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Note

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ABSTRACT Collection of fecal samples for use in a genetic capture-mark-recapture framework has become popular as a noninvasive method of monitoring wildlife populations. A major caveat to this process, however, is that fecal samples often yield low quality DNA that is prone to genotyping errors, potentially leading to biases in population parameter estimation. Therefore, considerable care is required to identify robust genetic markers, especially in hot or humid conditions that may accelerate DNA degradation. We identified microsatellite loci in wild pig (*Sus scrofa*) fecal samples that were robust and informative within warm, humid ecosystems. To examine how degradation affected genotyping success, we sampled pig feces across 5 days and calculated how the number of quantitative polymerase chain reaction (qPCR) cycles required to reach the fluorescent threshold (C_t) changed over time. We identified 17 microsatellite loci that had high polymorphism and amplification success and low genotyping error rates (0–0.050 per locus). In the degradation experiment, C_t increased over the 5 days, but in the absence of rain, the majority of samples produced accurate genotypes after 5 days (2,211/2,550 genotypes). Based on the high amplification success and low error rates, even after 5 days of exposure to warm, humid conditions, these loci are useful for estimating population parameters in pig fecal samples. © 2016 The Wildlife Society.

KEY WORDS degradation, feces, microsatellites, noninvasive, South Carolina, *Sus scrofa*, wild pigs.

Fecal DNA is an important source of information for monitoring wildlife populations, and has been used successfully across many taxa (Jacob et al. 2010, Arandjelovic et al. 2011, Hettinga et al. 2012, Sharma et al. 2013, Lounsberry et al. 2015). Collection of feces does not require the capture of animals, and if appropriate genetic markers exist, can be used as a method of capture-mark-recapture (CMR) to estimate abundance or density (Mondol et al. 2009, Arandjelovic et al. 2011, Hedges et al. 2013). By identifying individuals via DNA from fecal samples, wildlife managers can estimate additional population characteristics, such as movement and dispersal rates (Sharma et al. 2013) and patterns of relatedness (Archie et al. 2006, Pinho et al. 2014), in species for which traditional mark-recapture would be difficult, stressful to animals, and expensive.

Despite the considerable promise collection of fecal samples holds for CMR studies, small amounts of starting template DNA from feces and subsequent degradation can result in low quality DNA, and ultimately, amplification failure, genotyping errors, and low sample size (Buchan et al. 2005, Broquet et al. 2007, Murphy et al. 2007, Brinkman et al. 2010). These errors can bias CMR-based estimates of abundance, density, movement, and parentage (Mills et al. 2000, Waits and Paetkau 2005). To mitigate these potential pitfalls, pilot studies are important to identify polymorphic genetic markers that can successfully amplify low quantities of template DNA and conditions that may increase genotyping errors within such loci. Consequently, multiple studies have investigated how species' diet (Murphy et al. 2007), field conditions (Maudet et al. 2004, Murphy et al. 2007, Brinkman et al. 2010), and laboratory protocols (e.g., preservation method, Murphy et al. 2002, Wultsch et al.

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2015; extraction protocol, Frantz et al. 2003, Espinosa et al. 2015) affect amplification of fecal DNA. Although common threads exist within these studies, particularly with regard to environmental conditions (e.g., success is highest in cold, dry conditions; Nsubuga et al. 2004, Piggott 2004, Murphy et al. 2007, Brinkman et al. 2010), great variability in amplification and genotyping success exists between genetic markers, species, and study areas. Therefore, species-specific pilot studies are needed to identify ideal conditions to minimize potential errors within fecal CMR studies, especially when environmental conditions are expected to accelerate DNA degradation.

Our study focused on optimizing amplification protocols and selecting robust markers to conduct CMR of invasive wild pigs (*Sus scrofa*) within the southeastern United States, an area characterized by an overall humid, subtropical climate with hot summers (>22°C; Peel et al. 2007). To date, the majority of CMR studies of wild pigs using fecal DNA have occurred in Germany (Fickel and Hohmann 2006, Ebert et al. 2012, Kolodziej et al. 2012) where fecal samples were collected during winter. Although these CMR studies provide insights about appropriate microsatellite loci for wild pigs in North America, conditions in the southeastern United States are much warmer overall (15.39–33.44°C in our study area vs. 8–9°C in Ebert et al. [2012]). The warmer temperatures and high humidity in the southeastern United States are likely to expedite DNA degradation (Nsubuga et al. 2004, Murphy et al. 2007), which could be problematic for genetic studies designed to estimate abundance and density of wild pigs.

Data on abundance and density are needed to evaluate the risks posed by wild pig populations and effectiveness of control measures because invasive wild pig populations in the southeastern United States have grown in the last 30 years (Bevins et al. 2014), causing extensive agricultural damage (Engeman et al. 2004, Campbell and Long 2009, Bevins et al. 2014) and threatening native ecosystems (Barrios-Garcia and Ballari 2012, Bevins et al. 2014). Therefore, we evaluated if microsatellite loci used in European and Asian studies (Fickel and Hohmann 2006, Ebert et al. 2012, Choi et al. 2014) could provide reliable genotypes when amplified from fecal samples collected in sub-tropical or tropical areas, such as those typical of the southeastern United States. Our goal for this study was twofold: first, we aimed to identify microsatellite loci that would be robust for wild pig genotypes from fecal samples collected in the warm, humid environments like the southeastern United States; and second, we examined how sample degradation affected our ability to obtain DNA from fecal material in similar environmental conditions.

STUDY AREA

We conducted this study at the Savannah River Site (SRS) on the South Carolina-Georgia border in the southeastern United States. The SRS is a 78,000-ha United States Department of Energy facility with limited public access. The SRS is located within the upper Atlantic Coastal Plain, a low-lying region (elevation: 36–104 m) with a warm (average monthly high temperatures: 15.39–33.44°C), humid (average monthly relative humidity: 63–80%) climate (Savannah River National Laboratory Atmospheric Technologies Group, unpublished data). Monthly average rainfall ranged from 7.11–13.92 cm/month. The degradation study occurred during 9–24 April 2015 where weather conditions were generally overcast and humid (relative humidity = 81.8–98.8% during the day) with high temperatures of 22.48–29.55°C during the sampling period.

Upland pine forests of long-leafed pine (*Pinus palustris*) occur across the majority of SRS (68%), and are managed by the United States Forest Service. Bottomland hardwood forests containing mixes of oak-hickory or cypress-tupelo (*Taxodium districhum-Nyssa aquaticus*) cover another 22% of habitat on site (White and Gaines 2000, Imm and McLeod 2005). The remaining habitat is sporadic nonforested habitat (e.g., developed and clear cuts; White and Gaines 2000). A variety of soils occur on site according to the different habitat types with sandy soils in the highlands and clay-loam soils in the lowlands (Rogers 1990). The SRS provides habitat for numerous game species such as white-tailed deer (*Odocoileus virginianus*), turkeys (*Melegris gallopavo*), bobcats (*Felis rufus*), and coyotes (*Canis latrans*) as well as endangered species (e.g., red-cockaded woodpecker [*Picoides borealis*]). High densities of wild pigs are present on the SRS, so hunting and trapping have occurred since 1952 to control wild pig numbers and limit negative impacts (Mayer and Brisbin 1991, Beasley et al. 2014).

METHODS

Microsatellite Screening

For all microsatellite screening, we used paired tissue and fecal samples from 40 individual pigs that were culled by United States Forest Service contractors on the SRS from January to April 2014. We scraped 2 fecal samples (~0.5 g each) from the outside of fecal pellets squeezed out of each pig's rectum shortly after death, and then placed them in 90% ethanol or dimethyl sulfoxide saline buffer (DET; Seutin et al. 1991). We also placed an ear biopsy from each culled pig in 90% ethanol for comparison to fecal samples, and stored both fecal and tissue samples in a −80°C freezer until extraction. We extracted each fecal sample in duplicate (i.e., 2 extraction replicates) via Nucleospin soil kits (Macherey-Nagel Inc, Bethlehem, PA). Fecal extractions consisted of 0.25 g of starting fecal material digested in SL-2 buffer, and ended with 2 elutions of 25 μL each. We processed tissue samples on different days than fecal samples, and extracted using Qiagen DNeasy blood and tissue kits (Qiagen, Valencia, CA). Following extractions, we used a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) to quantify DNA concentration and purity for all extractions, and then adjusted all samples to approximately 20 ng/μL.

Prior to microsatellite amplification, we amplified all fecal samples for a region smooth muscle protein 22-alpha gene using the primer set TAGLNsus (Ebert et al. 2012) in quantitative polymerase chain reaction (qPCR). Ebert et al. (2012) successfully used the TAGLNsus primer to remove

low quality samples prior to microsatellite amplification. We recorded the number of qPCR cycles required to amplify the TAGLNsus product to a concentration that was higher than the fluorescent threshold (C_t). Lie and Petropoulos (1998) and Morin et al. (2001) reported that C_t values are directly correlated with starting DNA template amount, and thus, a higher C_t indicates less starting template DNA and higher frequency of genotyping errors (Morin et al. 2001). Each 12-μL qPCR reaction contained 20 ng of template DNA and 9 μL of 2X iQ SYBR Green Supermix (Bio-RAD, Hercules, CA). Each run included a set of standards with known DNA concentrations (Hausknecht et al. 2010), 3 positive controls, and 1 negative control. Amplification conditions consisted of 95°C for 10 minutes, followed by 46 cycles of denaturation at 95°C for 15 seconds, annealing at 59°C for 25 seconds, and extension at 72°C for 35 seconds. We amplified all samples 3 times to assess consistency across qPCR runs and to validate any instances where samples failed to amplify (i.e., $C_t = 0$). We performed all qPCR reactions on a Bio-Rad CFX96 C1000 Touch.

Following qPCR, we initially screened fecal samples from 10 individual pigs (1 replicate extraction from ethanol and DET samples) on 31 loci (Table S1, available online at www.onlinelibrary.wiley.com) to identify loci that were amenable for fecal samples. In total, 21 loci were easy to call, polymorphic, and exhibited high amplification success in fecal samples (≥7/10 samples successfully amplified; Table S1, available online at www.onlinelibrary.wiley.com). We also found that ethanol-preserved samples always had better amplification success than DET-preserved samples regardless of the tested microsatellite locus. Similarly, ethanol preserved samples had higher DNA concentration in Nanodrop and lower qPCR cycles, so we decided to focus on ethanol-preserved fecal samples for the remainder of the microsatellite screening.

Based on the initial screen of putative microsatellite loci, we calculated amplification success and genotyping error rates for 21 microsatellite loci via paired tissue and ethanol-preserved fecal samples from 40 culled pigs. For the ethanol preserved samples, we amplified each fecal extraction 3 separate times for 6 amplifications per individual pig (2 replicate extractions for each pig, 3 amplifications per extraction). Polymerase chain reactions occurred in 12-μL volumes with 20 ng of template DNA, 10× AmpliTaq Gold buffer, 2.5 mM $MgCl_2$ buffer, 25.0 μg/mL bovine serum albumin, 0.2 mM of each dNTP, 5 pmol of each primer, and 0.5 U Amplitaq Gold (Applied Biosystems, Grand Island, NY). Amplification conditions included an initial 5-minute denaturation at 95°C followed by 20 cycles of touchdown PCR (95°C for 30 sec, 65°C for 30 sec with a −0.5°C drop each cycle, and extension at 72°C for 30 sec), then 20 cycles of standard denaturation (95°C), annealing (55°C), and extension (72°C) for 30 seconds each, and concluded with a final extension at 72°C for 5 minutes. We analyzed amplified products on an ABI 3170 Genetic Analyzer, and scored alleles in GeneMapper (Life Technologies, Grand Island, NY).

Fecal samples are prone to genotyping errors, particularly false alleles and allelic dropout (Taberlet et al. 1996, Taberlet and Luikart 1999). For our analyses, we defined a false allele as an allele that appeared in fecal samples and not in the paired tissue sample and vice versa for allelic dropout. To calculate error rates for a microsatellite locus, we did not allow missing genotypes for any individual pig, so all fecal extractions had to amplify 6 times (3 amplifications per extraction replicate, 12 amplifications per individual pig). If 12 successful amplifications did not occur in all 40 individuals for a locus, we removed the locus from the dataset. For the remaining loci, we counted the frequency of false alleles and allelic dropout within each locus. We defined acceptable rates of genotyping errors as 0.05 because error rates of ≤0.05 were considered normal for low quality DNA even after triplicate amplifications (Gagneux et al. 1997). An error rate of 5% across all loci will lead to large overestimations in population size in CMR studies (Waits and Leberg 2000), so we considered loci with the lowest error rates the most robust within our final suite. Also, for each locus we calculated probability of identity (P_{ID}), probability of identity assuming siblings (P_{SIB}), heterozygosity (expected [H_E] and observed [H_O]), and number of alleles (N_A) in GenAlEx 6.5 (Peakall and Smouse 2012).

Fecal DNA Degradation

Our second goal was to quantify how degradation affected amplification and genotyping in pig feces in humid conditions in the absence of rain. We excluded rain because multiple authors have recommended avoiding rain for sampling (Murphy et al. 2007, Brinkman et al. 2010), and rain is likely to wash off any epithelial cells containing DNA on the fecal sample. We collected additional paired fecal and tissue samples from 9 to 24 April 2015. We trapped 10 wild pigs (4 F, 6 M, 6 adults, 4 subadults) using baited corral traps and then culled the pigs. We included both males and females and different age classes to mimic variation in natural conditions because in practice, researchers will have no control over individual-based factors like diet, sex, and age of individuals captured in fecal studies. All trapping and euthanasia methods conformed to the American Society of Mammalogists guidelines (Sikes et al. 2011) and University of Georgia Animal Care and Use Committee policies (Protocol 12-010-Y3-A4). We immediately squeezed fecal material out of the rectum to mimic defecation. We separated fecal pellets from each individual into 2 groups (replicates A and B). We then placed all fecal pellets (10 per time period, 2 replicates per individual) separately in Tomahawk live traps (Tomahawk Live Trap LLC, Hazelhurst, WI) covered with plastic lids (i.e., traps containing fecal pellets were covered on the top, but not sides). Tomahawk traps create a cage via crisscross wire mesh, so fecal pellets rested within a hole of the mesh touching the native soil without touching the metal wire itself. Fecal pellets were, therefore, protected from rain, while remaining in contact with the ambient environment and the native soil substrate, allowing natural degradation by invertebrates, fungi, and bacteria.

We sampled all 20 fecal pellets (10 individuals, 2 replicates per individual) immediately following extraction from the

rectum (T_0) and then after 12 (T_{12}), 24 (T_{24}), 36 (T_{36}), 48 (T_{48}), 72 (T_{72}), 96 (T_{96}), and 120 (T_{120}) hours. Each DNA sample (hereby called fecal samples) consisted of 0.5 g of scat scraped from the outside of the fecal pellet using tweezers and carbon steel razor blades and placed in 90% ethanol. Fecal pellets physically degraded quickly, and beyond 120 hours, were no longer suitable to sample for DNA. Physical degradation generally occurred when a combination of insects, moisture, and fungus compromised the round structure of each fecal pellet, eventually resulting in a small pile of material indistinguishable from the native soil. We also placed a tissue sample from each pig in 90% ethanol upon capture as a reference sample for comparison to fecal genotypes, and stored all samples in a −80°C freezer until DNA extraction. DNA extraction and qPCR methods were identical to those described above for microsatellite screening. We recorded the number of cycles required to amplify the TAGLNsus product (or failure to amplify) for each sample as a measure of DNA quality. Although recording the number of cycles implies a whole number, qPCR cycles contain partial cycles, so they are amenable to linear models after log transformation.

We examined how time since collection (i.e., how long fecal samples were exposed to environmental conditions) affected the C_t values required for amplification via a repeated measures linear mixed model. The linear model contained 3 predictor variables: time since collection (in hours) as a fixed effect and 2 random effects for the repeated measures design with replicates nested within individuals. The first random effect accounted for the differences in slopes between individual fecal samples and the second allowed for differences among intercepts between individuals. We assumed that the slopes and intercepts varied independently (i.e., the starting C_t does not determine how fast a sample degrades), so we coded each random effect separately. We assessed model selection by comparing our full model with time since collection (hours) to 3 other reduced models. Two reduced models contained either 1 random effect (intercept only) or both random effects (intercept and slope), whereas the third reduced model contained time since collection (hours) and the intercept random effect. We conducted all mixed model calculations in the lme4 package (function lmer; Bates et al. 2015) in R (R Core Team 2013). We conducted model selection following Burnham and Anderson's (2002) methods via corrected Akaike's Information Criterion (AIC_c) where we considered all models with a $\Delta AIC_c < 2$ to be top models. We calculated the conditional R^2 value (variance explained by both random and fixed effects) and marginal R^2 value (variance explained by only fixed effects) for each top model following Nakagawa and Schielzeth (2013).

In addition to qPCR, we amplified tissue and fecal samples at the final suite of microsatellite loci that we retained after our initial screening. Erroneous genotypes or failures to amplify in fecal samples, therefore, could be attributed to DNA degradation. All PCR and allele sizing methods were the same as for the initial microsatellite screening, and we recorded the number of cases of failures to amplify, false alleles, and allele dropout within each locus (collectively called mismatched genotypes). We attempted to statistically test the effect of time since collection (hours) on whether amplification resulted in a mismatched genotype, but the low number of mismatched genotypes within each locus prevented meaningful statistical testing (see Results). Instead, we compared the mean number of C_t values in samples that either failed to amplify or had genotyping errors at a specific microsatellite locus to those that had accurate genotypes in the same microsatellite locus using Mann–Whitney *U*-tests in R (R Core Team 2013). We performed 17 Mann–Whitney *U*-tests, 1 for each microsatellite locus.

RESULTS

Microsatellite Screening

Of the initial 21 microsatellite loci selected (Table S1, available online at www.onlinelibrary.wiley.com), 17 were appropriate for fecal DNA samples. We eliminated 3 loci (S0068, Sw72, Sw632) based on low amplification success in fecal samples (144–192 successfully amplified out of 240 total amplifications per locus; 60–80%), and eliminated S0002 because of a high rate of genotyping errors (23/240 amplifications; 9.58%). Among the remaining 17 loci, 82 genotyping errors (6 false alleles, 76 allelic dropouts) occurred across 4,080 amplifications. Genotyping error rates (i.e., allelic dropout and false alleles combined) within each locus ranged from 0.000 to 0.050 (Table 1), and in all but 2 cases, the majority of amplifications (4–6 amplifications out of 6) resulted in the correct genotype for each individual.

Table 1. Description of the final set of 17 microsatellite loci from the microsatellite screening portion of this study sorted according to probabilities of identity (P_{ID}) for each locus. We calculated probability of identity (P_{ID}), probability of identity assuming siblings (P_{SIB}), observed (H_O), expected (H_E) heterozygosity, and genotyping error rates based on 40 culled wild pigs on the Savannah River Site, South Carolina, USA, 2014. Genotyping error rates include both allelic dropout and false alleles that were identified via comparison with tissue samples. We removed 4 loci (Sw632, Sw72, S0068, and S0002) from the original set of 21 microsatellite loci based on low amplification success across all fecal samples (Sw632, Sw72, S0068) or high genotyping error rates (S0002, 0.096).

Locus	P_{ID}	P_{SIB}	N_A	H_O	H_E	Error rate
FH2148	0.072	0.474	8	0.700	0.788	0.025
Sw949	0.074	0.373	6	0.791	0.801	0.004
Sw226	0.080	0.377	6	0.786	0.796	0.000
UMNp358	0.093	0.393	7	0.761	0.771	0.021
Sw2021	0.105	0.421	9	0.711	0.720	0.025
Sw911	0.130	0.432	5	0.701	0.710	0.029
S0228	0.132	0.427	5	0.713	0.722	0.000
FH1589	0.136	0.438	6	0.750	0.798	0.008
s0090	0.143	0.454	6	0.663	0.671	0.017
IGF1	0.152	0.447	6	0.650	0.682	0.033
UMNp445	0.209	0.489	5	0.628	0.635	0.029
S0101	0.251	0.517	6	0.591	0.598	0.025
Sw936	0.255	0.554	6	0.519	0.526	0.050
UMNp09	0.257	0.532	3	0.563	0.571	0.025
S0155	0.260	0.553	7	0.524	0.530	0.000
Sw240	0.354	0.619	4	0.438	0.444	0.038
Sw24	0.436	0.684	5	0.350	0.354	0.013

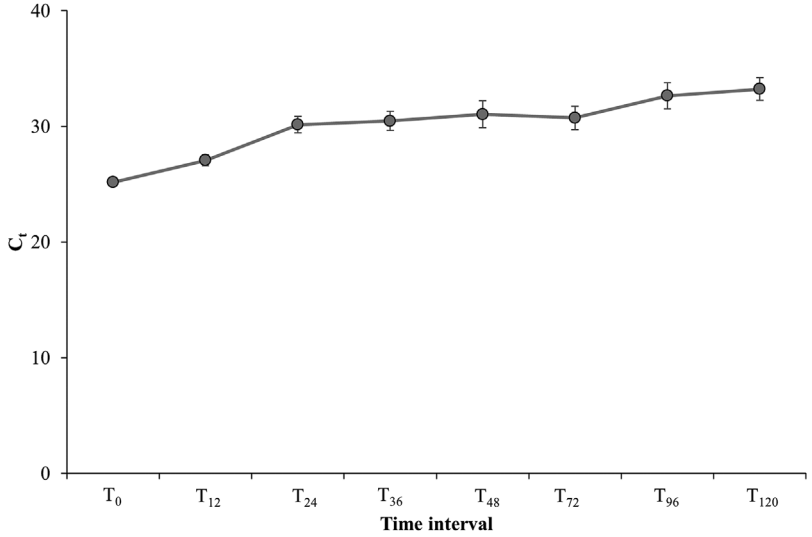

Figure 1. Number of quantitative polymerase chain reaction (qPCR) cycles required to amplify the TAGLNsus product above the fluorescent threshold (C_t) in wild pig fecal samples (Savannah River Site, South Carolina, 9–24 April 2015) versus number of hours fecal pellets were in the environment prior to sampling. Error bars correspond to 1 standard error around the average number of cycles per time period (T_0, T_{12}, T_{24}, T_{36}, T_{48}, T_{72}, T_{96}, and T_{120} hours).

All 17 loci were polymorphic with N_A ranging from 3 to 8 alleles/locus (Table 1). Observed heterozygosities ranged from 0.350 to 0.786. Probability of identity values were similar to other studies using noninvasively collected fecal DNA (Brinkman et al. 2010, Ebert et al. 2012), and over all loci, provided considerable power to discern individuals ($P_{ID} = 2.862 \times 10^{-14}$, $P_{SIB} = 2.485 \times 10^{-6}$). Based on the low error rates and high polymorphism of the screened microsatellites, we consider this suite of loci highly useful for CMR of wild pigs.

Fecal DNA Degradation

We extracted 150 fecal samples out of the potential 160 sampling events (10 individuals, 2 replicates per time period including T_0). Physical degradation prevented sampling of both replicates (A and B) for individuals #5 and #9 after 72 hours and 96 hours, respectively, resulting in a loss of 10 fecal samples. The ability to extract quality DNA from fecal samples decreased with time as evidenced by the qPCR and microsatellite results. All but 2 samples (148/150) successfully amplified in qPCR, with C_t values ranging from 23.194 to 43.104 from T_0 to T_{120} (Fig. 1). We observed an average increase of 7.648 (SD = 4.984) cycles/amplification to reach the threshold across the 5 days. Additionally, C_t values became more variable across the 20 fecal samples (10 individuals, 2 replicates each) after T_0. The linear mixed effect model indicated C_t values increased with number of hours, and the full model ($R^2_{marginal} = 0.467$, $R^2_{conditional} = 0.641$) provided a better fit than the reduced models (Table 2). In addition to time, 17.4% of the variation in degradation was due to variance among individuals and replicates (i.e., $R^2_{conditional} - R^2_{marginal} = 0.174$), which was surprising given the relatively constant weather over the 5 days.

Across the 17 microsatellite loci, amplification failures ($n = 271$) occurred more often than genotyping errors ($n = 69$) in 2,550 total genotypes (Fig. 2). In general, both failures to amplify and genotyping errors increased with time, especially after 72 hours (Fig. 2; Table S2, available online at www.onlinelibrary.wiley.com). Locus FH1589 had the highest success rate (141/150 correct genotypes; 94.23% correct) with 2 cases of genotyping errors (both false alleles) and 7 failures to amplify. In contrast, loci S0226 and Sw949 (121/150 correct genotypes each) had the lowest success rate among the 17 microsatellite loci; S0226 had the most cases of allelic dropout (7/150 total genotypes), whereas Sw949 had the highest failures to amplify (23/150 total genotypes).

The results from analysis of qPCR and microsatellite loci were congruent because samples with larger C_t values also failed to amplify or had genotyping errors with the

Table 2. Model selection results for linear mixed models examining the relationship between fluorescent threshold (C_t) values and 3 predictor variables: hours (time since sample collection) as a fixed effect and 2 random effects associated with the intercepts and slopes of replicates nested inside individual fecal samples. Fecal samples were from wild pigs in Savannah River Site, South Carolina, April 2015. Including a random intercept allows intercepts for both replicates (A and B) and individuals (1–10) to vary within the model, whereas a random slope allows slopes for both replicates and individuals to vary. Model selection with corrected Akaike's Information Criterion (AIC_c) indicated that the full model was the top model because it was the only model with a $\Delta AIC_c < 2.0$. We provide number of parameters (K), likelihoods, and model weights for each putative model.

Model	K	AIC_c	ΔAIC_c	Likelihood	Weight
Hours + random intercept + random slope	8	−275.087	0.000	1.000	0.911
Random intercept + random slope	7	−270.434	4.652	0.098	0.089
Hours + random intercept	6	−245.950	29.137	0.000	0.000
Random intercept	5	−178.694	96.391	0.000	0.000

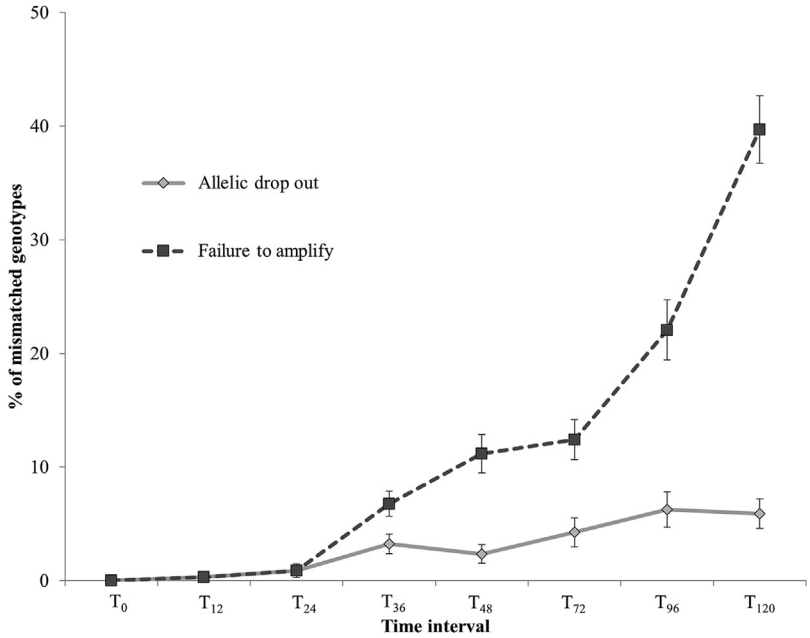

Figure 2. Average percentage of mismatched genotypes in wild pig fecal samples captured on the Savannah River Site, South Carolina (9–24 April 2015) across the suite of 17 microsatellite loci. Error bars correspond to standard errors around each percentage of mismatched genotypes for each time period (T_0, T_{12}, T_{24}, T_{36}, T_{48}, T_{72}, T_{96}, and T_{120} hours).

microsatellites (Fig. 3). Samples that did not amplify or had genotyping errors had higher average number of cycles than those with correct genotypes in all loci (all $U > 114$, all $P < 0.001$). Nonetheless, numbers of mismatched genotypes across the 17 loci were small (9–29 mismatched genotypes) compared to numbers of accurate genotypes across loci (121–141 correct genotypes).

DISCUSSION

Pilot studies like this investigation can provide critical insight for future CMR studies, particularly for methods to minimize genotyping errors that can lead to biased counts of recaptured individuals. We identified a suite of 17 microsatellite loci that exhibit high polymorphism and robust amplification success (>95% in fresh fecal samples) with low genotyping errors. Like all other studies using fecal DNA, the majority of our loci had some error, but with sufficient replication, we obtained accurate genotypes for all individuals within the 17 loci. Different recommendations exist for carrying out replication with low quality DNA samples like feces (e.g., multiple-tubes approach and modified versions; Taberlet et al. 1996, Frantz et al. 2003), but based on our data, ≥3 amplifications per extraction are needed for homozygotes to rule out allelic dropout. In contrast, false alleles were very rare, so a lower number of 2 amplifications is likely sufficient for

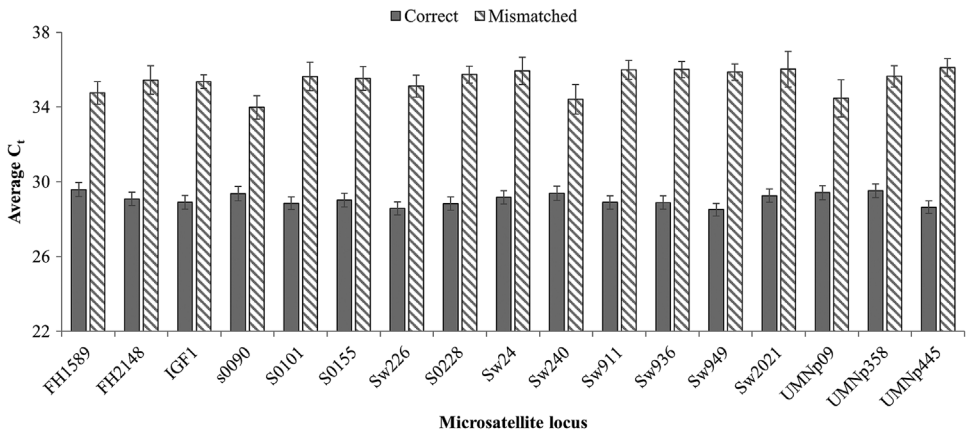

Figure 3. Average fluorescent threshold (C_t) values that occurred in wild pig fecal samples (Savannah River Site, South Carolina, 9–24 April 2015) that either had correct (solid gray) or mismatched (striped gray) genotypes across all 17 microsatellite loci. Correct genotypes correspond to those that matched the genotypes of the corresponding tissue samples for each individual pig. Error bars are standard errors around each average C_t values.

heterozygote genotypes. Another important finding from our screening is that we obtained baseline error rates for each locus. Pairing baseline error rates (i.e., those found in fresh fecal material) combined with techniques like McKelvey and Schwartz (2004) can aid in identifying problematic loci, and thus, provides another way to prevent errors, especially in conditions expected to degrade DNA and increase errors.

Based on our degradation study, we found relatively high amplification success (70–90% per locus) until 3 days. After 3 days, both C_t values and mismatched genotypes increased, but the majority of samples still yielded accurate genotypes in the tested microsatellites after 5 days (45–75% correct genotypes). We acknowledge that our method of squeezing feces out of the rectum may not perfectly mimic defecation, and therefore, may represent a best case scenario for degradation rates in wild pig feces. Multiple studies that collected relatively fresh fecal samples (<7 days) within warm, moist environments report relatively similar amplification rates to our microsatellites after 5 days (e.g., tigers [*Panthera tigris*]: 60%; Bhagavatula and Singh 2006, red wolves [*Canis rufus*]: 50%; Adams et al. 2007, chimpanzees [*Pan troglodytes*]: 46%; Arandjelovic et al. 2011). Certainly other species, locus, and habitat-based factors contribute to variation between studies, but we believe our results are realistic given these similar rates in amplification success.

Another important consideration for CMR studies is that individual variation accounts for a large portion of the variance in the C_t values over time. We captured pigs from across SRS, so it is likely that these individuals had different diets and other intrinsic factors that affected individual variation. It is not unusual to observe high variance between individuals because other factors besides climate may degrade DNA (e.g., diet and resultant inhibitors; Huber et al. 2002, Murphy et al. 2003, Maudet et al. 2004). Regardless, researchers in the field will likely have little control over what types of individuals they sample through fecal DNA (i.e., sex or sub-adult vs. adult), so minimizing other factors that can be controlled, such as time, is especially important to ensure quality genotypes for CMR studies.

An obvious solution to controlling time since defecation in wild pig samples is to only collect fresh feces. However, we were not able to find any reliable method to distinguish younger feces (i.e., <2 days) from older feces unless they had obvious signs of decomposers (e.g., fungus or substantial physical damage from insects). This inability to age feces will likely be problematic for studies based on opportunistic sampling, unless researchers have substantial prior knowledge on pig behavior as seen in other elusive species (Mondol et al. 2009, De Barba et al. 2010). One potential method to age feces is to conduct frequent transects across a study area. The initial sampling period would likely contain all types of samples, but clearing areas prior to sampling is probably not feasible. To offset the inevitable older samples within an initial transect, collecting feces along the same transects <5 days later should ensure the majority of subsequent samples are fresh (i.e., <5 days). Like most CMR studies regardless of the species (Adams et al. 2003, Mondol et al. 2009, De Barba et al. 2010, Hedges et al. 2013), field surveys would be required to identify areas that are conducive to clearing prior to collection of feces.

Following field collection, a highly useful step to identify fecal samples that likely will yield adequate DNA for robust amplification of microsatellite loci is to incorporate qPCR screening prior to genotyping. Our qPCR results supported the microsatellite results and previous studies (Morin et al. 2001, Ebert et al. 2012); samples with higher C_t values were more likely to produce mismatched genotypes. Additionally, samples that failed to amplify in our study averaged 35.423 cycles across all loci, which was similar to the cut-off reported in Ebert et al. (2012) of 32 cycles. By screening samples using this qPCR approach, researchers can avoid costly genotyping of low quality samples across multiple loci. This process may be particularly important for samples that experience rain because although they may not appear physically degraded, the performance of these samples is likely to be much lower (Farrell et al. 2000, Piggott 2004, Murphy et al. 2007, Brinkman et al. 2010).

MANAGEMENT IMPLICATIONS

With the considerable effort focused on controlling wild pig numbers throughout their introduced range, CMR studies using fecal DNA has potential to aid wildlife managers in understanding the population dynamics of wild pigs. For example, CMR studies have proven effective in estimating population sizes for evaluation of hunting regimes designed to control numbers in Germany (Ebert et al. 2012). The microsatellite suite identified in this study has power to discern individuals, which makes them able to estimate parameters such as abundance, density, and relatedness of wild pig populations. In particular, we recommend 9 loci for CMR studies because they had the lowest P_{ID} (FH2148, Sw949, and Sw226), lowest error rates (S0228 and S0155), or highest performance in the degradation study (FH1589, Sw911, Sw2021, and UMNp358). All these estimations require accurate genotypes to produce unbiased results, so we recommend the following steps for pig fecal samples: 1) conduct frequent sampling along transects (at most every 5 days) to ensure fecal samples are within 5 days of deposition in the absence of rain; 2) focus on sampling feces that are still round and easily discernable from the soil; 3) use screening via qPCR to remove low quality samples before genotyping; and 4) conduct repeated amplifications (2–4 per sample based on our study) to verify genotypes. With these steps, fecal samples can produce robust genotypes, which can help answer many questions that will aid in the research and management of wild pigs across their introduced range.

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