal as a model and molecular identification as a standard for accuracy of morphological identifications, within local context. While using only expert-based identifications for comparison with molecular identification standard, we have also compared the identifications performed by observers with different levels of experience. We extracted DNA from 63 scats (NE Portugal), which was successfully amplified/sequenced from 83% (n= 52) of the extracts: 38 were molecularly assigned to red fox (*Vulpes vulpes*), eight to stone marten (*Martes foina*), two to pine marten (*Martes martes*) and domestic dog (*Canis lupus familiaris*) and one to European badger (*Meles meles*) and common genet (*Genetta genetta*). There was a tendency for better performance by more experienced researchers, with 67% of scats being correctly assigned, but differences among observers were not significant. Due to the small sample size, only for foxes and stone martens was possible to estimate the error rate in species assignment, based on morphological criteria. False positive rates (% of times a scat was misassigned to a given species) were 4% for fox samples and 62% for stone marten. False negative rates (i.e. the rate at which a scat of a given species was assigned to another species) reached 29% for fox (scats that were initially assigned to stone marten and domestic dog were in fact from fox) and 25% for stone marten (originally misassigned to weasel, *Mustela nivalis*), respectively. The results support the need to implement molecular methods in ecological studies based on scat identification, so researchers can determine the error rates associated with morphological discrimination to develop accurate monitoring studies.

**Keywords:** Mesocarnivore, Mitochondrial DNA, Monitoring, Non-invasive sampling, Species molecular identification.

### Resumen

La identificación de especies mediante técnicas no invasivas, como la genética molecular, se ha revelado de importancia en la investigación ecológica. Durante décadas, la identificación de excrementos en estudios ecológicos se basó casi exclusivamente en la identificación morfológica de heces, que a menudo tenían en cuenta la información sobre el hábitat y la ubicación del excremento. Sin embargo, este enfoque planteaba serios debates, por la elevada probabilidad de error específico y por la falta de validación. El objetivo

de este estudio es comprobar el grado de precisión a la hora de identificar heces de mesocarnívoros mediante criterios morfológicos, validando estos resultados con la identificación molecular. Mientras utilizamos solo identificaciones realizadas por expertos para comparar con el estándar de identificación molecular, también comparamos las identificaciones realizadas por observadores con diferentes niveles de experiencia. Se extrajo ADN de 63 heces de mesocarnívoros (NE Portugal), de las cuales fue amplificado y secuenciado con éxito el 83% (n= 52) de los extractos: 38 se identificaron molecularmente como de zorro (*Vulpes vulpes*), ocho como de garduña (*Martes foina*), dos de marta (*Martes martes*) y perro doméstico (*Canis lupus familiaris*) y uno de tejón (*Meles meles*) y gineta (*Genetta genetta*). Debido al pequeño tamaño de la muestra, tan solo fue posible estimar la tasa de error de identificación de excrementos según criterios morfológicos (% de veces que las heces fueran mal asignadas a una especie determinada en el campo o en el laboratorio) para zorro y garduña: 4% para muestras de zorro y 62% para las de garduña. La tasa de error cuando se asignó el excremento de una especie a otra en el campo o en el laboratorio (confirmada por técnicas moleculares) alcanzó el 29% para el zorro (heces identificadas inicialmente como de garduña y de perro) y del 25% para la garduña (heces asignadas a comadreja, *Mustela nivalis*). Los resultados confirman la conveniencia de incluir métodos moleculares en los estudios ecológicos basados en la identificación de excrementos, de forma que los investigadores puedan determinar la tasa de error asociada con la discriminación morfológica para desarrollar estudios de monitoreo más precisos. **Palabras clave:** ADN mitocondrial, identificación molecular especies, mesocarnívoro, monitoreo, muestreo no invasivo.

## Introduction

Surveying wild species in the wilderness is a challenging and often time-consuming and expensive activity, depending on the species or species groups considered (Davison *et al.* 2002). Mammalian carnivore data collection is especially challenging because species are mostly nocturnal and/or crepuscular, have high mobility, often occupy large home ranges, present low densities and are frequently sensitive to disturbance (Gese 2001, Wilson & Delahay 2001).

Carnivores play a key role in the structure and functioning of ecosystems (Gittleman 1989). The accuracy and reliability of species identification are essential for estimating several biological and ecological parameters. This is then the baseline for the implementation of conservation plans (Heinemeyer *et al.* 2008). Furthermore, carnivores' elusive behaviour, body size and conservation status impose an additional difficulty in implementing methods that involve the capture and/or handling of individuals, which may become stressful and potentially dangerous for handlers and wildlife (Kelly *et al.* 2012). As a result, gathering ecological information about these animals depends on non-invasive sampling, specifically on indirect evidences of the presence of a species (scats, footprints, claw-marking; Waits & Paetkau 2005, Gompper *et al.* 2006, Kelly *et al.* 2012). Among these methods, scats' identification is one of the most informative and frequently used methods for the detection and

monitoring of small and medium-sized carnivores (mesocarnívoros) in Europe (Davison *et al.* 2002, Barea-Azcón *et al.* 2007, Rosellini *et al.* 2008). This is due to scat abundance and conspicuousness, and also the diversity of information that can be obtained from its use (e.g. diet, parasite burden, species-habitat associations; Putman 1984).

However, the information obtained from mesocarnívoros' scats can only be useful if based on correct species identification. Success rate estimation becomes even more relevant when the faeces are produced by sympatric and similar size species – e.g. red fox *Vulpes Vulpes* (Linnaeus, 1758), stone marten *Martes foina* (Erxleben, 1777) and pine marten *Martes martes* (Linnaeus, 1758) (Laguardia *et al.* 2015) – that may share the same food resources (Foran *et al.* 1997) and produce highly similar scats, that are often deposited in analogous structures (e.g. along dirt roads). High identification error rates are reported as a recurring problem (e.g. Davison *et al.* 2002, Birks *et al.* 2004, Lonsinger *et al.* 2015, Morin *et al.* 2016, Monterroso *et al.* 2019), and to overcome this problem, non-invasive molecular methods have been applied (Weiskopf *et al.* 2016). The implementation of those methods allow accurate species identification, namely through the use of mitochondrial DNA (mtDNA) (Palomares *et al.* 2002, Beja-Pereira *et al.* 2009) or nuclear DNA fragments (Oliveira *et al.* 2010), and the assessment of field identification error rates is crucial to account for misidentification during field work.

Based on the research needs highlighted above, the main objective of this study is to test the accuracy of mesocarnivores' scat identification based on a conventional approach (morphological criteria), using a Mediterranean mesocarnivore guild as model. As a standard, for accuracy, we will use the results of scats' molecular identification. By providing identification error estimates for each species, we expect to provide relevant insights for carnivore ecological studies, but also, practical information for the conservation of mesocarnivore species in Mediterranean habitats.

## Material and Methods

### Field sampling

The study was implemented in Northeast Portugal (41°30'33.3" N, 6°56'57.5" W) – Bragança and Vila Real districts – covering a total area of 8,395 km<sup>2</sup>. The study area was divided into grid cells of 10x10 km and, using a knight chess moving pattern, starting on the northeast corner of the area, we selected 15 squares of 10x10 km. Each one of these squares was once again divided into 4 grid cells of 5x5 km. Only two of 5x5 km grid cells, per 10x10 km grid square, were randomly selected to be sampled. Within the area of these 5x5 km squares, we monitored a total of 2,5 km of transects, to adequately sample all existing habitats. Transects were located along trails or dirt roads, surveyed on foot by one or two observers to search for mesocarnivores' signs of presence (scats). Samples were collected in summer (July–September, 2016), when the offspring of most mesocarnivores begins to be independent (Loureiro *et al.* 2012).

All the scats were initially identified in the field based on their location and morphology with the support of field guides (Sanz 2003, Bang & Dahlstrom 2006) and later, by other observers who were not present during the fieldwork, but used the same morphological identification criteria, through the analysis of photographic records (of scats and surrounding area) and with access to all information collected in field (e.g. scat position/location within the trail, surrounding habitat). Four observers with two different levels of experience in scats' morphological identification (i.e. experienced – several years of field experience – and not experienced researchers), identified all the mesocarnivores' scats.

A total of 96 scat samples were carefully collected, with disposable sterile gloves, to avoid contamination, and stored in plastic containers (identified with the sample code) in 96% ethanol, until DNA extraction. Attempts were made to collect the most recent samples (nearly intact and moist), since it increases the probability of success in the identification by molecular methods (Taberlet *et al.* 1996, Foran *et al.* 1997). We also targeted samples with different morphology, in order to increase the possibility of belonging to different species (Bang & Dahlstrom 2006). Collected samples were maintained at room temperature, in ethanol, to prevent sample degradation, until arriving in the laboratory, where were conserved in frozen (-20°C).

### Laboratory procedures

DNA was isolated from scats using the QIAamp DNA Stool Mini Kit protocol (QIAGEN®), and polymerase chain reactions (PCR) were performed using Platinum™ II Taq Hot-Start DNA Polymerase, following manufacturer's instructions. A fragment corresponding to a non-coding region (D-loop region) of mitochondrial DNA (mtDNA) was amplified for all samples. This region contains the main regulatory elements for the replication and expression of the mitochondrial genome (Sbisà *et al.* 1997). While methods for molecular identification using nuclear markers do exist (Oliveira *et al.* 2010), mitochondrial genes are likely to be present in higher frequency in non-invasive samples (each cell has numerous mitochondria but only one nucleus); are haploid in mammals (heteroplasmy does occur but is rare), facilitating the sequencing procedures; and mtDNA markers have been proven to perform better than nuclear DNA markers for molecular identification of scats (Monterroso *et al.* 2013). Care was taken at all times to avoid cross-contaminations (e.g. use of UV-light equipped chamber, aerosol resistant pipette tips, negative controls and dedicated rooms for DNA isolation, pre and post-PCR procedures).

Samples were initially submitted to amplification with L-Pro (Mucci *et al.* 2004) and MelCr6 primers (Marmi *et al.* 2006), resulting in a fragment of around 600bp (depending on the species). Samples identified in the field as belonging to fox or wildcat, but not successfully amplified with the first pair of primers, were amplified using the primers Thr-L 15926 and DL-H 16340 (Vilà *et al.* 1999), resulting in a fragment of about 350bp (n=

4), and CR1 and CR2R (Palomares *et al.* 2002), resulting in a fragment of about 300bp (n= 3). Amplifications were performed in a final volume of 25µL using: 1-5µl of DNA template; 2µg/µl of BSA; 2mM MgCl<sub>2</sub>; and 0,12µM of each primer solution, with annealing temperatures of 48°C (for the two first pairs of primers) or 58°C (for CR1 and CR2, following Monterroso *et al.* (2013).

PCR success was confirmed through electrophoresis and visualization of DNA fragments under UV light. Successfully amplified fragments were enzymatically purified (ExoSap-IT®). Sequences were obtained for both strands using the above-mentioned primers and were used to generate consensus sequences for each sample. Consensus sequences were analysed using the software MEGA (Molecular Evolutionary Genetics Analysis) version 7 (Kumar *et al.* 2016) and identification was performed by comparing generated sequences with sequences deposited in GenBank® using BLAST searches (NCBI 2017). Prior to laboratorial procedures, we had confirmed that sequences were available in GeneBank® for all mesocarnivore species likely to occur in the area. Sequences were assigned to a species when matched > 98% to a Genbank record (Hebert *et al.* 2003, Hubert & Hanner 2015).

### Data analysis

The DNA isolation and amplification success were estimated by the number of successfully amplified samples, relatively to the number of samples from which we tried to isolate DNA. Successfully amplified samples were then sequenced. Morphological identification of the collected scats was performed in two ways: by four individual researchers (observers 1-2-3-4) and by two teams of two researchers (non-experienced researchers – observers 1 and 2 – *vs* experienced researchers – observers 3 and 4). For identification based in more than one observer, when different observers assign the same sample to different species, the identification was based on the identification of the most experienced observer. The precision of this conventional approach was expressed as the proportion of correct identifications (“matches” with molecular identification), over the total number of samples successfully identified by molecular methods.

The identification success rate from different observers (individual and team) were compared

using Chi-square proportion tests (Armitage 1966). The p-values of the multiple tests were adjusted using Bonferroni correction, in order to reduce Type I errors due to multiple testing (Gordon *et al.* 2007). The success rate of identification per species was estimated based on the number of morphological identifications that matched the molecular identification, over the total number of samples assigned to the species, based on morphological criteria.

### Results

We collected 96 scats that were initially assigned to seven species: red fox, Iberian wolf *Canis lupus signatus* Cabrera, 1907, weasel *Mustela nivalis* Linnaeus, 1766, stone marten, European badger *Meles meles* (Linnaeus, 1758), common genet *Genetta genetta* (Linnaeus, 1758) and wildcat *Felis silvestris* Schreber, 1777). Efforts were made to collect the most recent and less degraded samples, to increase the probability of success in the molecular identification. However, DNA isolation was only attempted in 66% of the collected scats (63 samples), because the decomposition level of the remaining 33 samples was too advanced and could influence the results of molecular identification. Of the 63 analysed samples, 52 were successfully amplified, sequenced and assigned to mesocarnivore species based on molecular criteria, corresponding to a 83% identification rate of the scats submitted to genetic analysis. Most of the 52 genetically identified scats belonged to red foxes (n= 38). The remaining samples were molecularly assigned to stone marten (n= 8), domestic dog (*Canis lupus familiaris* Linnaeus, 1758; n= 2), pine marten (n=2), common genet (n=1) and European badger (n=1). Most of the sequenced scats have been previously identified by morphological characteristics as belonging to the red fox (54%, n= 28) and to the stone marten (31%, n= 16), but other species were also genetically identified in one or two of the collected scats (Table 1): *Martes martes* (n= 2), *Meles meles* (n= 1), *Genetta genetta* (n= 1) and *Canis lupus familiaris* (n= 2). Agreement between morphological and molecular identification of scats varied among species.

The success of morphological identification among the researchers varied between 48% and 67% (52% to 33% error rates, respectively). Although there were no significant differences



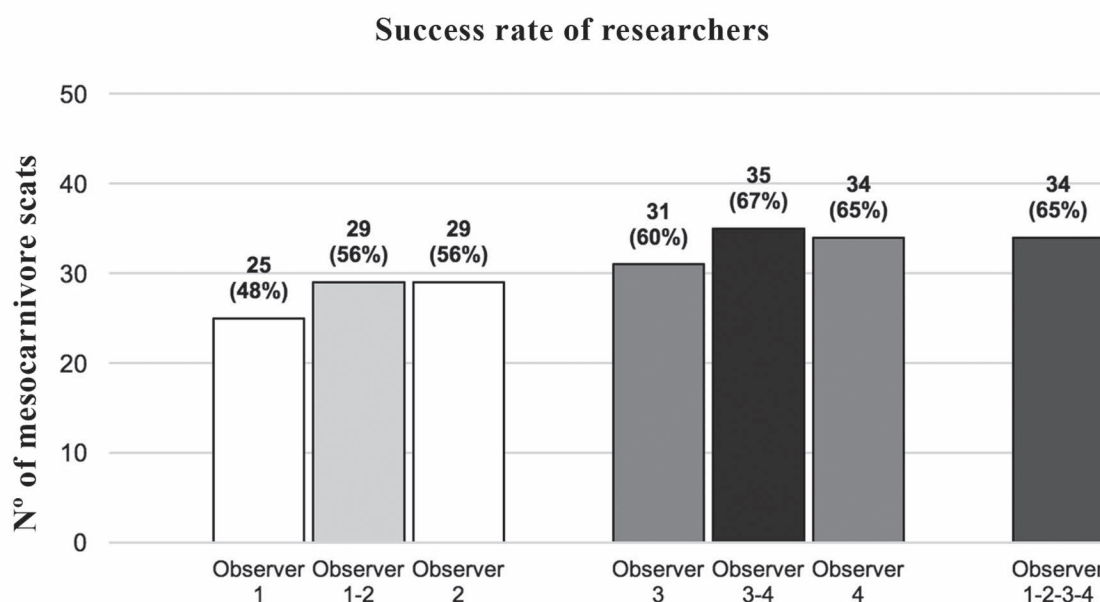
between the success rates among experienced and non-experienced researchers ( $\chi^2= 1.016$ ,  $df= 1$ ,  $p> 0.05$ ), in order to calculate the success of identification in each species, we selected the morphological identification data with the highest species identification success rate, i.e. association of the identifications of experienced researchers, corresponding to 67% (Fig. 1).

The samples that were identified by the observers (morphological identification) as belonging to the red fox, had an accuracy rate of over 96% (27 out of 28), and the molecular identification revealed that the only misidentified sample belonged to the domestic dog. However, 29% (11/38) of scats that were identified as belonging to the red fox by molecular analysis, were misassigned to another species by field observers, i.e. false negatives – Iberian wolf ( $n= 1$ ), stone marten ( $n=8$ ) and wildcat ( $n= 2$ ). The samples that were identified as belonging to the stone marten were accurately identified, by morphological characteristics, in 38% (6 out of 16) of the occasions. The molecular analysis revealed that some samples were morphologically misidentified as belonging to the stone marten, when in fact they belonged to the red fox ( $n= 8$ ), pine marten ( $n= 1$ ) and domestic dog ( $n= 1$ ). It was also revealed that, 25% (2/8) of scats that were molecularly identified as stone marten, were misassigned to the weasel (Table 1).

Error rates in scats' species assignment based on morphology were highly variable and can happen in two ways: 1) when a scat is misassigned to a given species – false positive (the species is overrepresented); and 2) when a scat of a given species is misassigned to another species – false negative (the species is underrepresented). For example, false positive rate was much higher in the case of stone marten (*Martes foina*, 62%) than in the case of red fox (*Vulpes Vulpes*, 4%), which means that the risk of overestimating stone marten abundance or presence would be higher. On the other hand, false negative rates were very similar in both species (red fox – 29%; stone marten – 25%) and thus, the risk of underestimating the abundance or presence of the red fox would be only slightly higher than in the case of the stone marten.

## Discussion

The analysis of non-invasive samples, namely scats, is a very useful tool in ecology, conservation and monitoring of carnivore species (Foran *et al.* 1997, Lonsinger *et al.* 2016). However, if not accurately identified, scat-based information can result in the identification of inaccurate and misleading ecological patterns. Our results show that molecular genetic tools can be efficiently



**Figure 1.** Individual and team (observers 1-2 and observers 3-4) success rate of researchers, in the morphological identification of mesocarnivore scats. According to the level of expertise, the observers were grouped in teams of different levels of knowledge: non-experienced researchers (observers 1 and 2 – light coloured bars) and experienced researchers (observers 3 and 4 – dark coloured bars). Bar representing more than one observer show the success rate of morphological identification by teams.

**Table 1.** Matches (within dashed lines) and mismatches among identifications based on morphological and molecular criteria, for all the 52 samples for which molecular information was available. Percentages of times that: a scat is misassigned to a given species – false positive – (species is overrepresented, weighted across row); and a scat of a given species is assigned to another species – false negative – (species is underrepresented, weighted across column).

		Species inferred by genetic analysis					False positive rate	
		<i>Vulpes vulpes</i> (n=38)	<i>Martes foina</i> (n=8)	<i>Martes martes</i> (n=2)	<i>Meles meles</i> (n=1)	<i>Genetta genetta</i> (n=1)		<i>Canis lupus familiaris</i> (n=2)
Species inferred by morphological criteria	<i>Vulpes vulpes</i> (n=28)	27	-	-	-	-	1	1/28 (4%)
	<i>Canis lupus signatus</i> (n=1)	1	-	-	-	-	-	1/1 (100%)
	<i>Mustela nivalis</i> (n=3)	-	2	1	-	-	-	3/3 (100%)
	<i>Martes foina</i> (n=16)	8	6	1	-	-	1	10/16 (63%)
	<i>Meles meles</i> (n=1)	-	-	-	1	-	-	0/1 (0%)
	<i>Genetta genetta</i> (n=1)	-	-	-	-	1	-	0/1 (0%)
	<i>Felis silvestris</i> (n=2)	2	-	-	-	-	-	2/2 (100%)
<b>False negative rate</b>		11/38 (29%)	2/8 (25%)	2/2 (100%)	0/1 (0%)	0/1 (0%)	2/2 (100%)	

coupled to non-invasive scat sampling, allowing for species-specific assignment and the estimation of success rates in scats' species assignment through morphological methods. Our results show a high percentage of error (33%) in species assignment and add up to an increasing body of evidence supporting the need for error estimation in non-invasive scat surveys (Lonsinger *et al.* 2015, Lonsinger *et al.* 2016, Monterroso *et al.* 2019).

The genetic identification can be an adequate case-solving approach (Beja-Pereira *et al.* 2009, Harrington *et al.* 2010) and is strongly advisable, in particular for species for which morphological identification success rates are very low. In this study, we used a mitochondrial DNA marker that is widely used in carnivore identification. As mentioned at the beginning of the paper, alternatives using nuclear markers do exist (Oliveira *et al.* 2010), but have been proven less successful than mitochondrial markers (Monterroso *et al.* 2013; 27% against 77%

of identification success, respectively, standardized for an overall 78.4% success identification rate). In our study, we were able to genetically identify 83% of the scats submitted to genetic analysis, within the range of values reported by other researchers: 72% in Fernandes *et al.* (2008); 78.4% in Monterroso *et al.* (2013); 80% in Lonsinger *et al.* (2015); 83.9% in Ruiz-Gonzalez *et al.* (2013). While not being a 100% effective procedure, it allows for species confirmation of around 4 out of 5 analysed scats, on average. High temperatures, such as the ones occurring at our study area, during summer season, may contribute to the rapid degradation of scat DNA (Santini *et al.* 2007), as well as high humidity rates. Additionally, and despite we had no signs of contamination in the field or lab, DNA obtained from the non-invasive samples method is generally in low quantity and is often contaminated and degraded (Broquet *et al.* 2007). Optimization of the field and laboratory methods is essential to achieve

greater and faster success (Nakamura *et al.* 2017), and success rates might be improved by optimizing scat selection procedures.

Genetic identification allowed us to successfully identify 35 out of 52 scats (67%), based on morphological characteristics, considering the identifications made by observers with best identification performance. These results showed that two species identified by morphological characters (*Mustela nivalis* and *Felis silvestris*), actually had not been sampled. On the other hand, *Martes martes* – not identified from morphological analysis – was only reported after genetic analysis. The only scat assigned to *Canis lupus signatus* was actually confirmed to belong to *Canis lupus familiaris*, a common confusion in areas where the two subspecies coexist (Torres *et al.* 2017).

As a secondary objective, we tried to evaluate if we would find differences on the identification success depending on the level of expertise of the observers. We found no significant differences even if morphological identification success rate varied from 48% (least experienced observer) to 67% (combined results of the two most experienced observers). We must however call attention for the fact that our comparisons were based on a small number of samples. It is often assumed that more experienced observers will more often correctly identify species from their scats, but evidence for a no-effect from the level of expertise (Bulinski & McArthur 2000, Ruiz-González *et al.* 2013) does exist, or even a negative effect, as recently reported by Soller *et al.* (2000). Nevertheless, the level of experience might influence downstream procedures, such as individual identification (Ruiz-González *et al.* 2013) and inter-observer variation should be recognised, for it may introduce bias in identification and in subsequent data analysis based on this identification (e.g. Species Distribution Modelling; Molinari-Jobin *et al.* 2012). Poor identification is reported as a recurrent problem (Davison *et al.* 2002, Dalén *et al.* 2004, Monterroso *et al.* 2019), and this is a concern for researchers because of the uncertainty and bias that can be introduced in downstream procedures.

Taking into account the different errors that may occur in the identification of scats, some species may be overrepresented (false positives) or underrepresented (false negatives). Sources of error may include differences on abundance and distribution, with scats from widely distributed and abundant species being assigned to rarer species

(Monterroso *et al.* 2011, Lonsinger *et al.* 2015, Morin *et al.* 2016). Diet overlap can also result in more frequent confusion among species, such as in the case of red fox and wildcat (Urrea *et al.* 2014), as occurred in our study. Furthermore, as well as inter-species diet overlap, seasonal or intraspecific variation in diet might be a source of confusion, with individuals from the same population having different food searching strategies and eating different items, that will influence scat shape and composition (e.g. insects exoskeleton, fur or seeds; Monterroso *et al.* 2013).

Considering only the two species for which the number of samples is higher (red fox and stone marten), it becomes evident that frequency of false positives (observers and context being the same) may vary substantially among species (4% in red fox and 64% in stone marten, with an average of 33%). Such asymmetric and species-specific bias has already been reported by other authors (Lonsinger *et al.* 2015, Morin *et al.* 2016), and a species-specific identification success rates can really be a pattern for mesocarnivores. Frequency of false positives was extensively reviewed by Monterroso *et al.* (2019) and, even if a median value of 18% of false positives was found across carnivore taxa, values above 50% were found in 17% of all reviewed studies, and values as large as 75% were reported for carnivore guilds similar to the one under study.

Contrarily to false positives, false negative rates are less often reported (Monterroso *et al.* 2019), but are equally relevant for some species that may indeed be underrepresented. Considering again only the red fox and stone marten, false negative rates were more balanced and averaged 27%. We might be tempted to think that false positive and negative rates could help to neutralize their effect and minimize the source of bias, for the number of scats erroneously assigned to a species would partially counterbalance the number of scats of that species that were erroneously assigned to a different species. However, in ecological studies, most often this is not the case, for scats carry additional relevant information. For example, scats are sampled in specific locations and false positives and negatives introduce cumulative bias into the inference of distribution ranges by potentially overestimating the distribution in some areas (and habitats) and underestimating in others, respectively. The effects would also be cumulative in diet studies – and all studies that rely on such data for predator-prey relations – with preys or food items being erroneously introduced in the case

of false positives, and removed, in the case of false negatives. Such mismatches and biases may induce misleading counterproductive, or even deleterious, decisions by managers and conservationists.

Interspecific variation in the magnitude and direction of species identification bias using scats, as evidenced in this study and others before, highlights the relevance and need of accurate identification methods that allow for error estimation. Molecular identification techniques can be a very useful tool in ecological research, by helping to identify and quantify the bias and generate a clearer picture on different aspects of carnivore ecology (e.g. diet, habitat preferences, distribution), complementarily to morphological identification of scats. In a time when wildlife faces continuous and multifactor conservation problems, managers cannot afford to base conservation actions on erroneous ecological data. While genetic identification methods are often considered very expensive, costs of analysis are continuously decreasing, turning it an effective alternative to other non-invasive (camera-trapping) or invasive (capture-mark-recapture, GPS or telemetry monitoring). Avoiding sequencing procedures and implementing finger-printing approaches, such as RFLP, can substantially reduce the costs of genetic analysis (Livia *et al.* 2006, Ruiz-Gonzalez *et al.* 2013). Additionally, just being able to estimate identification error rates for the identification of a given species (namely, false positive and negative rates) would be extremely useful and can be done by using just a random subset of collected samples. Insights from molecular identification also allow for the development of alternative, and more cost-effective, non-genetic approaches (Lonsinger *et al.* 2015), that can be assessed, optimized and then implemented in a long-term basis, with known error rate and without the need for further genetic analysis.

## Acknowledgements

We are grateful to all the people and institutions that provided valuable assistance, particularly *Instituto da Conservação da Natureza e das Florestas* (ICNF). Co-author Eduardo Ferreira was supported by a post-doctoral grant from FCT (Program POPHQREN, ref: SFRH/BPD/72895/2010). We would like to thank for the financial support to University of Aveiro (Department of Biology), to CESAM (UID/AMB/50017; UID/AMB/50017/2019) and cE3c (UIDB/00329/2020), to FCT/MEC through national funds and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. We

also thank Sergio Chozas and Xose Pardavilla for their help with the title and abstract translation into Spanish. Xose Pardavilla also contributed to the definition of the sampling design.

## References

- Armitage P. 1966. The chi-Square test for heterogeneity of proportions, after adjustment for stratification'. *Journal of the Royal Statistical Society. Series B (Methodological)*, 28(1): 150-163. DOI: [10.1111/j.2517-6161.1966.tb00629.x](https://doi.org/10.1111/j.2517-6161.1966.tb00629.x)
- Bang P. & Dahlstrom P. 2006. *Animal tracks and hunter signs*. Oxford University Press, Oxford, 264 pp.
- Barea-Azcón J.M., Virgós E., Ballesteros-Duperón E., Moleón M. & Chiroso M. 2007. Surveying carnivores at large spatial scales: a comparison of four broad-applied methods. *Biodiversity and Conservation*, 16(4): 1213-1230. DOI: [10.1007/s10531-006-9114-x](https://doi.org/10.1007/s10531-006-9114-x)
- Beja-Pereira A., Oliveira R., Alves P.C. Schwartz M.K. & Luikart G. 2009. Advancing ecological understandings through technological transformations in noninvasive genetics. *Molecular Ecology Resources*, 9(5): 1279-1301. DOI: [10.1111/j.1755-0998.2009.02699.x](https://doi.org/10.1111/j.1755-0998.2009.02699.x)
- Birks J., Messenger J., Braithwaite T., Davison A., Brookes R. & Strachan C. 2004. Are scat surveys a reliable method for assessing distribution and population status of pine martens?. Pp. 235-252. In: D.J. Harrison, A.K. Fuller & G. Proulx, G. (eds). *Martens and fishers (Martes) in human-altered environments: an international perspective*. Springer, New York.
- Broquet T., Ménard N. & Petit, E. 2007. Noninvasive population genetics: a review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates. *Conservation Genetics*, 8(1): 249-260. DOI: [10.1007/s10592-006-9146-5](https://doi.org/10.1007/s10592-006-9146-5)
- Bulinski J. & McArthur C. 2000. Observer error in counts of macropod scats. *Wildlife Research*, 27(3): 277-282. DOI: [10.1071/WR98061](https://doi.org/10.1071/WR98061)
- Dalén L., Götherström A. & Angerbjörn, A. 2004. Identifying species from pieces of faeces. *Conservation Genetics*, 5(1): 109-111. DOI: [10.1023/B:COGE.0000014060.54070.45](https://doi.org/10.1023/B:COGE.0000014060.54070.45)
- Davison A., Birks J.D.S., Brookes R.C., Braithwaite T.C. & Messenger, J.E. 2002. On the origin of faeces: morphological versus molecular methods for surveying rare carnivores from their scats. *Journal of Zoology*, 257: 141-14. DOI: [10.1017/S0952836902000730](https://doi.org/10.1017/S0952836902000730)
- Fernandes C.A., Ginja C., Pereira I., Tenreiro R., Bruford M.W. & Santos-Reis M. 2008. Species-specific mitochondrial DNA markers for identification of non-invasive samples from sympatric carnivores in the Iberian Peninsula. *Conservation Genetics*, 9(3): 681-690. DOI: [10.1007/s10592-007-9364-5](https://doi.org/10.1007/s10592-007-9364-5)



- Foran D.R., Crooks K.R. & Minta S.C. 1997. Species Identification from scat: An unambiguous genetic method. *Wildlife Society Bulletin*, 25: 835-839.
- Gese E.M. 2001. Monitoring of terrestrial carnivore populations. Pp. 372-396. In J.L. Gittleman, S.M. Funk, D.W. MacDonald & R.K. Wayne (eds). *Carnivore Conservation*. USDA National Wildlife Research Center - Staff Publications, New York.
- Gittleman J.L. 1989. *Carnivore Behavior, Ecology, and Evolution*. Springer US, New York.
- Gompper M.E., Kays R.W., Ray J.C., Lapoint S.D., Bogan D.A. & Cryan J.R. 2006. A comparison of noninvasive techniques to survey carnivore communities in Northeastern North America. *Wildlife Society Bulletin*, 34(4): 1142-1151. DOI: [10.2193/0091-7648\(2006\)34\[1142:ACONTT\]2.0.CO;2](https://doi.org/10.2193/0091-7648(2006)34[1142:ACONTT]2.0.CO;2)
- Gordon A., Glazko G., Qiu X. & Yakovlev A. 2007. Control of the mean number of false discoveries, Bonferroni and stability of multiple testing. *The Annals of Applied Statistics*, 1(1): 179-190. DOI: [10.1214/07-AOAS102](https://doi.org/10.1214/07-AOAS102)
- Harrington L.A., Harrington A.L., Hughes J., Stirling D. & Macdonald D.W. 2010. The accuracy of scat identification in distribution surveys: American mink, *Neovison vison*, in the northern highlands of Scotland. *European Journal of Wildlife Research*, 56(3): 377-384. DOI: [10.1007/s10344-009-0328-6](https://doi.org/10.1007/s10344-009-0328-6)
- Heinemeyer K.S., Ulizio T.J. & Harrison R.L. 2008. Natural sign: tracks and scats. Pp. 45-75. In: R.A. Long, P. MacKay, W.J. Zielinski & J.C. Ray (eds). *Noninvasive survey methods for carnivores*. Island Press, Washington.
- Kelly M.J., Betsch J., Wulsch C., Mesa B. & Millis S. 2012. Noninvasive sampling for carnivores. Pp. 47-69. In: L. Boitani & A. R. Powell (eds). *Carnivore Ecology and Conservation*. Oxford University Press, Oxford.
- Kumar S., Stecher G. & Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33: 1870-1874. DOI: [10.1093/molbev/msw054](https://doi.org/10.1093/molbev/msw054)
- Livia L., Francesca V., Antonella P, Fausto P. & Bernardino R. (2006). A PCR-RFLP method on faecal samples to distinguish *Martes martes*, *Martes foina*, *Mustela putorius* and *Vulpes vulpes*. *Conservation Genetics* 8, 757-759. DOI: [10.1007/s10592-006-9203-0](https://doi.org/10.1007/s10592-006-9203-0)
- Lonsinger R.C., Gese E.M., Knight R.N., Johnson T.R., Lisette P., Lonsinger R.C., Gese E. M., Knight R.N., Johnson T.R. & Waits L.P. 2016. Quantifying and correcting for scat removal in noninvasive carnivore scat surveys. *Wildlife Biology*, 22: 45-54. DOI: [10.2981/wlb.00179](https://doi.org/10.2981/wlb.00179)
- Lonsinger R.C., Gese E.M. & Waits L.P. 2015. Evaluating the reliability of field identification and morphometric classifications for carnivore scats confirmed with genetic analysis. *Wildlife Society Bulletin*, 39(3): 593-602. DOI: [10.1002/wsb.549](https://doi.org/10.1002/wsb.549)
- Loureiro F., Pedroso N.M., Santos M.J. & Rosalino L.M. (eds) 2012. *Um Olhar sobre os Carnívoros Portugueses*. Carnivora, Lisboa.
- Marmi J., López-Giráldez F., Macdonald D.W., Calafell F., Zholnerovskaya E. & Domingo-Roupa X. 2006. Mitochondrial DNA reveals a strong phylogeographic structure in the badger across Eurasia. *Molecular Ecology*, 15(4): 1007-1020. DOI: [10.1111/j.1365-294X.2006.02747.x](https://doi.org/10.1111/j.1365-294X.2006.02747.x)
- Molinari-Jobin A., Kéry M., Marboutin E., Molinari P., Koren I., Fuxjäger C., Breitenmoser-Würsten C., Wölf S., Fasel M., Kos I., Wölf M. & Breitenmoser U. 2012. Monitoring in the presence of species misidentification: the case of the Eurasian lynx in the Alps. *Animal Conservation*, 15(3): 266-273. DOI: [10.1111/j.1469-1795.2011.00511.x](https://doi.org/10.1111/j.1469-1795.2011.00511.x)
- Monterroso P., Alves P.C. & Ferreras P. 2011. Evaluation of attractants for non-invasive studies of Iberian carnivore communities. *Wildlife Research*, 38(5): 446-454. DOI: [10.1071/WR11060](https://doi.org/10.1071/WR11060)
- Monterroso P., Castro D., Silva T.L., Ferreras P., Godinho R. & Alves P.C. 2013. Factors affecting the (in)accuracy of mammalian mesocarnivore scat identification in South-western Europe. *Journal of Zoology*, 289(4): 243-250. DOI: [10.1111/jzo.12000](https://doi.org/10.1111/jzo.12000)
- Monterroso P., Godinho R., Oliveira T., Ferreras P., Kelly M.J., Morin D.J., Waits L.P., Alves P.C. 2018. Feeding ecological knowledge: the underutilised power of faecal DNA approaches for carnivore diet analysis. *Mammal Review*, 49: 97-112. DOI: [10.1111/mam.12144](https://doi.org/10.1111/mam.12144)
- Morin D.J., Higdson S.D., Holub J.L., Montague D.M., Fies M.L., Waits L.P. & Kelly M.J. 2016. Bias in carnivore diet analysis resulting from misclassification of predator scats based on field identification. *Wildlife Society Bulletin* 40(4): 669-677. DOI: [10.1002/wsb.723](https://doi.org/10.1002/wsb.723)
- Mucci N., Pertoldi C., Madsen A.B., Loeschcke V. & Randi E. 2004. Extremely low mitochondrial DNA control-region sequence variation in the otter *Lutra lutra* population of Denmark. *Hereditas*, 130(3): 331-336. DOI: [10.1111/j.1601-5223.1999.00331.x](https://doi.org/10.1111/j.1601-5223.1999.00331.x)
- Nakamura M., Godinho R., Rio-Maior H., Roque S., Kaliontzopoulou A., Bernardo J., Castro D., Lopes S., Petrucci-Fonseca F. & Álvares F. 2017. Evaluating the predictive power of field variables for species and individual molecular identification on wolf noninvasive samples. *European Journal of Wildlife Research*, 63(3): 53. DOI: [10.1007/s10344-017-1112-7](https://doi.org/10.1007/s10344-017-1112-7)
- NCBI - National Center for Biotechnology Information. 2017. Basic Local Alignment Search Tool (BLAST). Available at: <http://blast.ncbi.nlm.nih.gov/Blast.cgi> (Accessed: 1 January 2017).
- Oliveira R., Castro D., Godinho R., Luikart G. & Alves P.C. 2010. Species identification using a small

- nuclear gene fragment: application to sympatric wild carnivores from South-western Europe. *Conservation Genetics*, 11(3): 1023-1032. DOI: [10.1007/s10592-009-9947-4](https://doi.org/10.1007/s10592-009-9947-4)
- Palomares F., Godoy J.A., Piriz A., O'Brien S.J. & Johnson W.E. 2002. Faecal genetic analysis to determine the presence and distribution of elusive carnivores: design and feasibility for the Iberian lynx. *Molecular Ecology*, 11(10): 2171-2182. DOI: [10.1046/j.1365-294X.2002.01608.x](https://doi.org/10.1046/j.1365-294X.2002.01608.x)
- Putman R.J. 1984. Facts from faeces. *Mammal Review*, 14(2): 79-97. DOI: [10.1111/j.1365-2907.1984.tb00341.x](https://doi.org/10.1111/j.1365-2907.1984.tb00341.x)
- Rosellini S., Osorio E., Ruiz-González A., Piñeiro A. & Barja I. 2008. Monitoring the small-scale distribution of sympatric European pine martens (*Martes martes*) and stone martens (*Martes foina*): a multievidence approach using faecal DNA analysis and camera-traps. *Wildlife Research*, 35(5): 434-440. DOI: [10.1071/WR07030](https://doi.org/10.1071/WR07030)
- Ruiz-González A., Madeira M.J., Randi E., Urra F. & Gómez-Moliner B.J. 2013. Non-invasive genetic sampling of sympatric marten species (*Martes martes* and *Martes foina*): assessing species and individual identification success rates on faecal DNA genotyping. *European Journal of Wildlife Research*, 59: 371-386. DOI: [10.1007/s10344-012-0683-6](https://doi.org/10.1007/s10344-012-0683-6)
- Sanz B. 2003. *Huellas y Rastros de los Mamíferos Ibéricos (Mamíferos Semiurbanos)*. Ed. Certeza, Zaragoza.
- Sbisà E., Tanzariello F., Reyes A., Pesole G. & Saccone C. 1997. Mammalian mitochondrial D-loop region structural analysis: identification of new conserved sequences and their functional and evolutionary implications. *Gene*, 205: 125-140.
- Soller J.M., Ausband D.E., Gunther M.S. 2020. The curse of observer experience: Error in noninvasive genetic sampling. *PLOS One* 15(3): e0229762. DOI: [10.1371/journal.pone.0229762](https://doi.org/10.1371/journal.pone.0229762)
- Taberlet P., Griffin S., Goossens B., Questiau S., Manceau V., Escaravage N., Waits L.P. & Bouvet J. 1996. Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, 24(16): 3189-3194. DOI: [10.1093/nar/24.16.3189](https://doi.org/10.1093/nar/24.16.3189)
- Torres R.T., Ferreira E., Rocha R.G. & Fonseca C. 2017. Hybridization between wolf and domestic dog: First evidence from an endangered population in central Portugal. *Mammalian Biology*, 86: 70-74. DOI: [10.1016/j.mambio.2017.05.001](https://doi.org/10.1016/j.mambio.2017.05.001)
- Urra F., Lozano J., Fernandes M., España A.J. & Monterroso P. 2014. El gato montés *Felis silvestris* (Schreber, 1777). In: J. Calzada, M. Clavero & A. Fernández (eds). *Guía virtual de los indicios de los Mamíferos de la Península Ibérica, Islas Baleares y Canarias*. Sociedad Española para la Conservación y Estudio de los Mamíferos (SECEM), Málaga. <http://www.secem.es/guiadeindiciosmamiferos/>
- Vilà C., Amorim I.R., Leonard J.A., Posada D., Castroviejo J., Petrucci-Fonseca F., Crandall K.A., Ellegren H. & Wayne R.K. 1999. Mitochondrial DNA phylogeography and population history of the grey wolf *Canis lupus*. *Molecular Ecology*, 8(12): 2089-2103. DOI: [10.1046/j.1365-294x.1999.00825.x](https://doi.org/10.1046/j.1365-294x.1999.00825.x)
- Waits L.P. & Paetkau D. 2005. Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. *Journal of Wildlife Management*, 60(4): 1419-1433.
- Weiskopf S.R., Kachel S.M. & McCarthy K.P. 2016. What are snow leopards really eating? Identifying bias in food-habit studies. *Wildlife Society Bulletin*, 40(2): 233-240. DOI: [10.1002/wsb.640](https://doi.org/10.1002/wsb.640)
- Wilson G.J. & Delahay R.J. 2001. A review of methods to estimate the abundance of terrestrial carnivores using field signs and observation. *Wildlife Research*, 28: 151-164.

Submitted: 10 February 2020

Accepted: 8 August 2020

Associate editor was Pablo Ferreras