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# Noninvasive individual and species identification of jaguars (*Panthera onca*), pumas (*Puma concolor*) and ocelots (*Leopardus pardalis*) in Belize, Central America using cross-species microsatellites and faecal DNA

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## Abstract

**There is a great need to develop efficient, noninvasive genetic sampling methods to study wild populations of multiple, co-occurring, threatened felids. This is especially important for molecular scatology studies occurring in challenging tropical environments where DNA degrades quickly and the quality of faecal samples varies greatly. We optimized 14 polymorphic microsatellite loci for jaguars (*Panthera onca*), pumas (*Puma concolor*) and ocelots (*Leopardus pardalis*) and assessed their utility for cross-species amplification. Additionally, we tested their reliability for species and individual identification using DNA from faeces of wild felids detected by a scat detector dog across Belize in Central America. All microsatellite loci were successfully amplified in the three target species, were polymorphic with average expected heterozygosities of $H_E = 0.60 \pm 0.18$ (SD) for jaguars, $H_E = 0.65 \pm 0.21$ (SD) for pumas and $H_E = 0.70 \pm 0.13$ (SD) for ocelots and had an overall PCR amplification success of 61%. We used this nuclear DNA primer set to successfully identify species and individuals from 49% of 1053 field-collected scat samples. This set of optimized microsatellite multiplexes represents a powerful tool for future efforts to conduct noninvasive studies on multiple, wild Neotropical felids.**

*Keywords*: cross-species microsatellites, individual identification, molecular scatology, noninvasive genetic sampling, *Panthera onca*, scat detector dogs

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## Introduction

Jaguars (*Panthera onca*) and co-occurring Neotropical felids, such as pumas (*Puma concolor*) and ocelots (*Leopardus pardalis*), are extremely difficult to study in the wild due to their elusive nature. As landscapes change and human impacts increase, felid populations become increasingly fragmented and ecological processes such as dispersal, intraguild competition and top-down trophic impacts (Linnell & Strand 2000) are affected, but remain understudied. Consequently, there is a great need for efficient survey methods that can be applied to multiple co-occurring species instead of focusing research efforts on a single species. Noninvasive genetic monitoring has been of increasing importance in the field of wildlife management and conservation in recent years (e.g. Waits & Paetkau 2005; Beja-Pereira *et al.* 2009), providing the potential to gain valuable information on multiple species existing across a fragmented landscape. Molecular scatology, in which individuals are genotyped using DNA isolated from sloughed intestinal epithelial cells in field-collected faecal samples (Hoss *et al.* 1992; Kohn & Wayne 1997), is especially suitable for wild felids, which often deposit scat at prominent sites for intra- and interspecific communication (Sunquist & Sunquist 2002). Additionally, DNA extracted from faeces can be obtained without physically capturing or disturbing animals of interest (Taberlet *et al.* 1999). Noninvasive genetic sampling also holds great promise for providing large sample sizes for multiple species simultaneously and identification of species, gender and individuals, which is beneficial for a wide array of analyses (e.g. Waits & Paetkau 2005; Kelly *et al.* 2012; Rodgers & Janecka 2013).

To efficiently monitor threatened species, robust identification of individuals is crucial for various types of

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studies (e.g. capture–recapture analysis). However, individual-based molecular scatology studies of single and especially of multiple wild felid species are rare, particularly in tropical regions. Multispecies felid studies often do not go beyond the species level (e.g. Zuercher *et al.* 2003; Cossios & Angers 2006; Haag *et al.* 2009; Michalski *et al.* 2011; Roques *et al.* 2011), with a few exceptions (e.g. Singh *et al.* 2004; Trigo *et al.* 2008; Mondol *et al.* 2012). One of the challenges to conducting individual-based genetic population monitoring using faecal DNA collected in tropical environments is the rapid removal of faecal samples by insects and rain and the rapid degradation of DNA. DNA degradation is affected by various environmental factors (high temperatures, precipitation and UV radiation) (e.g. Brinkman *et al.* 2010; Vynne *et al.* 2012), which are particularly problematic in tropical climates where excessive heat and humidity cause DNA to deteriorate rapidly. Generally, low DNA quantity and quality are major concerns for molecular scatology studies in the tropics (e.g. Michalski *et al.* 2011; Vynne *et al.* 2012), as they decrease polymerase chain reaction (PCR) amplification success and cause genotyping errors (Taberlet *et al.* 1996). Genotyping errors increase the chance of misidentification of individuals and potentially bias population estimates (Taberlet & Luikart 1999; Taberlet *et al.* 1999; Mills *et al.* 2000; Waits & Leberg 2000).

To conduct a noninvasive genetic study of multiple Neotropical felid species simultaneously, a set of highly polymorphic microsatellite markers, also known as simple sequence repeats (SSRs), are needed that amplify multiple target species and have the potential to identify species and individuals from faecal DNA. Cross-species amplification of microsatellites depends on the conservation of primer sequences, which has been described for several mammalian taxa (e.g. Moore *et al.* 1991). Cross-species microsatellites are transferrable between closely related species (e.g. Barbara *et al.* 2007), which makes them a cost-effective and efficient approach for conservation genetic studies of multiple target species. Menotti-Raymond and O'Brien (1995) and Menotti-Raymond *et al.* (1999) characterized microsatellite loci for the domestic cat (*Felis catus*) and described their utility for cross-species amplification due to conserved flanking primer sequences across the family Felidae. Cross-species microsatellites subsequently were used for several felid studies (e.g. Menotti-Raymond & O'Brien 1995; Johnson *et al.* 1999; Carmichael *et al.* 2000; Williamson *et al.* 2002; Singh *et al.* 2004; Buckley-Beason *et al.* 2006; Moreno *et al.* 2006; Grisolia *et al.* 2007; Trigo *et al.* 2008). Their application is diverse and has helped facilitate comparisons among closely related feline species for assessing levels of genetic diversity and phylogeographic patterns (Johnson *et al.* 1999; Moreno *et al.* 2006; Grisolia *et al.* 2007), to recognize new feline species (Wilting *et al.* 2007), to assess hybridization between wild and domestic cats (e.g. Oliveira *et al.* 2008; Trigo *et al.* 2008) and to detect illegal hunting and trafficking of threatened felids (Maudet *et al.* 2004; Singh *et al.* 2004). Nevertheless, cross-species amplification of microsatellites has not often been used for demographic and genetic monitoring of multiple felids in the wild, which could ultimately improve conservation and management activities for these elusive species. Besides the comparison of population genetic parameters among multiple closely related species, cross-species microsatellites can also be applied for species identification based on species-specific allele sizes at multiple loci, a technique that has rarely been used for noninvasive studies of wild carnivores (e.g. Pilot *et al.* 2007; Wilting *et al.* 2007).

We conducted a 4-year noninvasive genetic study of three co-occurring felids (jaguar, puma and ocelot) across several study sites in tropical Belize, Central America. We initially screened 20 microsatellite loci developed in earlier feline studies (Menotti-Raymond *et al.* 1999) and identified 14 microsatellite markers for a molecular scatology study of three Neotropical felids suitable for answering a variety of questions relevant to conservation and management. Specifically, our objective was to define a set of highly polymorphic microsatellite loci applicable for reliable and cost-effective species and individual identification across three target species. Furthermore, we evaluated the reliability of genotypes from faecal samples of highly variable DNA quality and quantity as a primary DNA source by determining PCR amplification success, genotyping accuracy and error rates.

## Methods

### *Study area*

We conducted 2- to 3-month long scat surveys across five main study sites (Mountain Pine Ridge Forest Reserve – MPR, Rio Bravo Conservation and Management Area – RB, Cockscomb Basin Wildlife Sanctuary – CBWS, Chiquibul Forest Reserve and National Park – CFRNP and Fireburn/Balam Na Nature Reserve – FB) and 2- to 10-day surveys at several other sites (Big Falls – BF, Bladen Nature Reserve – BNR, Boden Creek Ecological Preserve – BC, Bull Run Farm – BRF, Golden Stream Corridor Preserve – GS, Hidden Valley Reserve – HVR, Machaca Hills – MH, Manatee Forest Reserve – MFR, Sarstoon-Temash National Park – STNP, Shipstern Nature Preserve – SNP, Tiger Sandy Bay – TSB) from 2007 to 2010 across Belize, Central America (17°15′N, 88°45′W; Fig. 1). All sites except BF, BRF, MH and TSB are part of the national system of protected areas in Belize. The study sites also fall within the forests of La Selva Maya

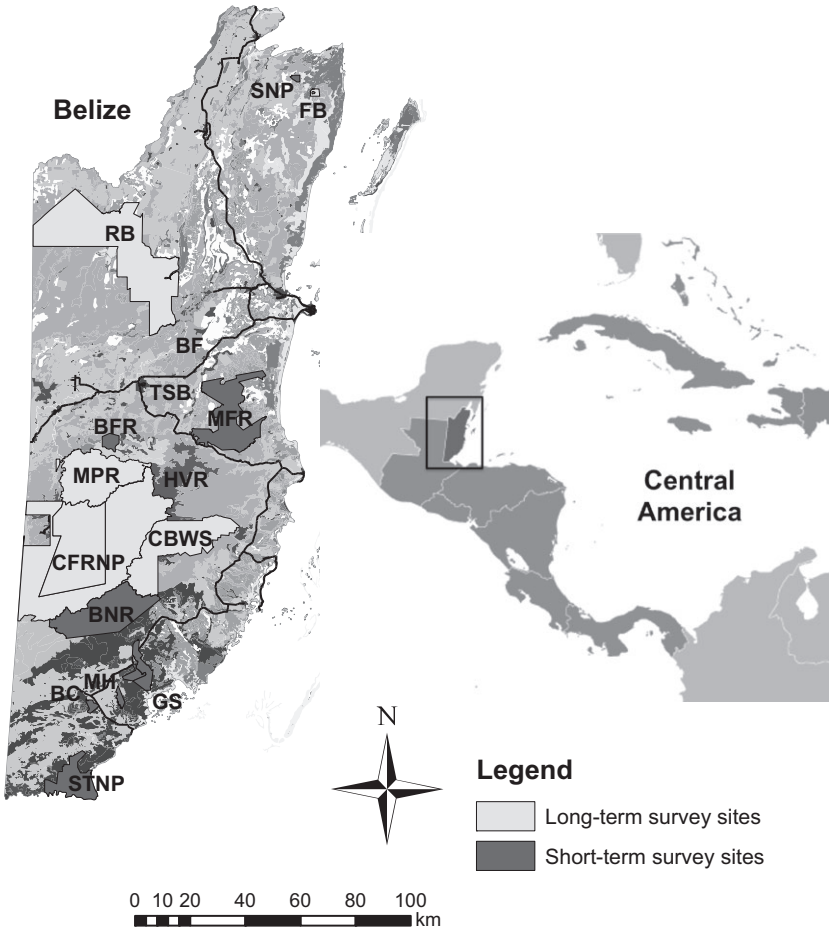


**Fig. 1** Map of short- and long-term survey sites across Belize, Central America, including Mountain Pine Ridge Forest Reserve (MPR), Rio Bravo Conservation and Management Area (RB), Cockscomb Basin Wildlife Sanctuary (CBWS), Chiquibul Forest Reserve and National Park (CFRNP), Fireburn/Balam Na Nature Reserve (FB), Big Falls (BF), Bladen Nature Reserve (BNR), Boden Creek Ecological Preserve (BC), Bull Run Farm (BRF), Golden Stream Corridor Preserve (GS), Hidden Valley Reserve (HVR), Machaca Hills (MH), Manatee Forest Reserve (MFR), Sarstoon-Temash National Park (STNP), Shipstern Nature Preserve (SNP) and Tiger Sandy Bay (TSB).

(The Mayan Forest), a recognized biodiversity hot spot that forms part of the northern section of the Mesoamerican Biological Corridor. Across study sites, elevation ranges from 0 to 1120 m. Mean annual rainfall varies from 1524 mm in the north to 4064 mm in the south, with a pronounced wet season from June to December. Average annual temperatures fluctuate between 17.7 and 31.3 °C. A high diversity of native habitat types is represented within the study sites, including lowland and submontane broad-leaved moist and wet forests, lowland and submontane pine forests, mangrove and littoral forests, lowland savannah, shrub land and wetland.

### *Sample detection*

Faecal samples were detected using a professionally trained scat detector dog (PackLeader LLC, Gig Harbor, WA, USA). Opportunistic searches with the dog off-leash were conducted within all study sites (Wasser *et al.* 2004) to detect scat samples along roads, trails, game trails, off-trail, and across various landscape features (e.g. streams) and habitat types. The scat detector dog was trained to locate scat samples of all five native feline species (jaguars, pumas, ocelots, margays, *Leopardus wiedii*; jaguarundis, *Puma yaguarondi*). All scat samples located by the scat detector dog, regardless of their appearance or suspected age, were collected and genotyped.

### *Faecal DNA storage and extraction*

From each scat, approximately 0.5 mL sample was collected and stored at room temperature in sterile 2-mL screw-top tubes filled with dimethyl sulphoxide saline solution (DETs buffer, Seutin *et al.* 1991) at 1: ≥4 volume scat-to-solution ratio. Scat samples were collected using disposable gloves and wooden sampling sticks. Faecal DNA extractions were conducted in a separate room at the Laboratory for Ecological, Evolutionary and Conservation Genetics (LEECG) at the University of Idaho (Moscow, ID, US), dedicated to noninvasive genetic studies, in order to avoid contamination while working with low-concentration DNA samples. The QIAamp DNA Stool Mini Kit protocol (Qiagen, Inc.) was used to extract DNA from all faecal samples. An extraction negative was added to each extraction run to control for contamination.

### *Microsatellite screening and selection*

A total of 20 microsatellite loci (F53, F85, F98, F124, FCA008, FCA032, FCA043, FCA090, FCA096, FCA100, FCA124, FCA126, FCA132, FCA212, FCA225, FCA229, FCA275, FCA391, FCA441 and FCA741) originally identified for the domestic cat (Menotti-Raymond *et al.* 1999, 2005) were tested on faecal samples from wild Neotropical felids (19 jaguars, 18 pumas and 12 ocelots) collected in the first two study sites (MPR and RB). Microsatellite performance was evaluated by assessing allelic variation, probabilities of identity for unrelated individuals and siblings (Waits *et al.* 2001), PCR amplification success, genotyping accuracy and genotyping error rates.

### *Microsatellite amplification and genotyping*

Following microsatellite screening, 14 highly polymorphic loci were selected, labelled with fluorescent dyes and arranged in three PCR multiplex reactions (multiplex 1 – F124-PET, FCA391-NED, FCA043-NED, FCA275-VIC, FCA096-6-FAM, FCA126-PET, FCA090-6-FAM; multiplex 2 – F85-VIC, F98-6-FAM, FCA741-PET, FCA225-PET, FCA008-6-FAM; and multiplex 3 – F53-NED and FCA441-6-FAM) to enhance performance and efficiency. The three multiplexes each contained 5.2 μL PCR mixture and 1.8 μL of DNA. Multiplex 1 included 3.5 μL 1× concentrated Qiagen Master Mix (Qiagen, Inc.), 0.7 μL of 0.5× concentrated Qiagen Q solution (Qiagen, Inc.), 0.88 μL of primers (0.10 μM F124, 0.34 μM FCA391, 0.07 μM FCA043, 0.13 μM FCA275, 0.21 μM FCA096, 0.20 μM FCA126, 0.20 μM FCA090), 0.12 μL $H_2O$ and 1.8 μL DNA extract. Multiplex 2 consisted of 3.5 μL 1× concentrated Qiagen Master Mix, 0.7 μL of 0.5× concentrated Qiagen Q solution, 0.65 μL of primers (0.20 μM for F85, 0.09 μM for F98, 0.11 μM for FCA741, 0.43 μM for FCA225, 0.10 μM for FCA008), 0.35 μL $H_2O$ and 1.8 μL DNA extract. Multiplex 3 only differed in the amount of water (0.76 μL) and primers (0.24 μL; 0.20 μM for F53, 0.14 μM for FCA441) added. Microsatellite PCR amplifications were conducted using a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc.) starting 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 60 °C with a decrease in annealing temperature of 0.8 °C in each cycle and 1-min elongation at 72 °C; followed by 30 cycles of 30 s at 94 °C for denaturation, 1.5 min at 50 °C for annealing and 1 min elongation at 72 °C; and 30 min at 60 °C for final elongation. A polymerase chain reaction negative was included in each group of PCRs to indicate the presence/absence of contamination. We visualized PCR products using an ABI PRISM® 377 automated DNA sequencer (Applied Biosystems™), and genotypes were identified using the software GENEMAPPER, version 3.7 (Applied Biosystems™).

Initially, we performed a minimum of two PCRs for all scat samples using the screening multiplex 1 (seven loci). Depending on the number of successfully amplified loci within multiplex 1, we placed the scat samples in three categories describing amplification success: (i) bad (0–2 loci amplified), (ii) medium (3–5 loci amplified) and (iii) good (6–7 loci amplified). We conducted 1–4 additional PCR runs for scat samples placed into the medium and good quality categories. Scat samples with ≤2 loci amplified were discarded from the study. The total number of PCRs ranged from 2 to 6 replicates per locus and averaged 4.29 ± 1.84 (SD) per locus for samples with finalized genotypes. To finalize the consensus genotypes, a multitube approach was used where at least three identical homozygote PCR results were required for homozygote genotypes, and each allele had to be observed in two independent PCRs to record a heterozygous genotype.

### *Species and individual identification*

Scat samples were assigned to feline species based on species-specific alleles and allelic ranges initially identified across several loci from known faecal DNA samples of 12 jaguars, four pumas, 12 ocelots, 30 margays and four jaguarundis obtained from captive facilities (Belize Zoo, Belize; Feline Conservation Center, CA, US; Naples Zoo, FL, US, Profelis, Costa Rica). Additionally, to verify species assignment of all unique jaguar, puma and ocelot individuals, we conducted assignment tests with the Bayesian clustering software STRUCTURE, version 2.3.3 (Pritchard *et al.* 2000). Species assignment for all individual genotypes detected was verified by analysing clustering patterns based on distinctive allele frequencies and the most likely number of genetic clusters (*K*). The admixture model was used (predefined $K = 1–5$ corresponding to the five native feline species; 10 runs per *K* value; length of burn-in period: $10^5$ iterations; number of MCMC iterations after burn-in: $10^6$). The optimal K value was chosen by calculating the posterior probability for each *K* value, which is based on estimated maximum log-likelihood values. After finding the optimal *K*, individuals were assigned to distinct genetic clusters using the % of the genotype's ancestry (*Q*) attributed to each genetic cluster. For successful species identification, *Q* values had to be ≥95%. Also, mitochondrial sequencing was conducted for four DNA samples per species cluster to confirm species assignment. Four mitochondrial gene regions were amplified including cytochrome *b* (H15149, Kocher *et al.* 1989; Farrell-R, Farrell *et al.* 2000), *12S* (L1085, H1259, Kitano *et al.* 2007), *16S* (L2513, H2714, Kitano *et al.* 2007), *16Scp* (16Scp-F, 16Scp-F, Kitano *et al.*

2007) and adenosine triphosphate-6 (ATP6-DF3, ATP6-DR2, Chaves *et al.* 2012). DNA sequences were edited and matched with reference samples from the Global Felid Genetics Program at the American Museum of Natural History (New York, NY) using GENEIOUS, v. 6.1.5. (Biomatters Ltd., Auckland, New Zealand).

For individual identification, we estimated probabilities of identity (per locus and cumulative) for unrelated individuals ($P_{(ID)}$) and siblings ($P_{(ID)sib}$) per species as described by Waits *et al.* (2001) using GIMLET, version 1.3.3. (Valiere 2002). Cumulative $P_{(ID)}$ and $P_{(ID)sib}$ values were calculated per species for screening multiplex 1, and the minimum number of loci required for individual identification was estimated following a criterion of $P_{(ID)sib} < 0.010$ as suggested by Mills *et al.* (2000) and Waits *et al.* (2001).

Once consensus genotypes were finalized for multiplex 1, we used GENALEX, version 6.41 (Peakall & Smouse 2006) to determine the minimum number of individuals per species by checking for repeated matching genotypes, which if detected, were classified as recaptures of individual felids. Near-matching genotypes, which differed at only one or two loci, were genotyped two more times. We selected the scat sample with the highest PCR product quality for each detected individual and screened these using multiplex 2 (five loci) and multiplex 3 (two loci). We obtained consensus genotypes for the final two multiplexes after performing an additional 2–6 PCR replicates per locus. Genotypes, which were only detected once, were further examined using the software RELIOTYPE (Miller *et al.* 2002) and maximum likelihood to assess their reliability. If the accuracy of multilocus scores was below 95%, additional PCR replicates were conducted.

### *Data analysis*

GENALEX, version 6.41 (Peakall & Smouse 2006) was used to assess genetic variation per species at single loci and across all loci by calculating the number of alleles ($N_A$) and estimating observed ($H_O$) and expected heterozygosities ($H_E$). Additionally, we determined allelic richness ($A_R$) using the rarefaction method with HP-RARE, version 1.0 (Kalinowski 2005) and polymorphic information content (*PIC*) with CERVUS, version 3.0 (Kalinowski *et al.* 2007).

To calculate PCR amplification success, genotyping accuracy and genotyping error rates, we selected the last two PCR runs for all loci across all scat samples. We used only the last two runs to standardize the number included per sample. PCR amplification success rates were assessed by calculating the percentage of successful PCRs across all samples tested and for all samples with finalized species identification. Genotyping accuracy rates were estimated by calculating the percentage of successful PCRs, which matched the finalized consensus genotype. Genotyping error was quantified by calculating the rate of false alleles (FA) and allelic dropouts (ADO) following the protocols of Broquet and Petit (2004). False allele rates were calculated for all consensus genotypes, and allelic dropout rates were estimated only for heterozygous genotypes.

Hardy–Weinberg equilibrium and linkage disequilibrium (LD) were tested for all three species with GENEPOP, version 4.1 (Raymond & Rousset 1995) using default settings for Markov chain parameters. The presence of null alleles was examined with MICRO-CHECKER (Van Oosterhout *et al.* 2004). Data were tested for normality and homoscedasticity. Statistical differences between groups were evaluated using nonparametric Kruskal–Wallis and post hoc Wilcoxon rank-sum tests in program R, version 3.0.1 (R Development Core Team 2009). All results for multiple significance tests were adjusted by applying sequential Bonferroni correction (Rice 1989).

## Results

### *Sample detection*

The total number of faecal DNA samples located by the scat detector dog across all study sites was 1053, with 110 collected in MPR, 203 in RB, 223 in CBWS, 111 in CFRNP, 217 in FB and 189 samples during several short-term surveys across various sites (BF, BNR, BC, BRF, GS, HVR, MH, MFR, STNP, SNP and TSB) in Belize.

### *Microsatellite selection*

Microsatellite DNA from faecal samples of wild jaguars, pumas and ocelots collected in MPR and RB amplified across all 20 initially selected loci, confirming their cross-species utility. Nonetheless, six microsatellite loci (FCA032, FCA100, FCA124, FCA132, FCA212 and FCA229) were omitted from the analysis due to low levels of genetic variation ($N_A \leq 2$ and $H_E \leq 0.5$ for FCA100, FCA132 and FCA212 in jaguars; $N_A = 1$ and $H_E = 0$ for FCA032 and FCA212 in pumas; $H_E \leq 0.5$ for FCA132 in ocelots) and overlapping allelic size ranges (FCA124 with FCA043, FCA229 with FCA275). Based on $P_{(ID)sib}$ estimates, loci FCA132 for jaguars, FCA032 and FCA212 for pumas and FCA132 for ocelots were the least powerful loci for individual identification among those tested. We also assessed primer performance across 74 faecal samples collected in MPR and RB in terms of PCR amplification success and genotyping error rates. Loci FCA032 and FCA100 had generally low PCR amplification success rates. Loci FCA100 and FCA132 showed high rates

of allelic dropout, and loci FCA100 and FCA229 produced high rates of false alleles.

### *Species and individual identification*

After screening, 14 highly polymorphic microsatellite loci were selected and used for species and individual identification. Species-specific alleles and allelic ranges were described for all target species using faecal samples obtained from the wild in Belize (Table S1, Supporting information). Across all finalized loci, for pumas, 70.3% of the alleles described were species-specific followed by 64.1% for ocelots and 63.8% for jaguars. The most powerful loci for species assignment (100% of alleles were species specific) were F124, FCA391, FCA096, FCA126 and FCA741 for jaguars, F124, FCA096, F98 and FCA008 for pumas, and F124, FCA043, FCA096 and F85 for ocelots.

Species identification was additionally confirmed by sequencing four mitochondrial gene regions for a subset of samples and by applying Bayesian assignment methods for all samples identified down to individual level, which estimated the number of $K$ clusters with an increase of likelihood values, peaking at $K = 3$. At $K = 3$, all individuals tested were successfully assigned to one species cluster with an average proportion of membership or $Q$ of 0.997 ± 0.005 (SD) for jaguars, 0.995 ± 0.007 (SD) for pumas and 0.997 ± 0.001 (SD) for ocelots (Fig. 2).

For individual identification, cumulative $P_{(ID)}$ and $P_{(ID)sib}$ values were calculated per species for the screening multiplex 1 (seven loci), which helped to assess the minimum number of loci required for individual identification. With the criterion of $P_{(ID)sib} < 0.010$, a minimum of six finalized loci for jaguars and ocelots and 5 loci for pumas were needed to identify individuals with high statistical confidence (Fig. 3). The cumulative $P_{(ID)}$ and $P_{(ID)sib}$ values for seven loci of multiplex 1 were 2.5E-06 and 0.005 for jaguars, 1.5E-08 and 0.002 for pumas, and 4.6E-08 and 0.002 for ocelots. For all 14 loci, cumulative $P_{(ID)}$ and $P_{(ID)sib}$ estimates were 3.9E-11 and 5.3E-05 for jaguars, 4.3E-14 and 1.5E-05 for pumas, and 5.5E-15 and 7.1E-06 for ocelots.

In summary, of 1053 scat samples collected across all study sites, 512 (49%) were successfully identified to the species and individual level. In total, we detected 149 individual felids (65 jaguars, 54 pumas and 30 ocelots) in the wild. Jaguars were genetically identified 299 times, pumas 153 times and ocelots 60 times (Table 1). Due to a small sample size for margays ($n = 9$) and nondetection for jaguarundis, we decided to not include these two species into this manuscript.

### *Characterization of cross-species microsatellites*

For the finalized set of microsatellite loci, the number of alleles per locus ranged from 3 to 8 (5.00 ± 1.66, SD) for jaguars, 3–15 (8.00 ± 3.49, SD) for pumas and 3–10 (6.36 ± 1.87, SD) for ocelots. Mean allelic richness was highest for pumas (7.50 ± 3.19, SD), followed by jaguars (5.17 ± 1.56, SD) and ocelots (3.87 ± 0.87, SD). The average expected and observed heterozygosities for all loci were $H_E = 0.60 \pm 0.18$ (SD) and $H_0 = 0.57 \pm 0.20$ (SD) for jaguars, $H_E = 0.65 \pm 0.21$ (SD) and $H_0 = 0.61 \pm 0.21$ (SD) for pumas, $H_E = 0.70 \pm 0.13$ (SD) and $H_0 = 0.62 \pm 0.18$ (SD) for ocelots. Based on mean PIC values, another measure of polymorphism for marker loci, ocelots showed the highest diversity

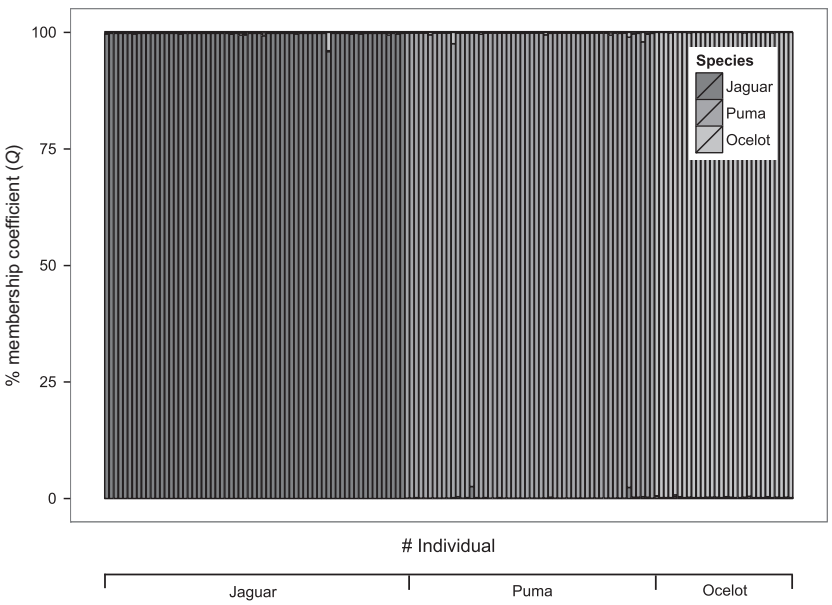


**Fig. 2** Species assignment using Bayesian clustering with STRUCTURE (Pritchard *et al.* 2000). Bar plot represents the assignment of the Neotropical feline species (jaguar, puma and ocelot) in Belize. Each vertical bar represents one individual. Each genetic cluster (coloured in a different grey shade) represents one species.

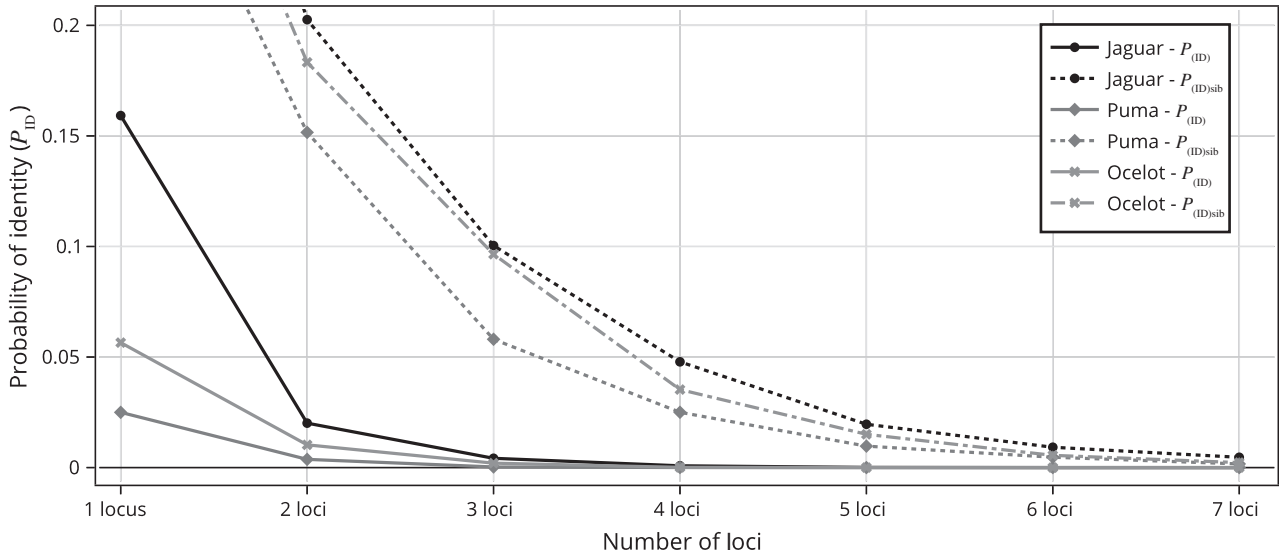


**Fig. 3** Relationship between the probability of identity (cumulative) for unrelated individuals ($P_{(ID)}$) and siblings ($P_{(ID)sib}$) for multiplex 1 (seven loci; used for screening, individual and species identification) in jaguars ($n$ = 65), pumas ($n$ = 54) and ocelots ($n$ = 30). DNA was isolated from faecal samples collected across several study sites in Belize. $P_{(ID)sib} < 0.010$ was used as criterion for individual identification.

**Table 1** Number of individual ($n$) jaguars, pumas and ocelots and number of captures per species detected across five long-term study sites (MPR, Mountain Pine Ridge Forest Reserve; RB, Rio Bravo Conservation and Management Area; CBWS, Cockscomb Basin Wildlife Sanctuary; CFRNP, Chiquibul Forest Reserve and National Park; FB, Fireburn/Balam Na Nature Reserve) and several other short-term survey sites in Belize from 2007–2010

| | | *Panthera onca* | | *Puma concolor* | | *Leopardus pardalis* | |
|---|---|---|---|---|---|---|---|
| Study sites | Survey year | $n$ | No. of captures | $n$ | No. of captures | $n$ | No. of captures |
| MPR | 2007 | 10 | 81 | 2 | 6 | 1 | 1 |
| RB | 2008 | 9 | 30 | 16 | 36 | 11 | 28 |
| CBWS | 2008 | 16 | 74 | 7 | 36 | 7 | 10 |
| CFRNP | 2008 | 8 | 49 | 6 | 9 | 6 | 10 |
| FB | 2009 | 8 | 24 | 11 | 55 | 3 | 8 |
| Other sites | 2010 | 14 | 41 | 12 | 11 | 2 | 3 |
| Total | | 65 | 299 | 54 | 153 | 30 | 60 |

(0.66 ± 0.14, SD) followed by pumas (0.62 ± 0.21, SD) and jaguars (0.55 ± 0.17, SD) (Table 2).

No loci deviated significantly from HWE for jaguars. Loci FCA043 ($P < 0.000$) and F98 ($P = 0.011$) revealed significant deviations from HWE for pumas after using sequential Bonferroni correction ($P \leq 0.015$). For ocelots, loci FCA391 ($P = 0.001$), FCA275 ($P < 0.000$) and FCA741 ($P = 0.011$) deviated significantly from HWE. Linkage disequilibrium was detected after using sequential Bonferroni correction ($P \leq 0.00055$) in only one case (FCA096/FCA441 for jaguars).

PCR amplification success across all samples was 61% ranging from 47% to 89% per locus. PCR amplification success for samples with finalized species ID differed significantly among species ($H = 17.53$, $P < 0.000$; Kruskal–Wallis rank-sum test) with highest mean rates for jaguars (90%), followed by pumas (88%) and ocelots (73%). Pairwise comparisons between species using post hoc Wilcoxon rank-sum tests revealed that PCR amplification success rates for ocelots were significantly lower compared to jaguars ($W = 183$, $P < 0.000$, $r = -1.14$) and pumas ($W = 102$, $P = 0.001$, $r = -0.88$). Genotyping accuracy across all samples was 90%, which also differed significantly among species ($H = 29.51$, $P < 0.000$; Kruskal–Wallis rank-sum test) with highest mean rates for jaguars (93%) followed by ocelots (86%) and pumas (75%). Post hoc Wilcoxon rank-sum tests revealed significant differences between all species pairs tested. Genotyping error was estimated by calculating mean allelic dropout and false allele rates for all samples collected (ADO, 15% ± 2.8; FA, 2% ± 1.0). Allelic dropout rates for samples with finalized species ID varied significantly among species ($H = 9.39$, $P = 0.009$; Kruskal–Wallis rank-sum test) with mean rates of 13% for jaguars, 12% for pumas and 17% for ocelots. Wilcoxon rank-sum tests showed that allelic dropout rates were significantly higher for ocelots than for jaguars ($W = 42$, $P = 0.012$, $r = -0.67$) and pumas ($W = 39$, $P = 0.007$, $r = -0.72$;

**Table 2** Characterization of 14 microsatellite loci for jaguars, pumas and ocelots in Belize, including number of alleles ($N_A$), allelic richness ($A_R$) using the rarefaction method (Kalinowski 2005), observed ($H_O$) and expected heterozygosities ($H_E$), polymorphic information content (PIC), *P*-value for the HWE test ($P_{HW}$) and frequency of null alleles ($F_{Null}$)

| | *Panthera onca* ($n$ = 65) | | | | | | | *Puma concolor* ($n$ = 54) | | | | | | | *Leopardus pardalis* ($n$ = 30) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | $N_A$ | $A_R$ | $H_O$ | $H_E$ | *PIC* | $P_{HW}$ | $F_{Null}$ | $N_A$ | $A_R$ | $H_O$ | $H_E$ | *PIC* | $P_{HW}$ | $F_{Null}$ | $N_A$ | $A_R$ | $H_O$ | $H_E$ | *PIC* | $P_{HW}$ | $F_{Null}$ |
| F124 | 6 | 5.78 | 0.63 | 0.64 | 0.60 | 0.31 | 0.02 | 15 | 13.23 | 0.81 | 0.87 | 0.85 | 0.41 | 0.03 | 8 | 4.45 | 0.72 | 0.77 | 0.75 | 0.60 | 0.04 |
| FCA391 | 6 | 6.00 | 0.62 | 0.71 | 0.66 | 0.25 | 0.07 | 7 | 6.88 | 0.52 | 0.65 | 0.61 | 0.11 | 0.10 | 5 | 3.04 | 0.43 | 0.64 | 0.57 | 0.00 | 0.14 |
| FCA043 | 3 | 6.00 | 0.52 | 0.62 | 0.55 | 0.38 | 0.07 | 9 | 8.17 | 0.74 | 0.78 | 0.74 | 0.00 | 0.01 | 5 | 3.14 | 0.59 | 0.56 | 0.53 | 0.81 | −0.04 |
| FCA275 | 3 | 3.00 | 0.54 | 0.65 | 0.57 | 0.21 | 0.08 | 7 | 6.61 | 0.69 | 0.70 | 0.67 | 0.72 | 0.01 | 8 | 4.67 | 0.46 | 0.80 | 0.78 | 0.00 | 0.21 |
| FCA096 | 7 | 6.92 | 0.74 | 0.74 | 0.69 | 0.79 | 0.00 | 7 | 7.00 | 0.75 | 0.77 | 0.74 | 0.19 | −0.01 | 6 | 3.67 | 0.58 | 0.71 | 0.67 | 0.05 | 0.08 |
| FCA126 | 6 | 5.90 | 0.76 | 0.64 | 0.61 | 0.48 | −0.12 | 6 | 5.96 | 0.60 | 0.62 | 0.59 | 0.88 | 0.02 | 8 | 4.58 | 0.73 | 0.80 | 0.77 | 0.64 | 0.04 |
| FCA090 | 5 | 5.00 | 0.59 | 0.59 | 0.55 | 0.75 | −0.01 | 9 | 8.34 | 0.79 | 0.83 | 0.80 | 0.07 | 0.03 | 5 | 3.72 | 0.76 | 0.74 | 0.70 | 0.98 | −0.01 |
| F85 | 4 | 3.95 | 0.40 | 0.55 | 0.46 | 0.04 | 0.13 | 9 | 8.85 | 0.71 | 0.85 | 0.83 | 0.03 | 0.09 | 8 | 3.81 | 0.85 | 0.74 | 0.69 | 0.78 | −0.08 |
| F98 | 3 | 3.00 | 0.50 | 0.56 | 0.50 | 0.36 | 0.05 | 3 | 2.94 | 0.22 | 0.26 | 0.24 | 0.01 | 0.07 | 6 | 3.76 | 0.70 | 0.73 | 0.69 | 0.79 | 0.02 |
| FCA741 | 3 | 3.00 | 0.05 | 0.05 | 0.05 | 1.00 | −0.03 | 11 | 9.13 | 0.71 | 0.73 | 0.68 | 0.07 | 0.01 | 5 | 5.00 | 0.25 | 0.78 | 0.75 | 0.01 | 0.33 |
| FCA225 | 6 | 5.90 | 0.58 | 0.63 | 0.58 | 0.27 | 0.05 | 4 | 3.86 | 0.25 | 0.26 | 0.24 | 0.69 | 0.00 | 10 | 5.23 | 0.87 | 0.84 | 0.83 | 0.52 | −0.02 |
| FCA008 | 4 | 3.90 | 0.41 | 0.48 | 0.43 | 0.23 | 0.07 | 5 | 4.35 | 0.31 | 0.33 | 0.31 | 0.17 | 0.05 | 7 | 3.83 | 0.70 | 0.72 | 0.68 | 0.79 | 0.02 |
| F53 | 8 | 8.00 | 0.75 | 0.77 | 0.75 | 0.40 | 0.01 | 14 | 14.00 | 0.80 | 0.84 | 0.82 | 0.31 | 0.03 | 5 | 3.34 | 0.62 | 0.63 | 0.59 | 0.52 | 0.00 |
| FCA441 | 6 | 6.00 | 0.89 | 0.79 | 0.76 | 0.09 | −0.07 | 6 | 5.63 | 0.67 | 0.67 | 0.61 | 0.93 | 0.00 | 3 | 1.99 | 0.38 | 0.32 | 0.28 | 1.00 | −0.21 |

Significance tests for HWE were adjusted by applying sequential Bonferroni correction ($P < 0.015$).

Table 3). Feline species did not differ significantly ($H = 0.68$, $P = 0.711$; Kruskal–Wallis rank-sum test) in mean rates for FA of jaguars (1% ± 1.0, SD), pumas (1% ± 1.2, SD) and ocelots (2% ± 1.3, SD) (Table 3).

## Discussion

### *Selection and performance of cross-species microsatellite loci*

Microsatellites represent a powerful type of neutral genetic marker commonly used to answer a variety of population genetic and ecological questions (e.g. Sunnucks 2000; Selkoe & Toonen 2006; Wang 2011; Allendorf *et al.* 2013). Due to many conservation challenges, species of concern often are studied in a comparative way and cross-species microsatellites have been identified for several different taxa (e.g. Barbara *et al.* 2007). We successfully cross-amplified 20 published microsatellite loci developed for the domestic cat in three Neotropical feline species. We conducted thorough screening of all loci tested and selected 14 polymorphic markers, which efficiently and reliably identified species and individuals from field-collected, faecal DNA samples of three felid species with highly variable DNA quality and quantity. Proper selection of genetic markers is crucial because it impacts all subsequent population genetic analyses (e.g. Taberlet & Luikart 1999). To increase the cost efficiency of our study, we arranged the 14 loci in three multiplexes and configured multiplex 1 with 7 highly polymorphic loci, which were used for sample screening, and species and individual identification. Multiplexes 2 and 3 were developed to add additional loci for fine-scale genetic structure or parentage analyses not reported here and were only run for one sample per individual.

The genotyping performance of the microsatellite loci was tested based on several parameters including genetic variation, PCR amplification success, genotyping accuracy and genotyping error rates. Results of the current study suggest that the cross-species microsatellite set optimized here is an efficient and powerful tool for conservation genetic studies of multiple Neotropical felids. The high variability indicates the potential usefulness for examining genetic structure of the target species. Although we conducted this study in tropical Belize and included all faecal samples detected by the scat detector dog ($n = 1053$), many of which were highly degraded, our analysis showed medium to high mean PCR amplification success rates using nuclear loci (61% ± 12.4, SD) compared with other studies of Neotropical felids with

**Table 3** Summary of PCR amplification success, genotyping accuracy and genotyping error rates for 14 microsatellite loci for all samples and all jaguar, puma and ocelot samples with finalized species ID detected across five study sites in Belize. PCR, % polymerase chain reaction amplification success; GA, % genotyping accuracy; ADO, % allelic dropout; FA, % false alleles

| | All samples $n = 1053$* | | | | *Panthera onca* $n = 299$* | | | | *Puma concolor* $n = 153$* | | | | *Leopardus pardalis* $n = 60$* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Primer ID | PCR | GA | ADO | FA | PCR | GA | ADO | FA | PCR | GA | ADO | FA | PCR | GA | ADO | FA |
| Multiplex 1 | | | | | | | | | | | | | | | | |
| F124 | 56.22 | 89.49 | 13.45 | 0.69 | 93.08 | 92.21 | 10.51 | 0.42 | 89.23 | 68.42 | 9.30 | 1.28 | 81.67 | 85.71 | 16.67 | 1.02 |
| FCA391 | 46.73 | 89.55 | 13.75 | 1.79 | 83.02 | 90.51 | 11.94 | 1.22 | 86.61 | 77.54 | 8.85 | 1.75 | 59.17 | 88.24 | 16.67 | 0.00 |
| FCA043 | 54.24 | 89.28 | 14.65 | 1.53 | 83.04 | 93.97 | 8.20 | 0.75 | 76.43 | 69.01 | 15.85 | 0.51 | 81.90 | 88.37 | 13.11 | 2.33 |
| FCA275 | 57.75 | 90.65 | 14.87 | 0.74 | 97.76 | 94.63 | 8.93 | 0.58 | 93.84 | 68.70 | 10.61 | 0.37 | 62.50 | 79.49 | 29.27 | 2.56 |
| FCA096 | 52.99 | 86.86 | 18.02 | 2.00 | 89.18 | 88.69 | 17.72 | 1.33 | 86.82 | 60.00 | 13.27 | 1.73 | 77.50 | 88.10 | 21.88 | 3.57 |
| FCA126 | 56.24 | 85.57 | 16.84 | 1.41 | 94.22 | 88.69 | 13.33 | 1.21 | 92.59 | 65.32 | 11.48 | 0.38 | 88.33 | 85.19 | 14.63 | 1.85 |
| FCA090 | 59.76 | 88.96 | 15.21 | 1.32 | 92.75 | 90.27 | 14.58 | 0.63 | 91.14 | 70.97 | 10.19 | 0.39 | 82.50 | 89.80 | 13.11 | 2.04 |
| Multiplex 2 | | | | | | | | | | | | | | | | |
| F85 | 77.81 | 89.63 | 14.18 | 2.94 | 86.75 | 94.66 | 8.16 | 2.29 | 87.03 | 77.04 | 12.04 | 1.97 | 74.55 | 84.93 | 15.79 | 2.74 |
| F98 | 80.77 | 91.24 | 17.62 | 1.39 | 88.55 | 93.23 | 12.12 | 1.59 | 89.02 | 92.92 | 16.33 | 0.00 | 83.93 | 87.50 | 16.36 | 0.00 |
| FCA741 | 66.93 | 93.78 | 15.67 | 0.50 | 88.18 | 96.60 | 26.92 | 0.00 | 79.10 | 73.23 | 9.71 | 1.50 | 15.74 | 87.50 | 0.00 | 0.00 |
| FCA225 | 76.17 | 86.19 | 20.45 | 2.53 | 85.24 | 89.31 | 15.48 | 1.53 | 86.77 | 81.30 | 16.87 | 2.56 | 74.11 | 81.69 | 19.23 | 2.82 |
| FCA008 | 88.39 | 92.81 | 12.19 | 0.51 | 94.34 | 95.68 | 7.01 | 0.36 | 95.29 | 84.33 | 14.77 | 0.58 | 87.72 | 85.42 | 16.05 | 1.04 |
| Multiplex 3 | | | | | | | | | | | | | | | | |
| F53 | 73.47 | 88.41 | 13.46 | 3.77 | 83.95 | 91.72 | 8.51 | 4.14 | 81.60 | 70.93 | 10.00 | 3.23 | 72.00 | 82.61 | 23.26 | 2.90 |
| FCA441 | 66.33 | 90.00 | 9.22 | 1.82 | 91.56 | 90.36 | 8.12 | 1.20 | 86.79 | 77.78 | 8.91 | 4.13 | 71.43 | 89.80 | 15.38 | 0.00 |
| Mean | 61.17 | 89.22 | 14.95 | 1.51 | 89.40 | 92.18 | 12.25 | 1.23 | 87.30 | 74.11 | 12.01 | 1.46 | 72.36 | 86.02 | 16.53 | 1.63 |
| SD | 12.37 | 2.30 | 2.75 | 0.95 | 4.68 | 2.64 | 5.32 | 1.03 | 5.37 | 8.45 | 2.88 | 1.22 | 18.43 | 3.08 | 6.48 | 1.27 |

*All samples were analysed with multiplex 1. Multiplex 2 and multiplex 3 were only used for samples with finalized individual ID.

scat detector dogs (jaguars, 26 - 41%: Michalski *et al.* 2011) or without detector dogs (pumas, 5–45%, Miotto *et al.* 2007). Other molecular scatology studies monitoring leopards or tigers without scat detector dogs in tropical environments (e.g. Bhagavatula & Singh 2006; Mondol *et al.* 2009a; Borthakur *et al.* 2011; Dutta *et al.* 2012) reported success rates similar to, or higher than, our study (>60%). Mean genotyping error rates differ widely among noninvasive felid studies focusing on tigers (ADO = 0–3.5%, FA = 0, Mondol *et al.* 2009a; ADO = 0–64.8%, FA = 0–9.0%, Bhagavatula & Singh 2006), leopards (ADO = 0–45.4%, FA = 0–7.6%, Dutta *et al.* 2012; ADO = 0–7.4%, FA = 0–1.5%; Mondol *et al.* 2009b) and pumas (ADO = 10.6%, Miotto *et al.* 2007). Mean genotyping error rates for this study (ADO = 15% ± 2.6; FA = 2% ± 1.0) were relatively low considering that low-quality faecal samples detected by the scat detector dog were included to the analysis. PCR amplification success and genotyping error rates for faecal DNA samples often vary greatly among studies depending on primer selection and design (e.g. Housley *et al.* 2006), and due to various other factors including faecal DNA quality and quantity, scat sample origin (e.g. species, environmental factors), and the choice of field and laboratory techniques (e.g. Wasser *et al.* 1997; Piggott 2004; Beja-Pereira *et al.* 2009; Soto-Calderon *et al.* 2009; Stenglein *et al.* 2010). The detection method used for scat sampling (humans or detector dogs) also affects success and error rates because studies using scat detector dogs have the potential to locate deteriorated scat samples not easily detectable by humans (e.g. Long *et al.* 2007).

### *Species and individual identification*

Recent studies of Neotropical felids suggested that techniques for individual identification of faecal samples need further improvement in field and laboratory techniques (e.g. Michalski *et al.* 2011). We addressed this by optimizing a powerful set of cross-species microsatellite loci, which can be used reliably for species and individual identification of co-occurring jaguars, pumas and ocelots based on field-collected faecal samples. In total, we collected 1053 scat samples from the wild and successfully genotyped 49% of the scats to the species level and individual level. We used probability of identity estimates to assess statistical confidence for individual identification with $P_{(ID)sib} < 0.010$ as the deciding criterion (Mills *et al.* 2000; Waits *et al.* 2001). The discriminatory power of the probability of identity estimates for this primer set was high, which indicates a strong resolving power between individuals, even when close relatedness among study animals may be an issue.

To increase the efficiency of this study, we used the same nuclear markers to identify species by examining species-specific alleles and applying Bayesian clustering analysis to verify species assignment. The application of species-specific alleles for species identification of faecal samples collected in the field has been mainly used for wildlife forensics to differentiate between several big cats (e.g. Singh *et al.* 2004) or to detect hybridization between two wild feline species (bobcat and lynx; Schwartz *et al.* 2004). Here, we demonstrated the potential of this approach for individual-based monitoring of multiple wild felid species on a local geographic scale; however, before application in other areas, it will be important to verify that these alleles remain species-specific. Besides species identification, cross-species microsatellite loci have a wide application and are especially useful when several closely related species at risk need to be managed and conserved simultaneously. Nonetheless, limitations of this approach (e.g. Barbara *et al.* 2007) need to be considered.

In conclusion, we strongly encourage the development of rigorous field and laboratory protocols especially for noninvasive genetic studies conducted in tropical environments hostile to DNA samples. The combination of the most informative markers and the assessment of locus-specific success and error rates both within and among three target species helped to optimize a set of polymorphic nuclear primers, which improved our ability to efficiently and accurately identify genotypes (at the species and individual level) from often highly degraded scat samples. Additionally, using a microsatellite marker set that amplifies across species represents an efficient and powerful way to study multiple co-occurring species on both the individual and population levels, and simultaneously to evaluate the conservation status (demographics, genetic diversity and connectivity) of potentially threatened species.

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C.W. conducted the field sampling, laboratory and data analysis, data interpretation and wrote the manuscript. L.P.W. assisted with laboratory protocols and study design, data interpretation, and the preparation of the manuscript. M.J.K. assisted with field study design, data interpretation and the preparation of the manuscript.

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## Data Accessibility

Structure input file and mtDNA sequence alignments are accessible via DRYAD database, doi:10.5061/dryad.j248q.

## Supporting Information

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

**Table S1** Species-specific allele size ranges and allele sizes for jaguars, pumas and ocelots in Belize using 14 microsatellite loci.