Research Article

Ethanol versus swabs: what is a better tool to preserve faecal samples for non-invasive genetic analyses?

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Abstract

Faecal non-invasive genetic sampling is one of the most practicable, ethical and applied tools to investigate the biology and the ecology of elusive or endangered mammal populations. However, the reliability, accuracy and effectiveness of this technique may be deeply conditioned by several factors such as climate, habitat characteristics, seasonality, sample freshness and storage conditions. In this study, we compared the practicality, efficiency, safety and cost-effectiveness of two preservation methods widely applied to collect and preserve wolf excremental DNA: scats in 96% ethanol and faecal swabs in ATL lysis buffer, to be genotyped in non-invasive monitoring projects. Forty-six wolf faecal samples were collected using both storage methods in three different areas of the Central-Northern Italy during two seasonal (cold and hot) periods and their DNAs were genotyped at 12 unlinked autosomal microsatellites through a multiple-tube approach. Genotyping performances and error rates obtained from the two methods resulted not significantly different. Nonetheless, faecal swabs showed to be more practical, safer and cost-effective than ethanol for the collection and analysis of faecal samples.

Our study, though conducted on a limited sample size, suggests that faecal swabs could represent a reliable alternative tool to routinely apply in non-invasive genetic projects to monitor the presence, distribution and dynamics of populations of elusive and endangered mammal species such as the Italian wolf, still threatened by illegal poaching, hybridization and conflicts with human activities.

Introduction

Non-invasive genetic sampling, in combination with other techniques such as occasional observations and camera trapping, is increasingly applied in long-term monitoring and conservation projects of elusive and endangered meso- and large carnivore species (Scandura et al., 2011; Galaverni et al., 2012; Caniglia et al., 2012; Anile et al., 2014; Velli et al., 2015; Aziz et al., 2017; Granroth-Wilding et al., 2017; Mysłajek et al., 2018). The genotyping of faecal DNA contained in the intestinal cells adhering to the faeces allows to estimate temporal trends of genetic and demographic parameters almost impossible to achieve using traditional field methods alone (Waits and Paetkau, 2005; Caniglia et al., 2014; Arandjelovic and Vigilant, 2018). However, accuracy and effectiveness of non-invasive genetic sampling applications can be limited by low amplification success and high genotyping error rates (allelic dropout and false alleles; Waits and Paetkau, 2005; Broquet al., 2007). There are, indeed, several factors, such as diet, rain, humidity, UV exposition, mould or bacterial activity, which could affect freshness and quality of faecal samples directly in the field before their collection (Waits et al., 2000; McKelvey and Schwartz, 2004; Waits and Paetkau, 2005; Agetsuma-Yanagihara et al., 2017). Moreover, especially when the storage period is too long and the collected samples

are not immediately processed, the amount and quality of faecal DNA may decrease, further influencing the reliability of the downstream molecular analyses (Beja-Pereira et al., 2009).

Nevertheless, amplification success may be maximized and genotyping errors minimized through well-planned sampling schemes aimed at favouring the collection of faeces as fresh as possible and by choosing the most appropriate storage method, which should be able to (1) prevent DNA degradation, blocking the activity of the endogenous nuclease enzymes, and (2) preserve DNA quality through time, granting its long-term reliability (Agetsuma-Yanagihara et al., 2017).

To date, a variety of methods, including desiccation (Wasser et al., 1997; Murphy et al., 2000), freezing (Wasser et al., 1997; Frantz et al., 2003) and storage in buffers (Santini et al., 2007; Panasci et al., 2011) or ethanol (Reddy et al., 2012; Caniglia et al., 2014), have been used to store up carnivore faecal samples for their molecular characterization (Tende et al., 2014). Nevertheless, though the effectiveness of preserving methods can depend on the environmental conditions of the monitored areas and may vary among taxa and diets (Tende et al., 2014; Miles et al., 2015), most of the studies carried out to compare their efficacy showed that ethanol usually performs better than the others, especially during extended storing periods (Panasci et al., 2011; Tende et al., 2014).

However, using ethanol for sample storage is not always efficient because of (1) shipping and travel restrictions due to its volatile and

highly flammable nature, and (2) the difficulty to transport the quantities needed for a large sampling in sites hard to reach (Beja-Pereira et al., 2009; Miles et al., 2015). Recently, alternative faecal sampling tools such as cotton swabs (Rutledge et al., 2009; DeMatteo et al., 2014; Ramón-Laca et al., 2015) have been increasingly used in non-invasive genetic studies. They allow to cleanly sample the epithelial cells directly from the mucous layer of scats, while avoiding to collect the faecal matrix that is rich of polymerase chain reaction (PCR) inhibitors (Ball et al., 2007; Rutledge et al., 2009).

Non-invasive genetic sampling approaches result particularly suitable also to study the biology and ecology of highly elusive, territorial carnivores such as the grey wolves (*Canis lupus*). Wolf scats can be easily collected, along selected trails where wolves defecate to actively mark pack territory boundaries, or along snow-tracking routes (Mech and Boitani, 2010), and their genotypes can be used to reconstruct familiar unit genealogies and monitor their dynamics (Caniglia et al., 2014; Standbury et al., 2016; Granroth-Wilding et al., 2017; Fabbri et al., 2018). To date, most of the non-invasive genetic monitoring and conservation studies on wolves were preferentially based on the analysis of DNA extracted directly from fragments of faecal material stored in ethanol (Creel et al., 2002; Scandura et al., 2011; Caniglia et al., 2014; Canu et al., 2017; Fabbri et al., 2018). Less frequently non-invasive wolf DNA was obtained using faecal swabs (Rutledge et al., 2009) and only in a few exceptions wolf faeces were conserved in silica gel (Marucco et al., 2009) or lysis buffers (Santini et al., 2007).

In this study we compared the effectiveness of two preservation methods, scats in 96% ethanol versus faecal swabs in ATL lysis buffer (Qiagen Inc., Hilden, Germany), for the collection and storage of wolf excremental DNA contained in fresh-looking scats sampled during non-invasive monitoring projects carried out in three habitats of the Central-Northern Apennines with different environmental conditions (Caniglia et al., 2014; Canu et al., 2017; Mattioli et al., 2018; Santostasi et al., 2018). Specifically, our experiment was designed aiming to evaluate which of the two methods better performed in terms of (1) rates of amplification success and genotyping errors at 12 autosomal microsatellite loci, which are commonly used to reconstruct individual genetic profiles of non-invasively collected samples and to assign them as wolves, dogs or their hybrids (Caniglia et al., 2014; Imbert et al., 2016; Fabbri et al., 2018); (2) their practicality during sampling, storage and DNA extraction steps and (3) risks and costs associated to the phases of sample collection and DNA extraction.

Materials and methods

Between April 2015 and January 2016 we collected 46 putative wolf scat samples, 15 during the cold (October-March) and 31 during the hot (April-September) seasons. Scats were sampled from three areas of the Central-Northern Italian Apennines with different climate and environmental characteristics: the Tuscan-Emilian Apennine National Park (TEANP; n=13), showing mountain environments with medium-high altitudes and a sub-humid climate range (mean annual temperatures 9.2 °C and mean annual rainfall 55.3 mm); the Grosseto province (GR; n=10) characterized by a lowland landscape with typical Mediterranean conditions (mean annual temperatures 16.2 °C and mean annual rainfall 60.2 mm); the Arezzo province (AR; n=23) showing a hilly environment with a sub-Mediterranean climate (mean annual temperatures 13.8 °C and mean annual rainfall 48.2 mm). Faeces were opportunistically collected along roads and trails showing known signs of wolf presence, as previously assessed by wolf-howling surveys or occasional direct observations, which were travelled on foot at least once per week. Scats were qualitatively aged at the time of their sampling according to travel and weather histories, external appearance and moisture content (Santini et al., 2007; Rutledge et al., 2009) and were categorized as older (n=6) or fresher (n=40) than seven days.

Each scat was sampled both a) by cutting about one cm^3 of faecal material with a mono-use scalpel and placing it in a plastic graduated 50 ml wide neck square bottle (series "600", Kartell™, Noviglio, Italy) containing 40 ml of 96% ethanol (ethanol scats; ES) stored at ambient temperature in the field and at −20 °C in the laboratory; and b) by rubbing the surface of all sides of the faecal sample with a sterile disposable plastic cotton-tipped swab (FLmedical, Torreglia, Italy) placed in a 2.0 ml safe-lock tube (Eppendorf, Hamburg, Germany) containing 180 µl of the ATL lysis buffer from the Qiagen Blood & Tissue Kit (Qiagen Inc., Hilden, Germany), stored at ambient temperature in the field and at 4 °C in the laboratory (faecal swabs; FS). In cases of dry scats, FS were obtained moistening samples with distilled water to facilitate the collection of epithelial cells from the superficial mucous layer (Rutledge et al., 2009).

DNA extraction was performed within three months from the collection using the genomic DNeasy Blood & Tissue Kit (Qiagen Inc., Hilden, Germany), following the manufacturer's instructions, in an automated Multiprobe iiex Robotic Liquid Handling System (Perkin Elmer, Weiterstadt, Germany). During the first step of the DNA extraction, for each ES c. 25 mg of air-dried external portion of the scats were put in a 1.5 ml safe-lock tube containing 180 µl of ATL lysis buffer and 20 µl of proteinase K, whereas for each FS 20 µl of proteinase K were directly added into the 2 ml safe-lock collection tubes. Then, the enzymatic digestion to solubilize cell membranes and protein structures was performed at 56 °C for 45 minutes. All subsequent extraction steps followed the manufacturer's instructions.

Each DNA sample was PCR amplified and genotyped through a multiple-tube approach at 12 unlinked autosomal microsatellites including seven dinucleotides (CPH2, CPH4, CPH5, CPH8, CPH12, C09.250, and C20.253) and five tetranucleotides (FH2004, FH2079, FH2088, FH2096, and FH2137). Extraction, amplification and post-amplification procedures were carried out in three separate rooms reserved to low-template DNA samples, adding a blank control (no biological material) during DNA extraction, and a blank (no DNA) and a positive (known wolf-DNA sample) controls during DNA amplification, following protocols described in Caniglia et al. (2014) and Fabbri et al. (2018). The four amplification replicates performed per sample and per locus were used by the software Gimlet v.1.3.3 (Valière, 2002) to reconstruct consensus genotypes and, following Pompanon et al. (2005), to estimate amplification success (the number of successful amplifications divided by the total number of amplifications across samples), allelic dropout and false allele rates.

We compared the proportions of reliably genotyped samples (i.e. the correct multilocus genotypes showing a probability threshold R>0.95 estimated by the software Reliotype; Miller et al., 2002) between (1) the two sampling methods, (2) cold and hot seasons and (3) geographic areas, using Fisher's exact tests which are highly recommended for analyses with reduced sample sizes.

We tested for the significance of the differences in mean amplification success, allelic dropout and false alleles rates between ES and FS using a two-way non parametric Mann-Whitney U test, since the normality tests performed on the data significantly rejected a normal distribution ($p<0.05$).

After verifying they satisfied the assumption of a normality distribution (see Fig. S1), we also analysed the successfully genotyped samples performing a two-way ANOVA and a Tukey's tests to evaluate the influence of the two approaches on amplification success compared to cold and hot seasons or different sampling areas.

All the statistical tests were performed using the software Past v.3.24 (Hammer et al., 2001) and the estimated average values were associated with their 95% confidential intervals (CI).

Given the limited number of analyzed samples, to verify the robustness of the results and determine their sensitivity (effect size), we performed a power analysis for each test using the software G*Power v.3.1.9.4 (Faul et al., 2007). The power of statistical tests was evaluated considering the complement of β (1-β) which, ranging between 1 (high power) and 0 (low power), denotes the Type II (or beta-error) probability of falsely retaining an incorrect null hypothesis (Faul et al., 2007).

Moreover, as faecal materials may contain pathogens and infectious agents, we indirectly estimated the possible risks associated to their manipulation by measuring the mean time the operator manipulated ES and FS during the preliminary phases of DNA extraction (handling
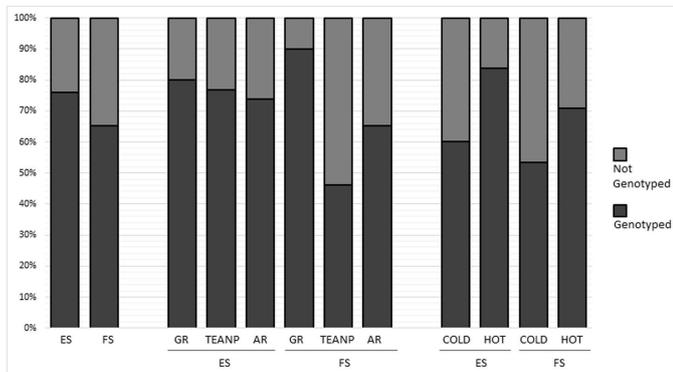
Figure 1 – Genotyping success rates (expressed in percentage) comparing results obtained from ES (ethanol scats) and FS (faecal swabs). Histograms show, comparing ES versus FS, percentages of overall rates of genotyped and not-genotyped samples (left side), percentages among different sampling areas (in the middle) and percentages between cold and hot seasons (right side).

Figure 2 – Amplification success and error rates in ES (ethanol scats) and FS (faecal swabs). Histograms show mean amplification success and mean error rates and their respective 95% confidential intervals.
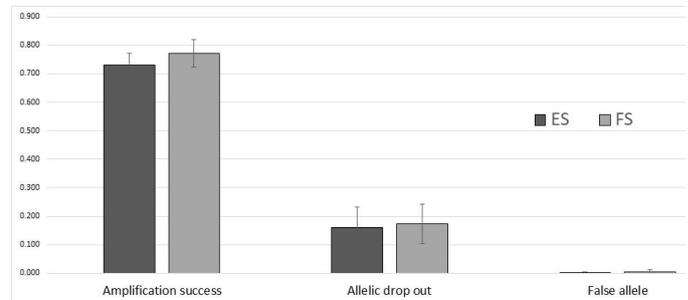
time) and the mean time faecal samples remained air-exposed (exposure time).

Finally, considering the necessary quantities of 96% ethanol, plastic bottles, 1.5 and 2.0 ml safe-lock tubes, cotton swabs, we roughly compared the mean costs for the collection and preservation of 100 ES and 100 FS samples. We obviously did not consider the ATL lysis buffer in the calculation since the same volume of this reagent was required for the enzymatic digestion of both ES and FS samples.

Results

We obtained reliable genotypes (Tab. 1) from 30 FS (65%) and 35 ES (76%), though the overall genotyping success did not significantly differ between the two methods (p=0.360). Coherently, FS and ES genotyping rates in cold (71% versus 84%) and hot (53% versus 60%) seasons were not significantly different (cold: p=0.999; hot: p=0.363; Fig. 1). Significant differences in genotyping rates were not detected between the two approaches (Fig. 1) even when comparing sampling areas (GS: 80% versus 90%, p=0.999; TEANP: 77% versus 46%, p=0.226; AR: 74% versus 65%, p=0.749). Interestingly, only 17% of FS and 33% of ES older than seven days were successfully genotyped against 73% of FS and 83% of ES fresher than a week.

We did not observe significant differences neither in amplification success nor in error rates (Tab. 1; Fig. 2): mean (μ) amplification success in FS (μ=0.772, 95% CI=0.049) was not significantly higher (U=421, p=0.17) than in ES (μ=0.730, 95% CI=0.042), as well as mean allelic dropout (μ=0.173, 95% CI=0.069) and false alleles (μ=0.006, 95% CI=0.007) rates in FS were not significantly different from allelic dropout (U=486, p=0.602) and false alleles (U=491.5, p=0.643) in ES (respectively μ=0.161, 95% CI=0.072 and μ=0.002, 95% CI=0.004). Amplification success in FS and ES was comparable also considering the influence of sampling periods and the three different sampling areas. There was neither a significant influence of sampling period nor a significant interaction of sampling period and storage method on amplification success (Tab. 2). However, we observed a significant influence of the sampling area on amplification success (F=5.71, p=0.005; Tab. 2) and the magnitude of the effect resulted dependent on the storage method with lower performances of FS in TEANP (Tukey's test for interaction FS – TEANP: Q=4.942, p=0.007; Fig. 1). The absences of significant differences for the considered parameters between the two methods, although extrapolated from limited sample sizes, appeared to be reliable enough since they were associated to a moderate statistical power for Fisher's exact (1-β=0.625) and Mann-Whitney U (1-β=0.660) tests, to a high statistical power for the ANOVA (1-β=0.819-0.715; Tab. 2) tests, and were further confirmed by the 95% CI of the mean values estimated from the statistical computations which were largely overlapping (Tab. 1; Fig. 2). FS handling during the preliminary phases of DNA extraction was much faster (μFS=927.5s, SE=±7.5s) than ES (μ ES=9,932s, SE=±4s) with a consequently shorter exposure time to possible pathogen and infectious agents for FS (μFS=0.9s, SE=±0.1s) than for ES (μES=77.5s, SE=±5.8s) (Tab. 1). FS showed a mean collection and preservation cost per 100 samples less than half cheaper (31.46 €) than ES (77.00 €) (Tab. 1). Finally, FS in 2 ml safe-lock tubes resulted more practical to transport or ship thanks to the lower individual weight (1.584 ± 0.035 g) than ES (43.520 ± 1.434 g) and each FS also required less storage space (3.00 m^3/pcs) than ES (87.75 cm^3/pcs).

Discussion

Faecal non-invasive genetic sampling represents an essential practical tool for integrating long-term monitoring projects aimed to effective conservational and management strategies, especially for elusive and endangered large carnivore species (Caniglia et al., 2014; Velli et al., 2015; Tsaparis et al., 2015; Bull et al., 2016; Aziz et al., 2017; Granroth-Wilding et al., 2017; Fabbri et al., 2018; Hollerbach et al., 2018; Karssene et al., 2018a,b; Chetri et al., 2019). However, the performances of such approach and the reliability of the derived information can be deeply conditioned by the low DNA quantity and quality contained in faecal samples (Waits et al., 2000). Nonetheless, such drawbacks can be reduced by collecting only samples as fresh as possible (Santini et al., 2007; Miles et al., 2015; Quasim et al., 2018; Schultz et al., 2018) and by using appropriate storage methods to preserve DNA integrity until molecular analyses (Tende et al., 2014). Several studies evaluated the efficiency of different methods to preserve mammalian faecal DNA (Wasser et al., 1997; Murphy et al., 2002; Santini et al., 2007; Panasci et al., 2011; Reddy et al., 2012; Tende et al., 2014; Goodman et al., 2017). However, most of them mainly focused on the success of the genetic analyses performed using a particular storage method compared to others, seldom considering the working times needed for the treatment of the collected samples or the possible biological risks associated to their manipulation (Miles et al., 2015; Goodman et al., 2017; Quasim et al., 2018). Differently, in this study we compared the genotyping performances as well as the associated costs, practicality and safety during sample collection and DNA extraction, of fresh wolf scats collected and stored using ethanol versus swabs in ATL lysis buffer. Although our sample size allowed us to account only for wide differences, our results were well-supported by the power analysis and demonstrated that both ES and FS well-performed in preserving DNA integrity, showing no significant differences in genotyping success rates, which were in both cases likely due to the preliminary accurate selection of mostly fresh samples, considerably higher than those usually obtained in most of the studies based on the analysis of mammal non-invasive materials (Lovari et al., 2009; Sugimoto et al., 2012; Ruiz-Gonzalez et al., 2013; Anile et al., 2014; Velli et al., 2015; Viglino et al., 2016), including those regarding canids (Caniglia et al., 2014; Fabbri et al., 2018; Quasim et al., 2018). Coherently, ES and FS did not show significant differences neither in amplification success nor in error rates, which were comparable to values reported in other non-invasive genetic studies (Ruiz-Gonzalez et al., 2013; Caniglia et al., 2014; Wultsch et al., 2014; Fabbri et al., 2018). Interestingly, though ES showed a slightly higher proportion of genotyped samples, FS yiel-

Table 1 – Summary of the parameters evaluated in this study comparing results obtained analysing ethanol scats and faecal swabs.

	Genotyped samples		Method Ampl. success		Allelic dropout		False Allele		Time Exposure		Handling		Costs (€) EtOH	Bottles	Tubes	Swabs	Total
Ethanol scats	35	(76%)	0.730	(0.042)	0.161	(0.072)	0.002	(0.004)	77.5	(5.8)	9932.5	(4.0)	31.50	38.00	7.5	0	77
Faecal swabs	30	(65%)	0.772	(0.049)	0.173	(0.069)	0.006	(0.007)	0.9	(0.1)	927.5	(7.5)	0	0	6.8	24.66	31.46

Method: applied collection and storage method, ethanol scats and faecal swabs; *Genotyped samples*: number of successfully genotyped samples (and corresponding percentages); *Ampl. success*: mean amplification success rates (and corresponding 95% confidential interval); *Allelic dropout*: mean allelic dropout rates (and corresponding 95% confidential intervals); *False alleles*: mean false allele rates (and corresponding 95% confidential intervals); *Exposure time*: mean exposure time (and corresponding standard errors), measured in seconds, for the operator to potential pathogen and infectious agents during the preliminary phases of DNA extraction; *Handling time*: the time (and corresponding standard errors), measured in seconds, necessary to carry out the preliminary phases of DNA extraction; *EtOH*: costs (€) for the 96% ethanol necessary to storage 100 faecal samples, considering a quantity of about 40 ml per faecal sample and a cost of 7.785 € per ethanol litre; *Bottles*: costs (€) for the plastic bottles necessary to storage 100 faecal samples, considering a cost of 0.38 € per bottle; *Tubes*: costs (€) for the 1.5/2.0 ml safe-lock tubes necessary for the collection and the preliminary phases of DNA extraction of 100 faecal samples; *Swabs*: costs (€) for the cotton swabs necessary for the collection of 100 faecal samples; *Total costs*: total costs necessary for the collection and the preliminary phases of DNA extraction of 100 faecal samples.

ded higher mean amplification success rates due to an overall higher number of positive amplifications per sample and per locus. Such a discrepancy, though not significant, might indicate that genotyped FS might have a better DNA quality than genotyped ES. All these findings suggested that both genotyping and amplification success rates were apparently independent from the storage methods, but they were mainly affected by the age of the analysed samples that significantly influenced DNA degradation and the consequent results (Piggott and Taylor, 2003; Santini et al., 2007; Rutledge et al., 2009; Anile et al., 2014). The similar performances showed by ES and FS were concordant with results obtained by Quasim et al., 2018, who did not find significant differences in the red fox (*Vulpes vulpes*) mitochondrial DNA amplification success rates between air-dried faecal swabs and scats. Interestingly, other canid non-invasive studies reported faecal swab amplification success rates at mitochondrial and nuclear loci comparable to those obtained analysing DNA extracted from good-quality biological samples (Rutledge et al., 2009) or significantly higher than those obtained from common DMSO-based extracts (Vynne et al., 2012).

However, the comparisons of genotyping success rates with those estimated in other studies evaluating the storage efficacy of faecal swabs should be treated with caution since (1) they analysed different sample

Table 2 – Results of the two-way ANOVA comparisons between cold and hot seasons and among the three sampling areas.

	F-value	df	p
ANOVA seasons			
Method	1.745	1	0.192
Season	0.238	1	0.627
Interaction	0.360	1	0.551
Within		61	
Total		6	
Statistical power: 0.819			
ANOVA areas			
Method	2.392	1	0.127
Area	5.713	2	0.005***
Interaction	1.797	2	0.175
Within		59	
Total		64	
Statistical power: 0.715			

Method: applied collection and storage method, ethanol scats and faecal swabs; *Season*: seasonal period in which samples were collected, cold (October – March) and hot (April – September); *Area*: investigated sampling areas, the Tuscan-Emilian Apennine National Park, the Grosseto province and the Arezzo province; *Interaction*: combined effects of factors (Method versus Season; Method versus Area) on the dependent measure (amplification success); *Within*: within-group variation; *Total*: sum of the statistics; *F-value*: F-statistic based on the ratio of Mean squares; *df*: degrees of freedom; *p*: probability value of the statistics; ***: highly significant *p*-value; *Statistical power*: power value of the statistical test (1-β) for the given sample size, considering an effect size of 0.5.

sizes, (2) applied different sampling and storage protocols (e.g. swabbing from frozen samples directly in lab, Vynne et al., 2012; stored swabs in empty vials at ambient temperature, DeMatteo et al., 2014; dried and stored in paper envelopes at ambient temperature, Miles et al., 2015) and (3) their results were clearly dependent on the trophic niche and diet of the examined taxa.

Nevertheless, in our study, we are confident of the reliability and repeatability of the comparisons executed between the two different storage methods we applied. In fact, though only tested on a limited number of samples (but comparable to other similar studies, e.g. Panasci et al., 2011; Tende et al., 2014), their performances were evaluated in terms of amplification success and error rates associated to genotypes obtained applying standardized and automated protocols, which have been already successfully used for the specific and individual identifications in long-term non-invasive monitoring projects of the Italian wolf (*C. l. italicus*) population (Caniglia et al., 2014; Randi et al., 2014; Fabbri et al., 2018). Interestingly, although not affecting genotyping success on average, our ANOVA analyses showed a significant influence of the sampling area on amplification success rates and a significant interaction between sampling methods and habitats with different climate and environmental characteristics. Conversely to other studies (Miles et al., 2015) which found that canid faecal swabs potentially yielded higher effectiveness when applied in humid habitat, our northernmost area (TEANP), characterized by mountain environments and sub-humid climate, showed the lowest amplification success rates for FS, whereas ES yielded similar results to the other two sampling areas. These findings let us to hypothesise that amplification success results might have been affected by the collectors' individual experience in swab sampling rather than by the operative ecological context per se. Such hypothesis would seem to be quite supported since, conversely to GR and AR areas, where sampling was performed only by the same trained collectors, in the TEANP sector sampling was carried out by a variety of collaborators, including not only expert personnel but also students, apprentices and volunteers. However, though concordant with other carnivore studies showing that the different expertise among collectors can significantly influence scat genotyping success rates (Ruiz-Gonzalez et al., 2013), our results are not conclusive because of the limited and not balanced number of analysed samples within different areas. Nonetheless, these findings suggest that, before starting any non-invasive genetic monitoring project, a proper training in sample collection techniques should be deeply encouraged, thus to standardize sampling methods and favour, whenever achievable, the collection of only fresh faecal depositions, possibly not older than one week (Santini et al., 2007; Caniglia et al., 2014; Miles et al., 2015; Canu et al., 2017; Quasim et al., 2018; Schultz et al., 2018). Conversely to genotyping performances, which were independent and not significantly affected by storage conditions, collection, processing and exposure times to potential pathogens were deeply different between the two preserving methods. For the first time, in our study we indeed evaluated such factors: FS, which required only few manual steps during both the sample collection in the field and the first DNA extraction phases in the laboratory, resulted extremely more practical and faster

to process than ES, whose faecal matrix needs several more steps to be collected and processed. Consequently, the use of FS revealed to be also a safer tool than ES since it required less prolonged exposure times, both in the field and in the laboratory, to eventual faecal canid pathogens and parasites such as *Echinococcus multilocularis* and *Echinococcus granulosus* (Guberti et al., 2004; Sobrino et al., 2006; Grosso et al., 2012; Poglayen et al., 2017; Massolo et al., 2018), thus decreasing the potential risks of echinococcosis for the operators. Finally, since they do not need the use of plastic bottles with ethanol in any step, FS confirmed to be a more cost-effective instrument than ES for carnivore non-invasive DNA-based monitoring projects (Quasim et al., 2018), showing overall processing prices reduced by more than 50% compared to ES. Additionally, being not flammable and characterized by much smaller storage volumes than ES, FS resulted to be also more practical to manipulate and low-cost to apply requiring much lower transport and shipping costs.

Conclusions

In this study, we empirically compared the genotyping efficiency, practicality, safety and cost-effectiveness of two broadly applied methods to collect and storage non-invasive wolf samples to be molecularly characterized for conservational and management purposes. Though currently conducted only on a limited sample size, our tests demonstrated that faecal swabs, when applied to well-planned sampling schemes ensuring an accurate selection of fresh samples, could represent a valid, rapid and reliable alternative tool for long-term carnivore non-invasive genetic monitoring projects. Our study showed that faecal swabs, compared to other classical storage methods, confirmed to be a more practical, safer and cost-effective tool, not significantly decreasing genotyping results, to actively monitor the presence, distribution and dynamics of populations of endangered mammal species such as the Italian wolf, still threatened by illegal poaching, hybridization and conflicts with human activities, and of other elusive carnivores such as the golden jackal (*C. aureus*), the lynx (*Lynx lynx*), the wildcat (*Felis silvestris ssp.*), the African golden wolf (*C. anthus*), the snow leopard (*Panthera uncia*), the jaguar (*Panthera onca*) (Wultsch et al., 2014; Velli et al., 2015; Bull et al., 2016; Hollerbach et al., 2018; Karssene et al., 2018a,b; Chetri et al., 2019). We are confident that future carnivore non-invasive studies based on the analysis of a larger number of faecal swabs, which should minimise possible effect sizes and increase the power of statistical computations, could definitively confirm our results. Additionally, their characteristics make faecal swabs a promising means to collect non-invasive samples as DNA source for future routinely studies based on the analysis of dozens of single nucleotide polymorphisms (SNPs), which represent the most widespread source of genome-wide variation, for the conservation and management of priority and endangered taxa.

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Supplemental information

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

Figure S1 Normality distribution plots of the mean amplification success values.