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Development of a Genotyping Protocol for Mojave Desert Tortoise Scat

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ABSTRACT. – Noninvasive fecal genotyping can be a useful tool for population monitoring of elusive species. We tested extraction protocols on scat samples from the threatened Mojave Desert tortoise, *Gopherus agassizii*, to evaluate whether scat-based mark–recapture and population genetic monitoring studies are feasible. We extracted DNA from *G. agassizii* scat samples collected in California and Nevada using several extraction protocols and evaluated the reliability of resulting genotypes using quality scores, maximum likelihood reliability estimates, and paired scat and blood genotypes from the same individuals. Finally, we assessed probabilities of identity and sibship, and locus amplification quality, and calculated genotyping error rates for 19 microsatellite loci to determine the best set of loci to use with *G. agassizii* scat extractions. We found that genotype quality depended more on the sample quality than on the extraction method, and that the Qiagen DNeasy Plant Mini extraction kit is an efficient method for extracting tortoise DNA from tortoise scat. We identified 6 *G. agassizii* microsatellite loci that can be used to generate a unique molecular tag for individual tortoises. We characterized the reliability of an additional 13 microsatellite loci for use in population genetic analyses where additional power at the expense of some increase in error may be advantageous. As proof of concept, with very low error rates, we matched 3 opportunistically collected scat samples to blood genotypes from animals captured during population surveys within the study area and discovered at least 3 new individuals, even after 2 yrs of extensive survey work. These results suggest that genotyping of field-collected scat can complement existing methods used in long-term demographic and movement studies of *G. agassizii* and other, closely related, tortoise species.

KEY WORDS. – California; *Gopherus agassizii*; genetics; microsatellites; Nevada; noninvasive sampling

The Mojave Desert tortoise, *Gopherus agassizii*, occurs in the Mojave Desert and in parts of the Sonoran Desert of the United States (US Fish and Wildlife Service [USFWS] 2011; Edwards et al. 2015). Because of decreases in population densities, the Mojave population of *G. agassizii* was listed as threatened under the federal Endangered Species Act in 1990 and also receives state protection in California, Nevada, Arizona, and Utah (USFWS 1990). Numerous factors threaten *G. agassizii* including loss, degradation, and fragmentation of habitat due to an expanding human footprint throughout its range (USFWS 2011). Currently, populations are monitored using traditional methods such as plot surveys, radiotelemetry, and genetic samples (i.e., blood) collected when animals are handled. However, these elusive herbivores can be difficult to detect because of their cryptic appearance, behavior, and propensity to use burrows. This results in many animals being missed during surveys (Freilich et al. 2000; Anderson et al. 2001; Nussear and Tracy 2007; Nussear et al. 2008). Therefore, to complement current methods implemented for studying *G. agassizii* populations, we explored the potential of using scat samples, which can be opportunistically collected during other survey efforts, to yield genetic data for monitoring.

Noninvasive scat sampling and individual identification through genotyping have become useful tools for population monitoring of wildlife species (Schwartz et al. 2007). Scat-based genotyping can provide information on individual movement, home range, relatedness, abundance, food habits, parasite load, and sex ratios (Waits and Paetkau 2005; Schwartz et al. 2007; Beja-Pereira et al. 2009; Luikart et al. 2010). Using scat-based genetic sampling without the need to handle individual animals is appealing for studies of elusive, rare, or endangered species (Piggott et al. 2008; Giambattista and Gentile

2018). While monitoring mammals using scat-based genetic methods has become fairly common (e.g., Piggott et al. 2008; Mitelberg and Vandergast 2016; Arandjelovic and Vigilant 2018), these methods have not been applied as often to herpetofauna (but see Pearson et al. 2015; Giambattista and Gentile 2018).

Scat-based genotyping must account for higher rates of genotyping errors such as allelic dropout (ADO), which is the failure to amplify one of the two true gene copies present at a locus, and false alleles (FA), which can arise through the amplification of spurious polymerase chain reaction (PCR) fragments (Taberlet et al. 1999; Bonin et al. 2004; Pompanon et al. 2005). These errors can result from degradation due to environmental exposure (Murphy et al. 2007; Brinkman et al. 2010; Panasci et al. 2011) and the presence of inhibitory compounds in scat (i.e., complex polysaccharides, bile salts, lipids, urate) that can interfere with sample extraction and PCR (Schrader et al. 2012). Genotyping errors have the potential to bias the conclusions of individual-based analyses, such as population size estimates (Waits and Leberg 2000) and parentage analyses (Gagneux et al. 1997), and can also influence population-level analyses (Pompanon et al. 2005). Therefore, it is important to conduct a pilot study to determine if reliable and informative DNA fingerprints can be obtained from *G. agassizii* scat.

In this study, we evaluated whether genetic monitoring using scat was feasible for *G. agassizii*. Specifically, we addressed 3 main questions. First, could a reliable multilocus genotype that is adequate for individual identification (a molecular tag) be obtained from DNA extracted from *G. agassizii* scat? Second, of the extraction protocols evaluated in the study, which was the most reliable and cost/time efficient? And third, which microsatellite loci were the most informative for population genetic analyses while limiting genotyping error? To address these questions, we tested 6 extraction protocols, evaluated the quality of genotypes derived from these extractions using several quality metrics, and estimated the cost of these methods. The extraction protocols tested in this study can be applied in long-term demographic and movement studies of *G. agassizii* and could extend to other, closely related, tortoise species.

METHODS

Collection and Storage. — We used scat and blood samples obtained from *G. agassizii* individuals, the majority of which were collected within the Ivanpah Valley in California and Nevada between March 2016 and October 2018 (Fig. 1). Paired scats and blood were collected directly from animals handled in the field for radio-tracking, marking, and health assessments. Additionally, to test the utility of genotypes obtained from field-collected tortoise scat, 11 fresh scats (recently deposited with sheath shiny, smooth, and intact) from unknown animals were collected opportunistically from the ground during the same sampling period.

Blood samples were stored at room temperature on Whatman cards. Scat samples were collected either in closed, 50-ml tubes with desiccating beads or in individual, non–air-tight plastic boxes or paper lunch bags and allowed to dry outdoors overnight (samples in boxes were dried with lids open). Increased air contact appeared to inhibit fungal development. A similar collection method has worked well in studies genotyping mule deer scat (Bohonak and Mitelberg 2014; Mitelberg and Vandergast 2016). To simulate the approximate age and quality of samples that would be collected in the field, a subset of 3 fresh samples were additionally exposed to 7–9 d of full sun. Scat samples were stored dry in the laboratory at room temperature and extracted within 3 wks of collection.

Blood Extractions. — All blood extractions were performed with the Qiagen DNeasy Blood and Tissue extraction kit, according to the manufacturer-provided protocol, with these minor modifications to improve yields: following addition of Buffer AL, samples were incubated at 70°C for 10 min; elution volume was halved to 100 µl, and the elution step was performed twice, for a total of 200 µl final elution volume.

Scat Extractions. — We experimented with 6 scat extraction protocols consisting of combinations of different pre-extraction surface washes, extraction kits, and postextraction clean-up kits to determine if any of these improved quality (Table 1). Five of these extraction protocols used the Qiagen QIAmp Fast DNA Stool Mini extraction kit (hereafter stool kit) on surface-washed cells, and one protocol used the Qiagen DNeasy Plant Mini extraction kit (hereafter plant kit) on whole, homogenized scat. Scats were split in half prior to extraction to increase sample sizes across extraction protocols and to provide paired samples for protocol comparisons. To minimize opportunities for contamination, we used new disposable gloves for each scat sample and performed all extraction steps (with the exception of centrifugation) under a PCR hood.

Stool Kit with Surface Wash and Clean-Up Steps. — Scat surface washes utilized Inhibitex buffer (included in the stool kit) by either 1) placing the scat segment into a 50-ml centrifuge tube with buffer and agitating on a nutating rocker for 10–15 min ("tube surface wash"; Table 1), or 2) placing the scat in a weighing dish with buffer and leaving the dish on the nutating rocker for 10–15 min ("dish surface wash"; Table 1). Following washes, remaining buffer and epithelial cells were transferred by pipette to 2-ml centrifuge tubes and extracted with the stool kit following the manufacturer's protocol. As an additional measure for removing PCR inhibitors from extracted DNA, an aliquot of each extraction was processed through either the OneStep PCR Inhibitor Removal kit (Zymo) or the Genomic DNA Clean & Concentrator-10 kit (Zymo). We followed

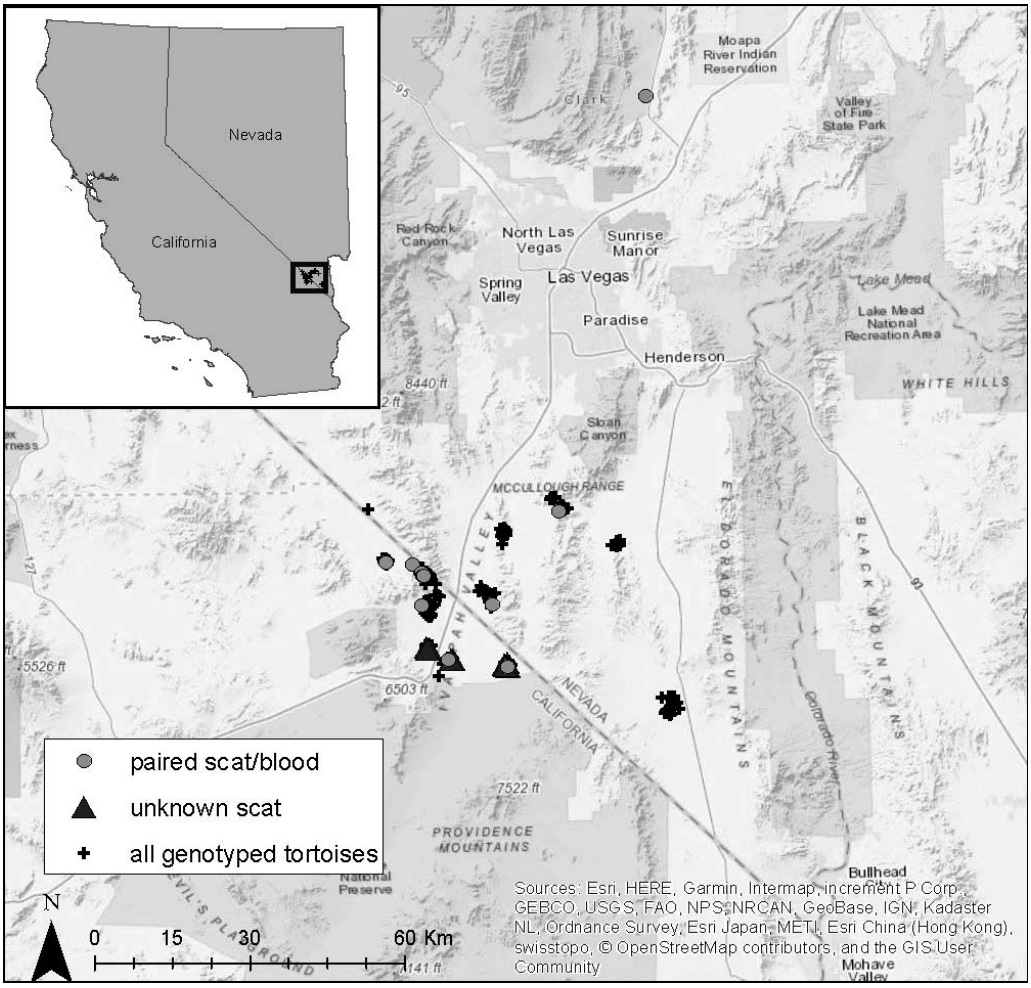

Figure 1. Study area in the Mojave Desert at the border of California and Nevada, USA. Locations of paired *Gopherus agassizii* scat and blood samples are shown as dots and unknown, opportunistically collected scats as triangles. Locations of all captured and genotyped tortoises used as reference samples are shown as black crosshatches.

manufacturer's protocols for the OneStep kit and made the following modifications to the Clean & Concentrator-10 kit: a 1-min spin step was added following the use of Wash Buffer (Zymo) to facilitate removal of all ethanol, and two sequential elution steps with 10-min incubation periods were performed using 20 µl warmed (60°C–70°C) Elution Buffer (Zymo).

Plant Kit Extractions. — The DNeasy Plant Mini kit (plant kit) includes steps to shear cell walls and reduce PCR inhibitors often present in plant tissues, which could be useful given the herbivorous diet of the desert tortoise. We cut off a small piece (< 300 mg) of each scat sample (making sure to include material from the surface and interior of the scat) and extracted the piece using the manufacturer's protocol. For a

Table 1. Extraction protocols including pre-extraction surface wash method, extraction kit, and clean-up kit (if used), number of *Gopherus agassizii* scat samples extracted, approximate time, cost, and number of steps per protocol.

Extraction protocol	Pre-extraction surface wash	Qiagen extraction kit	Zymo clean-up kit	No. of samples	Approx. time per sample (min)	Extraction protocol cost/sample (kits and reagents only) (US dollars)	No. of steps
1	Tube	Stool	None	4	45	5.38	15
2	Dish	Stool	None	6	45	5.38	15
3	Dish	Stool	OneStep	3	50	7.38	18
4	Tube	Stool	Clean & Concentrator	10[a]	55	6.88	21
5	Dish	Stool	Clean & Concentrator	11[b]	55	6.88	21
6	None	Plant	None	16[a,b]	40	4.58	13

[a] 8 scats were split and paired between protocols 4 and 6.
[b] 8 scats were split and paired between protocols 5 and 6.

paired comparison of the plant and stool kit protocols, the remainder of each of these scat samples was extracted with stool kit protocols 4 or 5 (Table 1).

Amplifications. — Blood and scat DNA extractions were amplified at either 6 or 19 microsatellite loci available for *G. agassizii* (Edwards et al. 2003; Schwartz et al. 2003; Hagerty et al. 2008). We assessed each locus individually in single-locus, 10-µl PCR reactions containing 5 µl 2× Multiplex PCR Plus cocktail (Qiagen), 1 µl 10 µM primer, 2.5 µl water, and 1.5 µl DNA (blood extractions were standardized to 4 ng/µl). Thermocycler conditions were as follows: enzyme activation for 5 min at 95°C followed by 30 and 40 cycles for blood and scat, respectively, of 30 sec at 95°C, and 3 min at 56°C, 45 sec at 72°C, with a final elongation at 68°C for 30 min. One microliter of PCR product was aliquoted into 10.5 µl Hi-Di™ formamide (Thermofisher) with 0.5 µl GeneScan™ 500 LIZ® size standard (Thermofisher) and was submitted for genotyping to Eton Bioscience (San Diego, CA). GeneMarker v.1.90 was used to score chromatographs. Negative controls were included with each round of PCR to monitor contamination and all PCR reactions were prepared under a PCR hood.

Deriving Consensus Genotypes. — To determine whether a reliable molecular tag could be obtained from *G. agassizii* scat, we amplified all extractions at a subset of 6 microsatellite loci yielding the shortest PCR products (referred to as screening loci from this point on). Loci with shorter amplicons generally have higher amplification and lower error rates (Frantzen et al. 1998; Broquet et al. 2006). Additionally, successful genotyping of at least 5 of these 6 loci satisfies the recommended minimum threshold for probability of identity ($P_{SIB} \leq 0.01$; Waits et al. 2001). Each scat extraction was genotyped 3 times at each locus, with extractions from the same scat sample before and after postextraction clean-up being treated as separate extractions. Because each scat sample was split in half and subjected to 2 different extraction treatments, this yielded a total of 6 replicate PCRs for each scat sample.

To arrive at an "extraction consensus genotype", we used the following guidelines of Frantz et al. (2003): 1) for heterozygotes, each allele must be present at least twice among replicate PCR reactions; and 2) for homozygotes, the allele must be present at least 3 independent times and all 3 times as a homozygote (to eliminate the possibility of ADO). In addition to a consensus genotype for each extraction, we were able to determine a "sample consensus genotype" for each scat sample using all 6 replicate amplifications. To arrive at the sample consensus genotype, we used the same rules as for the extraction consensus genotype.

Assessing Genotype Quality. — We used three approaches to estimate genotype quality across extractions, samples, and loci. First, we calculated genotype quality (Q-score) following Miquel et al. (2006). At each locus, genotypes were compared with the consensus genotype for that sample and a score of 1 was assigned for that replicate in the case of a match; all other genotypes (ADO, FA, PCR failure [FAIL], and lack of consensus genotype [UNK]) were assigned a score of 0. Q-scores were calculated for each extraction and sample, at each locus, by averaging scores for replicate genotypes. Second, we used the maximum likelihood software RELIOTYPE (Miller et al. 2002), which uses repeated amplifications to determine the probability that samples met certain reliability thresholds. We used default settings (95% reliability, 200 bootstrap replicates) and allele frequencies estimated from a larger sample of 159 tortoises collected throughout the Ivanpah Valley in 2016–2017 and genotyped from blood (Dutcher et al., in press). We applied the reliability criteria to the entire data set, limiting the incidence of false acceptances to less than 5% with 95% probability, and retained all instances of FA, as the focus of this study was to document potential issues with genotyping because of poor DNA quality. Samples accepted without further PCR replicates were deemed as suitable quality samples (SQS). Finally, for a subset of samples for which we had paired scat and blood samples available, we directly compared sample consensus genotypes of scat samples to their respective blood genotypes.

Evaluation of Scat Extraction Methods. — To evaluate extraction protocols, we compared Q-scores, cost, time, and steps involved in each extraction protocol. We used box plots to visualize Q-scores for the 6 extraction protocols we evaluated. We used a paired Wilcoxon signed-rank test to determine if there was a significant difference in Q-scores between samples extracted with the stool and plant kits. We limited this comparison to 16 samples that were divided and extracted with both kits (protocols 4, 5, and 6; Table 1).

Locus Suitability for Genotyping G. agassizii *Scat*. — To explore additional loci that may be useful for genotyping *G. agassizii* scat samples, we further genotyped 8 scat and 3 blood extractions at 19 loci, with 5 PCR replicates per locus. For scat, we limited this experiment to samples that were extracted with the plant kit (protocol 6; Table 1) and were deemed SQS samples when assessed at the 6 screening loci as described above. We calculated Q-scores and analyzed reliability with RELIOTYPE. We calculated error rates for all 19 loci, following Broquet and Petit (2004), based on consensus or blood genotypes when available (in the case of 3 of 8 scat samples). We visualized average Q-scores for loci using box plots. To assess our power to reliably identify individual tortoises from scat samples, probabilities of identity (P_{ID}) and probabilities of sibship (P_{SIB}) were calculated for these 19 microsatellite loci using allele frequencies estimated from the larger (159 sample) data set (Dutcher et al., in press).

Identifying Opportunistically Collected Scat. — To further test the utility of scat-derived genotypes, we conducted an identity analysis in CERVUS (v. 3.0.7; Kalinowski et al. 2007). We tested whether any of the opportunistically collected scat samples with SQS genotypes matched any tortoises genotyped from blood

Table 2. Quality of 25 *Gopherus agassizii* scat samples genotyped at 6 screening loci using 6 replicate PCR amplifications. Scat samples marked with E were exposed to 7–9 days of full sun. Quality metrics (averaged across loci) including Q-scores and reliability (per RELIOTYPE) are shown. Whether or not the sample met SQS (suitable quality sample) conditions, percent loci where a consensus genotype could be determined, and percent loci that matched paired blood genotypes are included. NA = not applicable for scat samples with no matching blood samples. Eleven samples had average Q-scores above 0.6 and met SQS standards without further replication.

Scat sample	Tortoise ID	Scat sample Q-score	Scat sample reliability	SQS	Percent loci with consensus genotype	Percent loci with matching blood genotypes
2017F_13	CN019	1.00	1.000	Yes	100	100
2017S_03	CN720	0.94	1.000	Yes	100	100
2017F_03B	BS589	0.92	1.000	Yes	100	100
2017F_03A	BS589	0.89	1.000	Yes	100	100
2016S_01	SS1067	0.89	1.000	Yes	100	100
2017S_01	CN605	0.86	1.000	Yes	100	100
2017S_06E	CN748	0.50	0.892	No	67	50
2017S_04E	CN507	0.36	0.860	No	50	50
2016S_02	FW6752	0.36	0.993	No	67	83
2017S_02	CN716	0.39	0.994	No	83	17
2017S_05E	CN704	0.06	0.173	No	17	17
2016S_03	CN812	0.03	1.000	No	17	17
2017F_06	Unknown	0.97	1.000	Yes	100	NA
2017F_01	Unknown	0.83	0.997	Yes	100	NA
2017F_08	Unknown	0.72	1.000	Yes	100	NA
2017F_09A	Unknown	0.69	0.999	Yes	100	NA
2017F_09B	Unknown	0.64	0.994	Yes	100	NA
2017F_02	Unknown	0.47	0.921	No	100	NA
2017F_05	Unknown	0.39	0.481	No	83	NA
2017F_04	Unknown	0.39	0.167	No	50	NA
2017F_11A	Unknown	0.36	0.852	No	100	NA
2017F_11B	Unknown	0.31	0.585	No	83	NA
2017F_10	Unknown	0.22	0.863	No	67	NA
2017F_12	Unknown	0.11	0.110	No	33	NA
2017F_07	Unknown	0.00	NA	NA	0	NA

samples in Ivanpah Valley to date (309 individuals; Dutcher et al., in press). For this analysis, we used allele frequencies from this larger data set, allowing up to 1 mismatching locus to avoid excluding matches due to genotyping error.

RESULTS

Quality of G. agassizii *Scat Sample Genotypes at Screening Loci*. — Overall, we evaluated 11 blood samples and 25 scat samples (in 50 extractions). Of 25 total scat samples, RELIOTYPE identified 11 (44%) as SQS samples (Table 2). Nine of 11 SQS samples qualified with all 6 loci genotyped. Two samples (2017S_01 and 2017F_09A) qualified with a 5-locus genotype. For these samples, locus GOAG4 was not evaluated by RELIOTYPE due to the presence of aberrant (previously unrecorded) alleles in at least one of the replicates. Of the 16 samples which were either field collected (with unknown identity) or for which field collection was simulated by exposure to field conditions (Table 2; samples identified with "E" in sample name), 5 (31%) were deemed SQS samples. Of the 9 fresh samples collected directly from animals being handled (and without exposure to field conditions), 6 (67%) were found to be SQS samples. SQS samples had average Q-scores that ranged from 0.64 to 1.

All SQS samples with average Q-scores ≥ 0.6 matched their blood genotypes at all loci, suggesting that samples that meet these quality criteria should have accurate genotypes (Table 2). In 3 samples that failed to meet the SQS and Q-score criteria above, we found 6 mismatches between the sample consensus genotype and the blood genotypes (Table 3). Five of these errors were identified as false alleles and one was a case of large allelic

Table 3. Mismatches between *Gopherus agassizii* scat and blood-derived genotypes (presented as allele sizes) and inferred error type. LADO = large allele dropout; FA = false allele.

Scat sample	Blood sample	Locus	Scat genotype	Blood genotype	Q-score	Error type
2017S_06E	CN748	GOA8	163/163	163/171	0.50	LADO
2017S_02	CN716	GOA2	207/210	210/213	0.83	FA
2017S_02	CN716	GOA12	148/152	111/152	0.17	FA
2017S_02	CN716	GOAG4	168/180	168/170	0.67	FA
2017S_02	CN716	GP30	207/211	207/207	0.33	FA
2016S_03	CN812	GOA12	142/158	111/142	0.17	FA

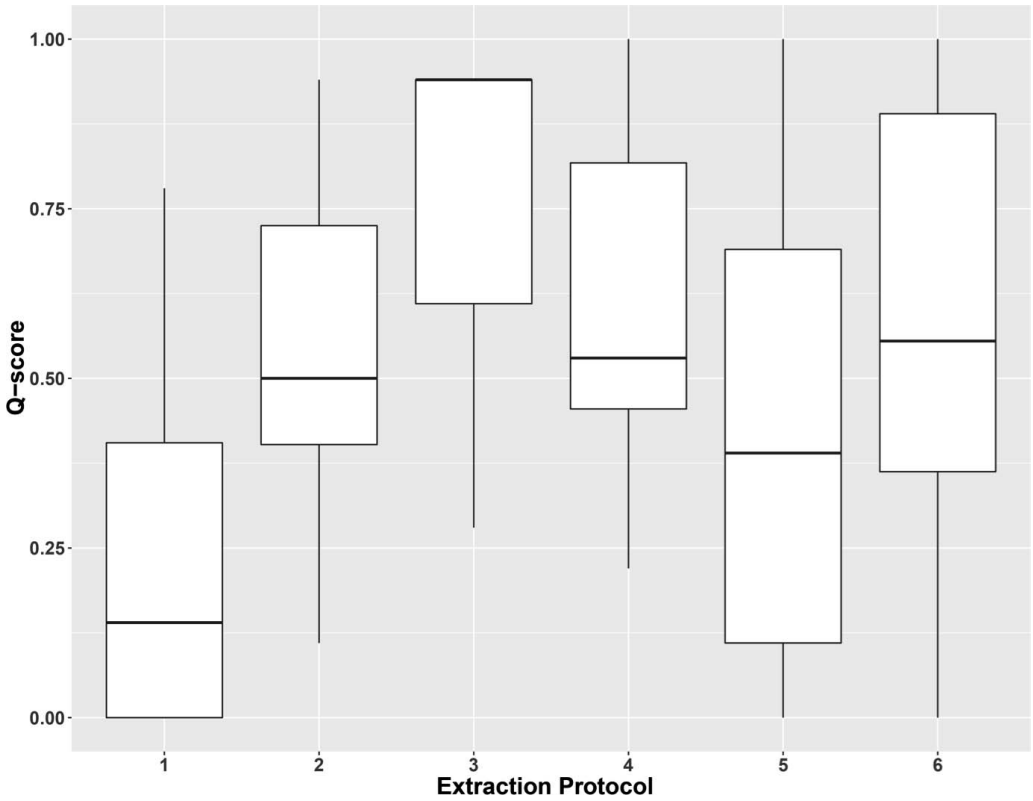

Figure 2. Box plots of the distributions of average Q-scores for the 6 scat extraction protocols tested on *G. agassizii* scat samples. Lines within boxes are placed at the median and the boxes extend between the 25% and 75% quartiles. Whiskers extend to the lowest and highest values within 1.5* interquartile range (IQR).

dropout (where the larger of two fails to amplify in a heterozygous individual).

Evaluation of Scat Extraction Methods. — Thirty-four scat extractions were performed using the stool kit (with 5 variations) and 16 with the plant kit. All extraction methods had overlapping distributions in average Q-scores, with protocol 1 performing slightly worse than the other protocols (Fig. 2). In addition, we found no significant difference in Q-scores between paired stool kit and plant kit extractions ($W_{15} = 58.5$; $p \leq 0.3815$). The cost and time investment for all stool kit protocols is higher than that of the plant kit protocol. The plant kit has fewer pipetting steps (limiting potential for contamination or operator error), consumes just a small portion of the scat sample (so multiple extractions could be performed to increase DNA yield), and extractions may also be used in the future for *G. agassizii* diet analyses.

Locus Suitability for Genotyping G. agassizii *Scat Extractions*. — For blood extractions genotyped multiple times, we encountered no PCR failures or erroneous genotypes at any of 19 loci (i.e., Q-score = 1.0). Of 8 scat extractions, each with 5 replicate PCR reactions, RELIOTYPE accepted the multilocus genotype of 4 extractions without additional PCR replicates (Supplemental Table S1; supplemental material is available at https://doi.org/10.2744/CCB-1394.1.s1). One of these samples qualified with a 14-locus genotype, 2 samples qualified with 18-locus genotypes, and 1 sample qualified with a 19-locus genotype. Error rates ranged from 0% to 47.4% for ADO and 0% to 5.9% for FA. Average Q-scores ranged from 0.23 for Locus GP81 to 1.0 for Locus GOA1 (Fig. 3; Supplemental Table S1). The 3 loci with the lowest average Q-scores (< 0.54), also had either exceptionally low successful allele call rates or low genotype accuracy rates. The cumulative probabilities of identity (P_{ID}cum and P_{SIB}cum) for the remaining 16 loci were 5.8×10^{-22} and 9.7×10^{-8}, respectively.

Identifying Opportunistically Collected Scat. — Three of the 7 unknown scat samples that met SQS standards were matched unambiguously at all 6 screening loci to previously captured tortoises. All three scats were found within 400 m of where the matching animal was captured. Additionally, we found 2 sets of samples with fuzzy matches (mismatched at 1 locus). One fuzzy match occurred between 2 blood samples taken approximately 8 km apart in different plots in the Ivanpah Valley data set, and so likely indicates these 2 individuals are close relatives. The other fuzzy match occurred between a blood and scat sample located within the same sampling site within 500 m. The 3 remaining high quality unknown scat samples did not match any individuals in our blood samples and likely represented previously unsampled individuals.

DISCUSSION

In this pilot study, we evaluated the feasibility of using *G. agassizii* scat to complement current methods

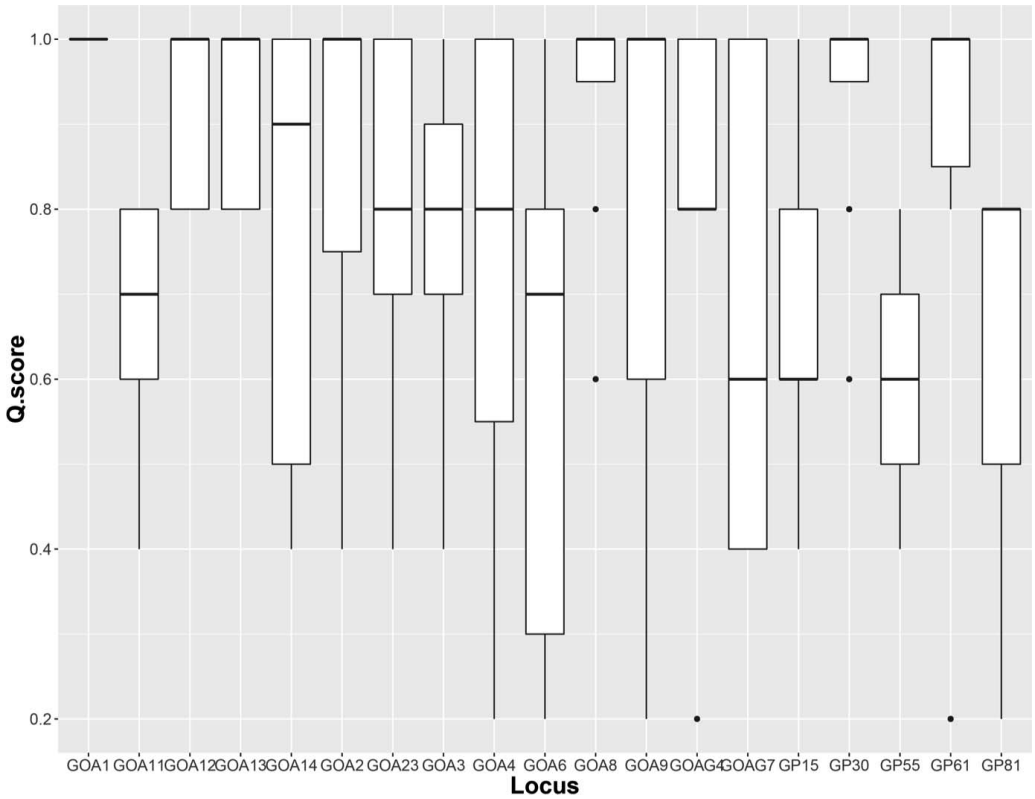

Figure 3. Box plots of Q-scores for 19 loci genotyped for 8 SQS (suitable quality samples) extracted with the Qiagen DNeasy Plant Mini kit. Five replicate PCR amplifications were performed for each *G. agassizii* scat sample and locus. Lines within boxes are placed at the median and the boxes extend between the 25% and 75% quartiles. Whiskers extend to the lowest and highest values within 1.5* IQR.

used to monitor this species. First, we found that a reliable molecular tag can be obtained from DNA extracted from *G. agassizii* scat. Notably, scat samples that met SQS criteria and had average Q-scores > 0.6 also exactly matched blood samples from the same individuals, suggesting that these 2 quality criteria are appropriate for retaining accurate tortoise genotypes from scat. Second, of the 6 extraction protocols evaluated in the study, we detected no differences in extraction quality, but we found the Qiagen DNeasy Plant Mini kit to be the most cost- and time-efficient method. Finally, we found that 16 of 19 loci tested could be genotyped from scat with relatively high confidence for population genetic analyses while limiting genotyping error.

Reliability of G. agassizii *Scat Sample Genotypes*. — As many as 7 amplifications per locus have been recommended to obtain reliable genotypes in noninvasive studies (Taberlet et al. 1999). Statistical approaches such as those employed by RELIOTYPE are designed to reduce replication number, without compromising accuracy, by taking into account data already available (i.e., allele frequencies and data from a limited number of initial replicates), inferring the reliability of the consensus genotype derived from those data and suggesting an appropriate number of additional replicates to arrive at a predetermined confidence level for the multilocus genotype. In our study, we found that the default settings of RELIOTYPE were stringent enough to accept without further replicates only those scat genotypes that matched their respective blood genotypes. We also found that approximately 31% and 67% of scat samples collected from the ground and directly from animals, respectively, yielded DNA of sufficient quality to obtain reliable genotypes. A multilocus genotype of at least 5 loci will yield a molecular tag with P_{SIB} below the recommended threshold of 0.01 (Waits et al. 2001); therefore, these multilocus genotypes can be used as molecular tags for purposes of mark–recapture studies or for population size estimates. As proof of concept, we were able to match several unknown scat samples to genotyped animals in the same plots with low P_{ID} and P_{SIB}.

Evaluation of Extraction Methods. — We observed high variability in genotype quality among samples regardless of extraction protocols. Sample quality is likely the most critical factor in determining whether a reliable genotype can be obtained from *G. agassizii* scat. Edwards et al. (submitted) evaluated a different scat extraction protocol on *G. agassizii* and *Gopherus morafkai* scat; they used epithelial cells swabbed from the surface of scats and reported similar success rates to our study (avg. 35% across field collected tortoise scats). Although we detected no difference in sample quality related to extraction protocol, the plant extraction

protocol has advantages in terms of cost, time, and yield efficiencies. Pearson et al. (2015) found that the Qiagen DNeasy Plant Mini kit performed well for individual genotyping from field-collected lizard scat when alternative extraction protocols optimized on captive lizard scat failed on wild samples. The authors attributed the failure to the diet of wild lizards, which was richer in vegetation than that of captive lizards. In our study, we did not find a significant difference between genotype quality for the subset of samples that were extracted with both the stool and plant kits. This may be because all stool kit extractions were performed on the surface washes, avoiding most of the plant material inside the scat (unlike alternative protocols in Pearson et al. 2015). We also note that, while pelleted herbivore scat lends itself well to surface washes (Flagstad et al. 1999; Wedrowicz et al. 2013), this was not the case with tortoise scat because 1) it is not pelleted, 2) size is variable, and 3) tortoise scat segments are larger and less compacted. At times, scat segments partially crumbled during the wash and absorbed significant amounts of buffer during the wash procedure (up to 4 ml of Inhibitex Buffer per sample). Overall, the plant kit protocol was less expensive and less labor-intensive, took less time, and had fewer steps (potentially reducing contamination risk). Finally, plant kit extractions could also be used to amplify and analyze the tortoise's herbivorous diet, the quality of which has been shown in controlled experiments to be directly linked tortoise health (Drake et al. 2016).

Locus Suitability for Genotyping G. agassizii *Scat Extractions*. — There is a trade-off in the amount and accuracy of information that can be obtained from noninvasively collected samples (Waits and Leberg 2000). For purposes of molecular tagging for estimates of census population size using capture–recapture methods, if the number of loci is too low some individuals will have the same molecular tag, resulting in an underestimate of population size. Conversely, introducing more loci increases the probability for error, which in turn creates false individuals resulting in population size overestimation. Thus, highly polymorphic loci with low genotyping error rates are recommended for studies using molecular tags to obtain estimates of population size (Waits and Leberg 2000). We found the initial 6 screening loci to be a reliable set of loci for molecular tagging, yielding a molecular tag with low probabilities of misidentifying 2 random individuals (P_{ID}) or 2 siblings (P_{SIB}) as the same individual.

In contrast to estimating census population size and paternity, genotyping error at low frequencies may not be as problematic in the estimation of population genetic diversity, differentiation, and effective population size, and the inclusion of more loci can increase precision in these estimates. In simulations, Smith and Wang (2014) found that, with genotyping error rates below 0.2, reasonable estimates of genetic variation and population subdivision could be obtained. Based on our tests of 19 loci for *G. agassizii*, we found that 16 loci could be genotyped with relatively high confidence.

Conclusions. — While sample sizes in this pilot study are small, the results are encouraging enough to warrant pursuing scat genotyping as a viable source of data for *G. agassizii* population monitoring. In particular, detection of 3 "recaptures" of previously genotyped animals indicates that scat genotyping could be used for recapture in regions with a good set of reference DNA samples. Finally, the developed scat extraction and genotyping protocol can be extended across the species range and possibly to other tortoise species for which these microsatellite loci cross-amplify. This reliable, scat-based genotyping method can complement ongoing efforts characterizing desert tortoise populations throughout the Mojave Desert.

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