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Tools and Technology

Optimizing Collection Methods for Noninvasive Genetic Sampling of Neotropical Felids

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ABSTRACT Field-sampling methods for molecular scatology studies must be optimized, especially when working on elusive species in challenging tropical environments where rates of DNA degradation are elevated because of hot and humid weather conditions. To maximize polymerase chain reaction (PCR) amplification success and genotyping accuracy rates and to minimize genotyping error rates for fecal DNA samples of jaguars (*Panthera onca*) and co-occurring Neotropical felids collected in Belize, Central America, we evaluated the performance of two fecal DNA storage techniques (dimethyl sulfoxide saline solution [DETs buffer] and 95% ethanol [EtOH]) suitable for long-term preservation at remote tropical sites. Additionally, we tested fecal samples collected from 4 different locations on the scat (top, side, bottom, inside) at 2 different tropical forest types (tropical broadleaf and tropical pine forests). DETs buffer was the superior fecal DNA preservation method, with 44% higher PCR amplification success ($P = 0.009$) and 17% higher genotyping accuracy ($P = 0.021$) than 95% EtOH-stored samples. Polymerase chain reaction amplification success of fecal DNA collected at the more open, pine-forest (*Pinus* sp.) site differed significantly across locations on the scat, with highest mean success rates obtained from the top (85% ± 6.5% SD), followed by the side (79% ± 9.4% SD), bottom (76% ± 11.9% SD), and inside (69% ± 10.3% SD) of scat samples. Scat samples collected at the more closed-canopy broadleaf site did not show any significant differences in amplification success rates across scat locations. We recommend that researchers optimize field-sampling methods, including collection and storage protocols, by conducting a pilot study prior to their molecular scatology research efforts. © 2015 The Wildlife Society.

KEY WORDS fecal DNA storage, genotyping error, jaguar, molecular scatology, neotropics, noninvasive genetic sampling, *Panthera onca*, PCR amplification success, puma, *Puma concolor*.

Molecular scatology is an advancing noninvasive genetic monitoring approach in which individuals are genotyped using DNA from exfoliated intestinal epithelial cells found in their feces (scat; Höss et al. 1992, Constable et al. 1995, Kohn and Wayne 1997). Without ever capturing elusive, wide-ranging carnivore species, and by simply collecting and analyzing their scat samples, researchers can determine species, gender, and individuals, making molecular scatology useful for ecological, demographic, and population genetic monitoring (e.g., Kohn and Wayne 1997, Kohn et al. 1999, Waits and Paetkau 2005, Kelly et al. 2012, Rodgers and Janečka 2013).

Although molecular scatology studies have been applied extensively in the northern temperate zone, there is a great need to standardize noninvasive genetic sampling techniques for warmer and more humid climates such as the tropics in order to study elusive and threatened species, including wild felids. Despite the potential power of this approach, several problems and limitations have been documented when applying noninvasive genetic sampling, including low quantity and quality of the host DNA due to enzymatic and bacteria-mediated DNA degradation, the presence of polymerase chain reaction (PCR) inhibitors (e.g., digestive enzymes, microorganisms, bile salts, bilirubin), and contamination with nontarget DNA (bacteria, dietary items, etc.; e.g., Kohn and Wayne 1997, Frantzen et al. 1998, Taberlet et al. 1999, Broquet et al. 2007). These factors may cause PCR failure or genotyping errors, leading to erroneous microsatellite genotypes and individual identification (e.g., Taberlet et al. 1996). Genotyping errors may result in either allelic dropout (ADO) due to nonamplification of 1 allele in a heterozygous genotype, or false alleles (FA) caused by PCR slippage errors or contamination with nontarget DNA (e.g., Taberlet et al. 1999, Broquet and Petit 2004).

In the tropics, fecal samples are exposed to high temperatures, elevated levels of humidity and ultraviolet light, and a high diversity of microorganisms, which can accelerate rates of DNA degradation (e.g., Lindahl 1993, Farrell et al. 2000,

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Piggott 2004, Vynne et al. 2012). The extent of degradation of fecal DNA prior to molecular analysis also is influenced by the target species itself and its diet (e.g., Murphy et al. 2003), scat sample condition (e.g., Piggott 2004, Murphy et al. 2007, Santini et al. 2007), and various field and laboratory techniques, including the choice of fecal DNA collection and storage methods (e.g., Wasser et al. 1997, Murphy et al. 2002, Piggott and Taylor 2003, Beja-Pereira et al. 2009, Stenglein et al. 2010).

DNA storage techniques aim to minimize DNA degradation prior to laboratory analysis. The variety of different preservation techniques for noninvasive genetic sampling makes it challenging for those initiating new studies to select the most efficient storage method (e.g., Beja-Pereira et al. 2009). The choice depends on a variety of factors, including the target species, its life history, study logistics, DNA storage time, DNA type, and environmental conditions (e.g., Frantzen et al. 1998, Piggott and Taylor 2003, Soto-Calderon et al. 2009). Comparative fecal DNA preservation studies for carnivores were conducted primarily for canids and ursids (e.g., Wasser et al. 1997, Murphy et al. 2000, 2002, Panasci et al. 2011). For felids, a wide variety of fecal DNA methods have been applied, including freezing (e.g., Ernest et al. 2002, Sugimoto et al. 2006), air drying (e.g., Farrell et al. 2000, Weckel et al. 2006), silica desiccation (e.g., Haag et al. 2009, Janečka et al. 2011), or liquid storage using buffer solutions (e.g., 20% dimethyl sulfoxide buffer, Vynne et al. 2012) or ethanol (EtOH; e.g., Mondol et al. 2009, Michalski et al. 2011, see also online Supporting Information Table S1). Yet, only a handful of comparative fecal DNA preservation studies examined the effectiveness of different methods on amplification of fecal DNA for felids (e.g., for mtDNA for wild tigers [*Panthera tigris*]; Bhagavatula and Singh 2006) and nuclear DNA (nDNA) markers for captive tigers (Reddy et al. 2012). An empirical evaluation of different fecal DNA storage methods to optimize multilocus nDNA genotyping and individual identification for wild felids sampled in challenging environments such as the tropics has not yet been conducted.

Fecal DNA is not uniformly distributed along the length of scat samples (Johnson et al. 2005); therefore, it is also important to consider the sampling location on the scat. Direct contact of fecal samples with soil and its decomposers (e.g., bacteria, fungi) increases rates of DNA degradation (e.g., Nsubuga et al. 2004, Hajkova et al. 2006, Santini et al. 2007), and direct exposure to ultraviolet light leads to DNA damage (Santini et al. 2007). Consequently, a wide variety of collection protocols for fecal DNA sampling have been used to maximize the success of noninvasive genetic studies of different species. Techniques for fecal DNA collection include collecting fecal material from the scat surface by swabbing (e.g., Frantz et al. 2003), scraping (e.g., Stenglein et al. 2010), washing (e.g., Palomares et al. 2002), or homogenizing fragments or entire scat samples prior to DNA extraction (e.g., Wasser et al. 1997). A few carnivore studies have empirically tested for differences in DNA quality using different sampling locations within a scat sample and uniformly recommended fecal DNA sampling from the outside or surface of the scat sample for higher PCR DNA amplification success (e.g., Pires and Fernandes 2003, Stenglein et al. 2010). An empirical evaluation of scat locations for felid DNA studies has not yet been conducted.

Molecular scatology studies of wild felids in tropical regions have been increasing in number, but PCR amplification success and genotyping error rates vary greatly, particularly when nuclear DNA markers are used for individual-based monitoring. We conducted a noninvasive genetic study of three co-occurring felids (jaguar [*Panthera onca*], puma [*Puma concolor*], and ocelot [*Leopardus pardalis*]) using a scat-detector dog, a set of highly polymorphic microsatellite loci, and fecal DNA samples collected across 2 environmentally contrasting study sites in Belize, Central America. Our main objective was to identify the most efficient protocol for fecal DNA collection, storage, and subsequent amplification. Specifically, we aimed to examine the effects of 1) 2 different liquid storage methods (dimethyl sulfoxide saline solution [DETs buffer] and 95% ethanol [EtOH]), and 2) 4 sampling locations within the scat sample (top, side, bottom, inside) on PCR amplification success, genotyping accuracy, and microsatellite genotyping error rates. We emphasize the importance of optimizing field-sampling protocols to increase the efficiency and reliability of noninvasive genetic monitoring techniques.

STUDY AREA

We conducted a 2–3-month-long scat survey at 2 study sites (Mountain Pine Ridge Forest Reserve, MPR; and Rio Bravo Conservation and Management Area, RB) from 2007 to 2008 in Belize, Central America (Fig. 1). The MPR site is located in Central-West Belize (16°57′N, 88°54′W), occupying approximately 430 km^2 of predominantly pine (*Pinus* sp.) forest with some broadleaf moist forest interspersed and a relatively open forest canopy. Elevation ranges from 120 m to 1,017 m, and annual rainfall averaged from 1,550 m to 2,108 m. Average temperatures fluctuated between 17° C and 29° C. The RB site, located in Northwestern Belize (17°42′N, 88°54′W), was the largest protected area (934.3 km^2) within the country, and had a relatively closed canopy consisting of a diversity of natural forest (broadleaf, pine, and mangrove forest), lowland savanna, and marsh ecosystems (Bridgewater et al. 2002). Elevation ranges from 4 m to 241 m, and average annual rainfall ranges from 1,549 mm to 1,600 mm. Average temperatures fluctuated between 26° C and 32° C.

METHODS

Fecal Sample Detection, Collection, and Storage

We detected fecal samples using a professionally trained scat-detector dog (PackLeader LLC, Gig Harbor, WA), which was trained to locate scat samples of all 5 wild felid species (jaguar, puma, ocelot, margay [*Leopardus wiedii*], and jaguarundi [*Puma yagouaroundi*]). We conducted opportunistic surveys within the study sites with the scat-detector dog searching off-leash following the protocols described in Wultsch et al. (2014). Upon detection in the field, we

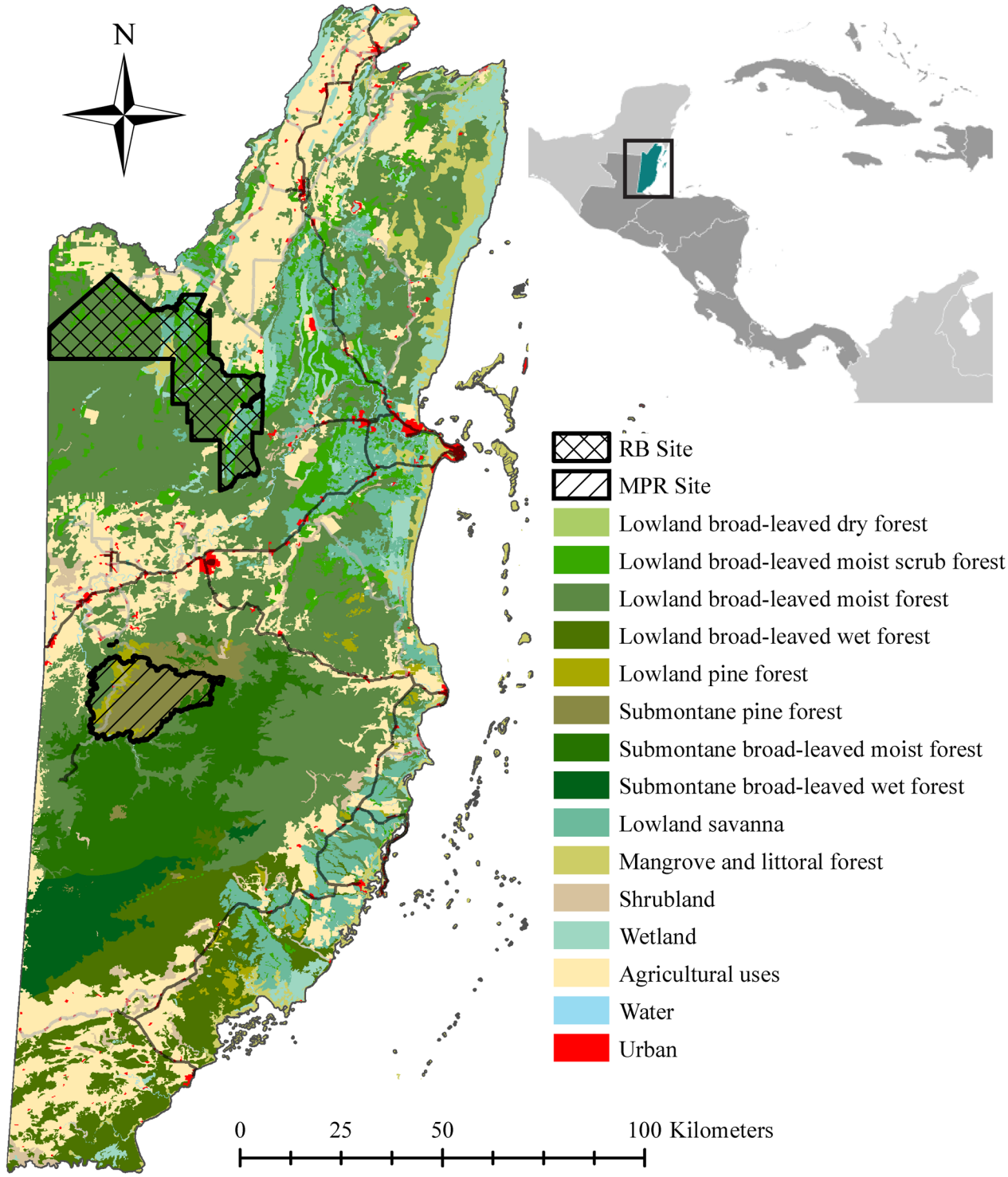

Figure 1. Locations of 2 study sites: the Mountain Pine Ridge Forest Reserve (MPR), and the Rio Bravo Conservation and Management Area (RB) in Belize, Central America, where we collected fecal DNA samples of jaguars (*Panthera onca*) and co-occurring Neotropical felids during 2007–2008, in order to evaluate the performance of fecal DNA collection and storage techniques.

categorized each scat as low or high quality based on overall appearance, color, odor strength, and presence of mold. Scat samples that looked visibly degraded, broken apart, and moldy were categorized as low-quality samples.

For each fecal sample, we collected approximately 0.5 mL fecal material and stored it at ambient temperature in 2 sterile, 2-mL screw-top tubes filled with either dimethyl sulfoxide saline solution (DETs buffer [20% dimethyl sulfoxide, 0.25M ethylenediaminetetraacetic acid, 100 mM Tris, pH 7.5, and NaCl to saturation]; Seutin et al. 1991) or 95% EtOH at a volumetric ratio of 1:4 scat-to-solution. We prefilled vials with storage liquids prior to fieldwork. We stored scat vials for up to 8 months under ambient or room temperature until extraction. Additionally, for each intact

scat located in the field, we collected approximately 0.5 mL of fecal material from 4 different locations (top, side, bottom, and inside) of the scat. For the top, side and bottom scat locations, we broke off small superficial fragments (2–3 mm thick) from the fecal sample using disposable wooden sampling sticks. For the inside scat location, we broke apart the fecal samples and collected small scat fragments.

Fecal DNA Extraction, PCR Amplification, and Microsatellite Genotyping

To avoid contamination, we conducted fecal DNA extractions in a separate room at the Laboratory for Ecological, Evolutionary and Conservation Genetics at the University of Idaho (Moscow, ID) dedicated to low-quantity DNA samples. We used the QIAamp DNA Stool Mini Kit protocol (Qiagen, Inc., Valenica, CA) to extract DNA from all fecal samples. We added an extraction negative to each extraction set to monitor for contamination.

We assigned scat samples to feline species based on species-specific microsatellite alleles and microsatellite allelic size ranges, and confirmed assignment by sequencing of 4 mitochondrial gene regions described in Wultsch et al. (2014). We amplified 10 microsatellite loci (FCA032, FCA096, FCA100, FCA124, FCA126, FCA132, FCA212, FCA225, FCA229, FCA275) originally developed for the domestic cat (*Felis catus*; Menotti-Raymond and O'Brien 1995, Menotti-Raymond et al. 1999) in 3 multiplexes (multiplex 1: FCA032, FCA100, FCA124; multiplex 2: FCA126, FCA212, FCA229; multiplex 3: FCA096, FCA225, FCA132, FCA275). Multiplexes 1 and 2 consisted of 3.5 μL 1 × concentration Qiagen Master Mix (Qiagen, Inc.), 1.54 μL of primers (0.4 μM for FCA032 F and R, 0.6 μM for FCA100 F and R, 0.1 μM for FCA124 F and R), 0.7 μL of 0.5 × concentration Qiagen Q solution, 0.26 μL H_2O, and 1.0 μL DNA extract. Multiplex 3 consisted of 1× concentration Qiagen 3.5 μL Master Mix, 1.96 μL of primers (0.2 μM for each F and R primer of multiplex 3), 0.7 μL of 0.5× concentration Qiagen Q solution, and 1.0 μL DNA extract. We conducted microsatellite PCR amplifications using a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). For multiplexes 1 and 2, we started with an initial denaturation step of 15 min at 95° C; followed by 13 cycles of 30 s at 94° C for denaturation, 1.5 min at 62.4° C with a decrease in annealing temperature of 0.3° C in each cycle, and 1 min elongation at 72° C; followed by 32 cycles of 30 s at 94° C for denaturation, 1.5 min at 60° C for annealing, and 1 min elongation at 72° C; and 30 min at 60° C for final elongation. For multiplex 3, we changed the annealing temperature to 57° C. A PCR negative was added in each group of PCR reactions to monitor for contamination.

We performed 3–6 PCR replicates/sample. To determine consensus genotypes, we followed protocols described by Wultsch et al. (2014). A multitube approach was used where ≥3 identical homozygote PCR results were required to finalize homozygote genotypes, and each allele had to be observed in 2 independent PCRs in order to record a heterozygous genotype. We visualized PCR products using an ABI Prism® 377 automated DNA sequencer (Applied Biosystems™, Waltham, MA), and identified genotypes using the software GENEMAPPER™, version 3.7 (Applied Biosystems).

Data Analysis

To calculate PCR amplification success, genotyping accuracy (GA), and genotyping error rates, we selected the last 2 PCR runs for all loci across all scat samples to standardize the number included per sample. We calculated rates across all loci and assessed them by calculating the percentage of successful PCR reactions across all samples tested and all samples with finalized species identification. We estimated genotyping accuracy rates by calculating the percentage of successful PCR reactions, whose outcomes matched the finalized consensus genotype. We quantified genotyping error by calculating the rate of ADO and FA following the protocols of Broquet and Petit (2004).

Comparison of 2 fecal DNA storage methods across scat samples of varying quality.—We stored a set of 30 scats (15 fresh or high- and 15 degraded or low-quality samples) from the MPR site using 2 liquid storage techniques (DETs buffer, 95% EtOH). We categorized the scat samples as low- or high-quality samples based on physical appearance, odor strength, color, and presence or absence of mold prior to collection. We calculated PCR amplification success, GA, ADO, and FA rates across all loci and compared them across fecal samples stored using the 2 preservation techniques. We evaluated statistical differences between groups using nonparametric Wilcoxon signed-rank tests using Program R, version 2.15 (R Core Team 2009).

Effect of 4 sampling locations on fecal DNA across 2 distinct tropical forest types.—For a subset of 40 intact scats, we collected fecal DNA samples from 4 different sample locations (top, bottom, side, and inside) within each scat sample. We collected the fecal DNA samples at 2 study sites ($n = 20$ at MPR, $n = 20$ at RB), which differed in vegetation types and environmental conditions. We stored scat samples for this study using DETs buffer. We evaluated statistical differences between groups (DNA scat location) using nonparametric Kruskal–Wallis, and *post hoc* Wilcoxon signed-rank tests using Program R, version 2.15 (R Core Team 2009).

RESULTS

The fecal samples included in the storage and collection study were identified genetically as Neotropical felids (storage study at MPR site, 30 scats [24 jaguars, 2 pumas, 4 failed]; collection study, 20 scats at each MPR [18 jaguars, 1 puma, 1 ocelot] and RB [3 jaguars, 8 pumas, 8 ocelot, 1 failed] sites).

Comparison of 2 Fecal DNA Storage Methods Using Scat Samples of Varying Quality

The PCR amplification success rate for all scat samples tested ($n = 30$) was significantly higher when stored in DETs buffer (62% ± 19.9% SD) than when stored in 95% EtOH (43% ± 14.9% SD). Likewise, the GA rate for DETs-stored samples (88% ± 8.2% SD) was significantly higher than for

EtOH-stored samples (75% ± 15.2% SD). Genotyping error rates were lower when using DETs storage (ADO, 22% ± 28.9% SD; FA, 3% ± 4.2% SD) than when using EtOH (ADO, 32% ± 17.6% SD; FA, 7% ± 7.5% SD), but variances were high and the differences detected were not statistically significant (Figs. 2 and 3; Table 1).

For high- ($n = 15$) and low-quality ($n = 15$) fecal samples, the mean PCR amplification success rate was significantly higher when stored in DETs buffer (67% ± 22.3% SD; 57% ± 18.5% SD) than when stored in EtOH (50% ± 21.7% SD; 35% ± 10.2% SD). Genotyping accuracy rates for high- and low-quality fecal samples were higher when stored in DETs (88% ± 11.5% SD; 88% ± 12.4% SD) than in 95% EtOH (75% ± 18.5% SD; 75% ± 18.8% SD). Allelic dropout and FA rates were lower using DETs (for high-quality samples [ADO, 21% ± 31.2% SD, FA, 3.0% ± 4.2% SD]; for low-quality samples [ADO, 20% ± 18.8% SD, FA, 3.0% ± 5.0% SD]) than for EtOH storage (for high-quality samples [ADO, 37% ± 23.7% SD, FA, 5% ± 6.1% SD]; for low-quality samples [ADO, 26% ± 17.2% SD; FA, 10% ± 10.9% SD]). Differences in results for GA, ADO, and FA rates, however, were not statistically significant (Figs. 2 and 3; Table 1).

Effect of 4 Sampling Locations on Fecal DNA Amplification Success Across 2 Distinct Tropical Forest Types

For overall comparisons of 4 different scat DNA sampling locations (top, side, bottom, or inside) within study sites, Kruskal–Wallis rank–sum tests showed that PCR amplification success across sampling locations from scats at the MPR site ($n = 20$) differed significantly, with highest success rates at the top (85% ± 6.5% SD) followed by the side (79% ± 9.4% SD), bottom (76% ± 11.9% SD), and inside (69% ± 10.3% SD) of scat samples. Genotyping accuracy rates also showed significant differences, with highest GA rates for the bottom (90% ± 5.2% SD), followed by the top (87% ± 8.7% SD), inside (85% ± 7.8% SD), and side (80% ± 10.0% SD) location. Differences for ADO and FA rates, however, were not statistically significant (Fig. 4; Table 2). For RB samples ($n = 20$), Kruskal–Wallis rank–sum tests showed that PCR amplification success, GA, ADO, and FA rates were not significantly affected by the 4 different sampling locations within a scat sample (Fig. 4; Table 2).

DISCUSSION

Fecal DNA sample preservation is a crucial component of every molecular scatology study, and several factors should be considered when choosing a DNA storage method. Optimization of sampling protocols is particularly important when scats are collected in tropical environments where DNA degradation rates accelerate because of extreme weather conditions. Yet, only a handful of molecular scatology studies have examined different preservation methods in tropical environments (e.g., Frantzen et al. 1998, Soto-Calderon et al. 2009), and only 2 have focused on felids. For tigers in India, mtDNA PCR amplification success rates did not significantly differ between EtOH and silica preservation (Bhagavatula and Singh 2006). Reddy et al. (2012) evaluated 3 storage methods (silica desiccation,

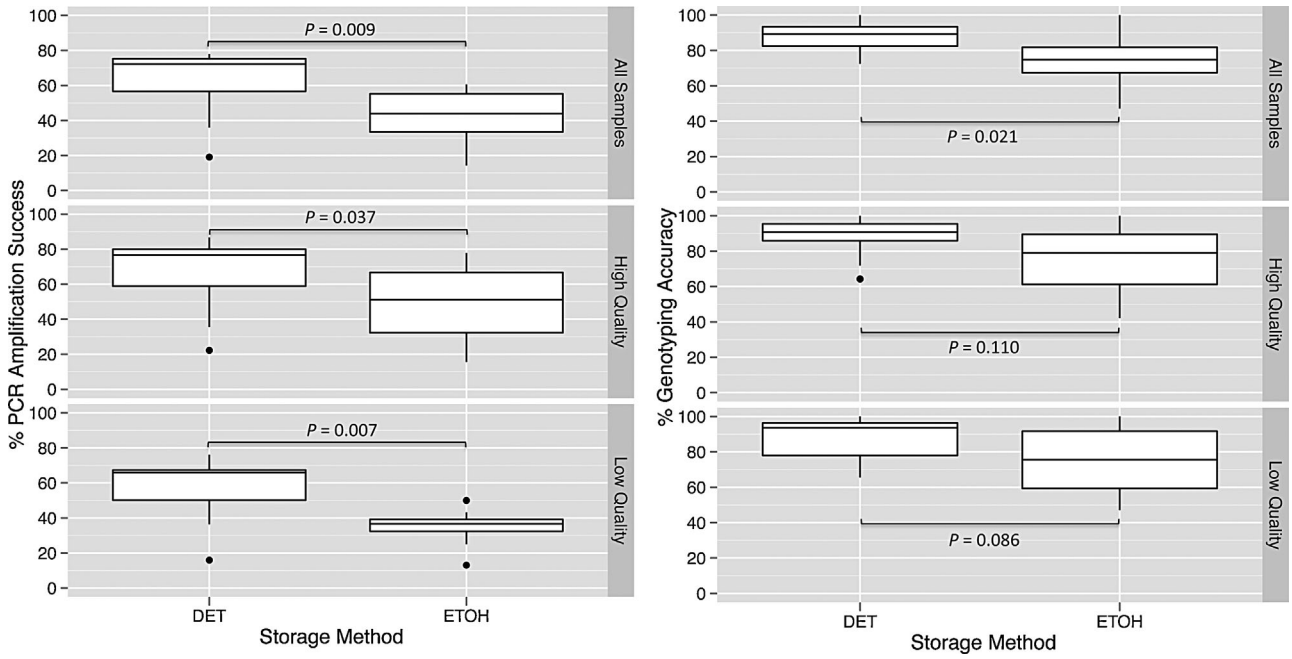

Figure 2. Polymerase chain reaction amplification success (PCR), and genotyping accuracy (GA) rates calculated across 10 microsatellite loci screened upon DNA isolated from scat samples from Neotropical felids collected in Belize, Central America, during 2007–2008, and stored using two fecal DNA storage techniques (dimethyl sulfoxide saline solution [DETs buffer] and 95% ethanol [EtOH]). Rates were assessed across all samples ($n = 30$), fresh or high-quality samples ($n = 15$), and degraded or low-quality scat samples ($n = 15$) collected at the Mountain Pine Ridge Forest Reserve. Statistical significance of differences was examined using pairwise Wilcoxon signed-rank tests (P-value <0.050). In the box-and-whisker plots, the central value represents the median and the central box represents the values from the 25th to the 75th percentiles.

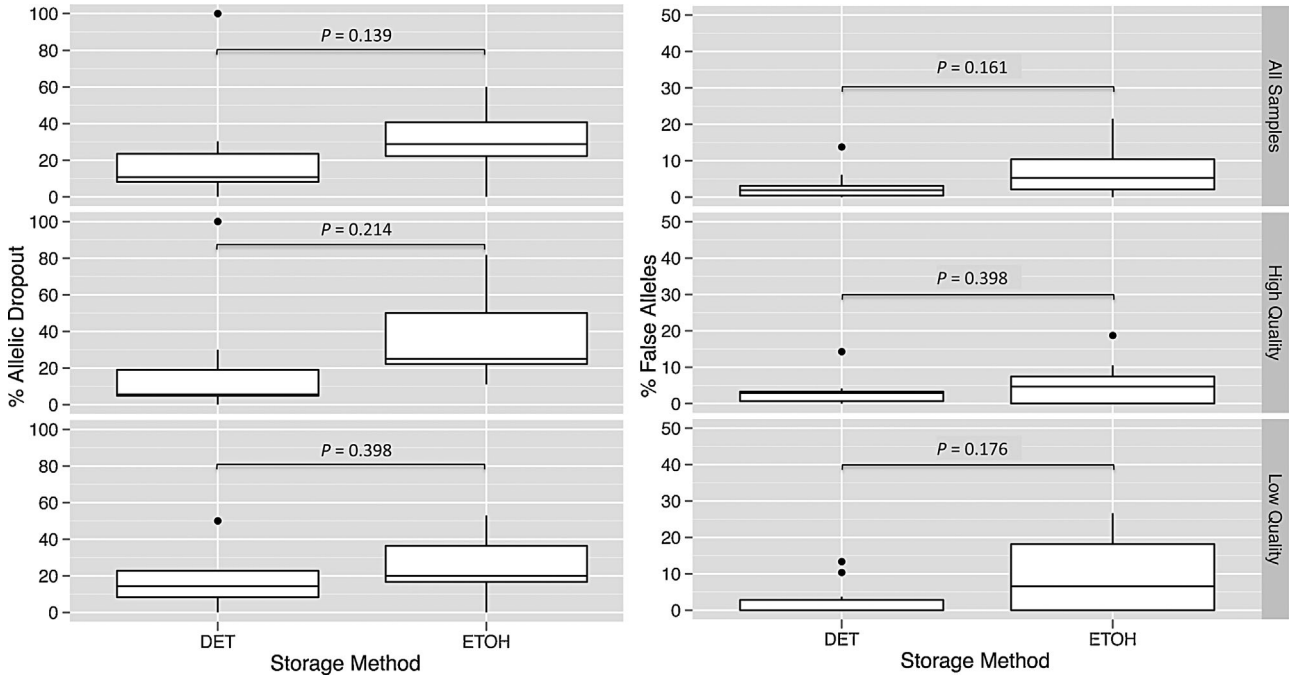

Figure 3. Allelic dropout (ADO) and false allele (FA) rates calculated across 10 microsatellite loci screened upon DNA isolated from scat samples for Neotropical felids collected in Belize, Central America, during 2007–2008, and stored using two fecal DNA storage techniques (dimethyl sulfoxide saline solution [DETs buffer] and 95% ethanol [EtOH]). Rates were assessed across all samples ($n = 30$), fresh or high-quality samples ($n = 15$), and degraded or low-quality scat samples ($n = 15$) collected at the Mountain Pine Ridge Forest Reserve. Statistical significance of differences was examined using pairwise Wilcoxon signed-rank tests (P-value <0.050). In the box-and-whisker plots, the central value represents the median and the central box represents the values from the 25th to the 75th percentiles.

EtOH, and 2-step [EtOH and silica desiccation] storage method) for fecal samples collected from captive tigers, and found that the 2-step method yielded 2–3 times more DNA than storage with silica or EtOH. Fecal DNA preservation studies for other carnivore species comparing PCR amplification success or genotyping error rates across different storage methods found significant differences between storage approaches, and overall recommended DETs buffer, EtOH, and silica desiccant (e.g., Wasser et al. 1997, Murphy et al. 2000, 2002, Frantz et al. 2003, Piggott and Taylor 2003, Panasci et al. 2011).

On account of the remoteness of most field sites and limited access to cooling and freezing facilities, choosing a long-term preservation method that allows reliable storage of fecal DNA samples under ambient or room temperature for several months is most practical. We found that the choice of a fecal DNA preservation method had a significant impact on PCR amplification success and genotyping accuracy rates and that DETs buffer was the superior fecal DNA preservation technique compared with 95% EtOH. Polymerase chain reaction amplification success and genotyping accuracy rates were significantly higher (by 44% and 17%, respectively), whereas genotyping error rates for allelic dropout and false alleles were lower for samples stored in DETs buffer. Polymerase chain reaction amplification success rates for low- and high-quality scat samples showed similar trends. In fact, PCR amplification success rates were higher for both low- and high-quality samples stored in DETs buffer, and the impact was more pronounced for low-quality samples, suggesting that the correct choice of a fecal DNA preservation method is even more crucial when low-quality and degraded scat samples are prevalent.

Genotyping error rates also decreased by storing samples in DETs buffer compared with EtOH preservation. Our

Table 1. Polymerase chain reaction amplification success (PCR), genotyping accuracy (GA), and genotyping error (allelic dropout, ADO; false allele, FA) rates calculated across 10 microsatellite loci screened upon DNA isolated from scat samples from Neotropical felids in Belize, Central America, during 2007–2008, and stored using 2 fecal DNA storage techniques (dimethyl sulfoxide saline solution [DETs buffer] and 95% ethanol [EtOH]). Rates were assessed across all samples ($n = 30$), fresh or high-quality samples ($n = 15$), and degraded or low-quality scat samples ($n = 15$) collected at the Mountain Pine Ridge Forest Reserve. Statistical significance of differences was examined using pairwise Wilcoxon signed-rank tests.

	All samples ($n = 30$)			High-quality samples ($n = 15$)			Low-quality samples ($n = 15$)		
Rates	DET	ETOH	P-value*	DET	ETOH	P-value*	DET	ETOH	P-value*
PCR	62 ± 19.9	43 ± 14.9	0.009	67 ± 22.3	50 ± 21.7	0.037	57 ± 18.5	35 ± 10.2	0.007
GA	88 ± 8.2	75 ± 15.2	0.021	88 ± 11.5	75 ± 18.5	0.110	88 ± 12.4	75 ± 18.8	0.086
FA	22 ± 28.9	32 ± 17.6	0.139	21 ± 31.2	37 ± 23.7	0.214	20 ± 18.8	26 ± 17.2	0.398
ADO	3 ± 4.2	7 ± 7.5	0.161	3 ± 4.1	5 ± 6.1	0.398	3 ± 5.0	10 ± 10.9	0.176

*P-value <0.050.

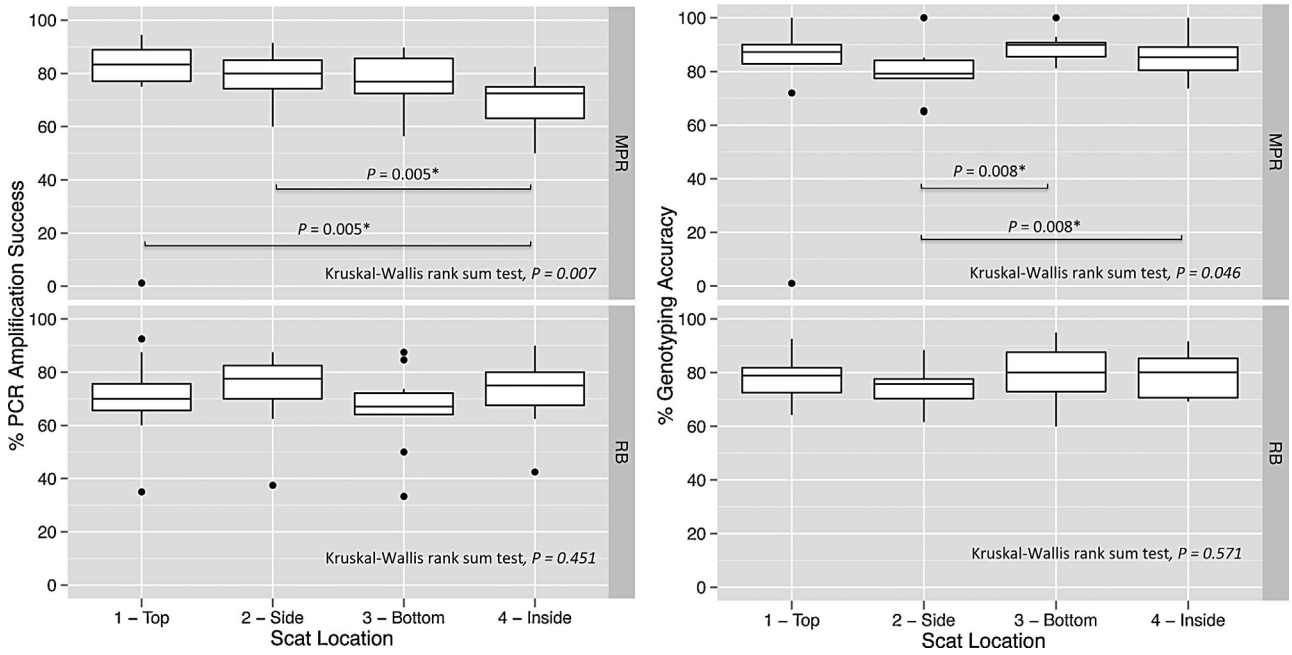

Figure 4. Polymerase chain reaction amplification success (PCR) and genotyping accuracy (GA) rates calculated across 10 microsatellite loci screened upon DNA isolated from scat samples from Neotropical felids collected in Belize, Central America, during 2007–2008. Rates are compared across four scat locations (1 top, 2 side, 3 bottom, 4 inside) at two sites, the Mountain Pine Ridge Forest Reserve (MPR, $n = 20$) and Rio Bravo Conservation Management Area (RB, $n = 20$). Kruskal–Wallis rank-sum tests and pairwise Wilcoxon signed-rank tests were used for statistical testing (P-value <0.050). In the box-and-whisker plots, the central value represents the median and the central box represents the values from the 25th to the 75th percentiles.

findings were consistent with those of several other studies examining the same preservation techniques. Seutin et al. (1991) recommended the use of DETs buffer to preserve DNA in avian tissue at ambient temperatures for extended times and suggested that DNA storage with 70% EtOH was less successful. Frantzen et al. (1998) found that DETs buffer was the most effective technique for nuclear DNA preservation in baboon (*Papio cynocephalus ursinus*) scats in a tropical environment. In contrast, Frantz et al. (2003) did not find differences in amplification success between DETs

Table 2. Polymerase chain reaction amplification success (PCR), genotyping accuracy (GA), and genotyping error (allelic dropout, ADO; false allele, FA) rates calculated across 10 microsatellite loci for wild felids in Belize, Central America, during 2007–2008. Rates were compared across four scat locations (top, side, bottom, inside; observed differences in %) at 2 study sites, the Mountain Pine Ridge Forest Reserve (MPR, $n = 20$) and Rio Bravo Conservation Management Area (RB, $n = 20$) using Kruskal–Wallis rank-sum tests (KW; P-value <0.050) and *post hoc* pairwise Wilcoxon signed-rank tests* (Bonferroni correction with P-value <0.008).

MPR site	Scat locations	Observed difference (%)	*P*-value*	RB site	Scat locations	Observed difference (%)	*P*-value
PCR	Top–Side	5.9	0.017	PCR	Top–Side	4.0	0.093
KW, $P = 0.007$	Top–Bottom	8.9	0.017	KW, $P = 0.451$	Top–Bottom	3.6	0.139
	Top–Inside	15.4	0.005		Top–Inside	3.3	0.154
	Side–Bottom	3.0	0.333		Side–Bottom	7.6	0.028
	Side–Inside	9.6	0.005		Side–Inside	0.8	0.722
	Bottom–Inside	6.6	0.028		Bottom–Inside	6.8	0.007
GA	Top–Side	7.4	0.015	GA	Top–Side	4.3	0.011
KW, $P = 0.046$	Top–Bottom	2.2	0.208	KW, $P = 0.571$	Top–Bottom	0.2	0.878
	Top–Inside	1.9	0.173		Top–Inside	0.3	0.959
	Side–Bottom	9.6	0.008		Side–Bottom	4.5	0.203
	Side–Inside	5.5	0.008		Side–Inside	4.5	0.110
	Bottom–Inside	4.1	0.066		Bottom–Inside	0.1	0.959
ADO	Top–Side	6.0	0.093	ADO	Top–Side	8.7	0.059
KW, $P = 0.104$	Top–Bottom	3.0	0.263	KW, $P = 0.330$	Top–Bottom	7.1	0.285
	Top–Inside	2.6	0.263		Top–Inside	1.8	0.959
	Side–Bottom	8.9	0.021		Side–Bottom	1.6	0.799
	Side–Inside	3.4	0.093		Side–Inside	10.5	0.074
	Bottom–Inside	5.5	0.069		Bottom–Inside	8.9	0.005
FA	Top–Side	5.4	0.012	FA	Top–Side	0.9	0.959
KW, $P = 0.106$	Top–Bottom	0.8	0.400	KW, $P = 0.852$	Top–Bottom	0.9	0.721
	Top–Inside	1.7	0.044		Top–Inside	1.1	0.575
	Side–Bottom	4.5	0.021		Side–Bottom	1.8	0.161
	Side–Inside	3.7	0.028		Side–Inside	0.3	0.767
	Bottom–Inside	0.8	0.173		Bottom–Inside	2.1	0.161

buffer and 70% EtOH for fecal DNA samples of European badgers (*Meles meles*). Panasci et al. (2011) had equal success for coyote (*Canis latrans*) fecal DNA storage in DETs buffer and 95% EtOH when diet of coyotes was not considered. However, when the effects of feeding habits were considered, DETs buffer preservation was the most efficient method for animals with a plant-based diet and 95% EtOH preservation was better for obligate and facultative meat-eaters. This was contrary to our findings, where DETs buffer had significantly higher success rates than 95% EtOH for carnivorous felids. Differences in findings between studies may be explained by multiple factors, including the target species, DNA sample type and condition, storage time, environmental influences, and the choice of field and laboratory techniques (e.g., Beja-Pereira et al. 2009, Panasci et al. 2011).

In addition to efficiency in producing reliable genotypes, we also considered several other factors when we chose a fecal DNA preservation method. For example, storage time for fecal DNA samples is often a deciding factor for long-term field studies, which do not have immediate access to laboratory facilities. Generally, concentrated EtOH (>70%) reduces water content from the scat, which decreases DNA degradation caused by bacteria, whereas DETs buffer uses high concentration of salts to inactivate the enzymes causing DNA degradation (Seutin et al. 1991, Kilpatrick 2002). Over time, EtOH does not efficiently preserve supporting components of tissues (e.g., proteins) and their DNA becomes acidified (Jackson et al. 2012). According to Kilpatrick (2002), DETs buffer provided the best protection from DNA degradation of high-molecular-weight DNA in tissues stored under room temperatures for up to 2 years, whereas DNA yield using EtOH storage was relatively low. Soto-Calderon et al. (2009) reported that microsatellite amplification success rates for tropical ungulate scats stored in EtOH declined significantly over time after 3 months of storage relative to success rates measured 1 week or 1 month after collection.

Noninvasive genetic studies of samples collected at remote field sites need simple fecal DNA preservation techniques, which require minimum storage space, easy portability in the field and transportation by air (if needed). We used sterile 2-mL screw-top tubes and showed that preservation of small fecal fragments (approx. 0.5 mL) is sufficient for microsatellite genotyping. Fecal DNA storage vials or containers of most other felid genetic studies using liquid storage techniques ranged from 10 mL to 50 mL (Table S1; e.g., Bhagavatula and Singh 2006, Michalski et al. 2011). Storage in 2-mL vials requires minimal space, which is important when handling a large number of scat samples. Additionally, no further treatment is necessary after fecal samples are placed into vials with the storage liquids. Generally, EtOH does not require any special precautions while it is handled in the field, although leakage can occur when used with plastic vials. Thus, frequent checks of EtOH levels in storage vials plus the use of alcohol-resistant markers for labeling are recommended. EtOH is available at most field sites, but transportation by air is regulated because it is flammable and classified as dangerous goods or hazardous materials. The International Air Transport Association allows transportation of limited quantities (5 L by passenger aircraft; e.g., Kilpatrick 2002). In contrast, DETs is not classified as dangerous goods or hazardous materials; thus, no special regulations for transporting DETs buffer by air apply. Nonetheless, it includes dimethyl sulfoxide, which must be handled with care (preferably with gloved hands), and contact with skin should be avoided (e.g., David 1972, Kilpatrick 2002). Seutin et al. (1991) described DETs buffer as a safe substance when handled with precautions.

Alternative fecal DNA storage techniques such as freezing and silica desiccation were not considered for this study for several reasons. Freezing is one of the most conventional preservation techniques for DNA, but was not a feasible method for our study because we had no access to reliable freezers. Preserving fecal DNA using silica desiccation, which is a common method, was not used because it was not considered practical for long-term storage of large amounts of fecal samples collected in tropical environments. Preservation with silica often requires additional drying of moist (fresh or rain-soaked) fecal samples using an oven, air, or sun prior to the actual preservation (e.g., Murphy et al. 2000). Further handling of fecal samples is time-consuming, increases the risk of contamination (particularly when a large number of samples is handled simultaneously), and requires designated areas for drying. In summary, we recommend the use of DETs buffer to preserve fecal DNA samples of Neotropical felids for large-scale field studies, which require storage under room temperature and handling of a large number of fecal samples.

Effect of 4 Sampling Locations Within Scat Sample on Fecal DNA

We found that specific sampling locations on or in a scat sample significantly affected PCR amplification success rates at one of our sampling sites. Fecal DNA collected from the surface of the scat is usually the last in contact with the intestinal lining and the 1st to desiccate; thus, collection of fecal DNA from the surface is most reliable, as suggested by the results of earlier studies (e.g., Stenglein et al. 2010). A few carnivore studies have empirically tested for differences in DNA quality using different sampling locations within a scat sample and uniformly recommended fecal DNA sampling from the outside or surface of the scat sample for higher PCR amplification success (e.g., Pires and Fernandes 2003, Stenglein et al. 2010). Fecal DNA collected from the top of a scat may be negatively affected by direct ultraviolet light and rainfall (e.g., Brinkman et al. 2010). The side location also is affected by these factors, but their impact may not be as direct as at the top. Soil decomposers may have a greater impact on fecal DNA samples collected from the bottom and inside locations. Santini et al. (2007) suggested that direct contact with the soil keeps the fecal sample moist, which accelerates the invasion of decomposer organisms.

We concluded that the scat sampling location within scats had a significant impact on PCR amplification success rates and genotyping accuracy for fecal DNA collected at the more

open, submontane MPR (pine forest) site. Generally, we found that PCR success rates were significantly higher when fecal DNA was collected from the outside (top, side, or bottom) of the scat compared with the inside, a result similar to those of past studies (Stenglein et al. 2010). Success rates were highest for samples collected from the top of the scat, followed by the side, bottom, and inside locations of scats. This outcome may be explained by the degree of desiccation, which varies across locations within freshly deposited samples (<2 days), but potentially also across older scats depending on the environment. Desiccation by air in more open and dry ecosystems preserves fecal DNA (e.g., Murphy et al. 2007). Tops of scats are most likely to dry first in open environments because of exposure to heat, ultraviolet light, and wind, followed by the sides, bottom, and inside of scats. Most scat samples sampled in this study were dried and of hard consistency; therefore, collection methods other than scraping (e.g., swabbing) were not considered practical.

Polymerase chain reaction amplification success rates did not differ significantly for scat locations from RB samples, where scats overall had higher levels of moisture and were detected predominately in closed-canopy lowland broadleaf forest. We hypothesize that the closed canopy and higher levels of moisture would minimize differences between sampling locations and thus homogenize DNA degradation rates. However, we cannot rule out the possibility that differences in species composition may have also affected the results. In summary, we recommend thorough inspection of fecal samples upon detection in the field, to select the most promising location for the fecal DNA sample. In our study, the top and side locations, which were usually the driest, resulted in highest PCR amplification success rates, and thus can be considered the preferred sampling location for fecal DNA.

Noninvasive genetic sampling (i.e., molecular scatology) has the potential to provide valuable information for conservation and management of elusive and difficult-to-study wildlife species. To increase the efficiency of this approach in tropical environments, we recommend optimizing field-sampling methods used for molecular scatology studies of any particular species prior to molecular analysis. We predict that our results will be consistent with those of molecular scatology studies of felids and potentially other carnivores in similar tropical environments, and we ultimately recommend conducting a pilot study prior to any molecular scatology study to verify performance of field-sampling protocols chosen for a particular species and geographical region.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Table S1: Molecular scatology studies of wild felids across temperate, desert-steppe, tropical–subtropical, and highland climate zones, including description of fecal DNA storage method (preservation type, scat amount, storage container, fecal DNA sample location within scat sample).