EVALUATION OF DNA EXTRACTED FROM TIMBER RATTLESNAKE (*CROTALUS HORRIDUS*) CLOACAL AND BLOOD SWABS FOR MICROSATELLITE GENOTYPING

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***Abstract.*—Genetic research is a key component to modern wildlife conservation, but it is contingent on the collection of reliable and high-quality genetic samples. Invasive genetic sampling techniques have potential to negatively impact individuals, which may be prohibitive when working with threatened and endangered species. Prior to sample collection, project managers must try to balance the negative impact on individuals included in the study with the demand for DNA and the difficulty of obtaining samples. Although established methods for blood and tissue collection in reptiles meet the need for high-quantity and quality DNA, they inherently require longer handling times and more skill to obtain. Thus, non-invasive DNA collection methods, such as cloacal swabs, may be preferred when animal welfare is a priority. Cloacal swabs are quicker, easier, require less training and reduce handling time. To evaluate cloacal swabbing as an alternative to collecting blood, we obtained both cloacal and blood swabs. We extracted DNA from cloacal and blood cells that were collected from 23 Timber Rattlesnakes (*Crotalus horridus*). We assessed DNA by purity (A260/A280), concentration, and microsatellite genotyping. Our results show high-quality DNA can be obtained from both cloacal swabs**

and blood samples, but quality and concentration of DNA was significantly lower from cloacal swabs. Further, degradation and contamination affects the performance of cloacal DNA when compared to blood DNA in microsatellite-based genotyping. Although we recommend collecting blood samples whenever possible to obtain the highest-quality DNA, cloacal swabs represent a viable alternative for genetic sampling when using microsatellite loci as genetic markers.

Key Words.— conservation genetics; microsatellites; non-invasive sampling; reptile; snake;

INTRODUCTION

Wildlife conservation programs incorporating population genetics typically seek to use the least invasive DNA collection methods possible to limit the amount of direct contact with the study organism (Taberlet et al. 1999; Smith and Wang 2014). Handling wildlife can have measurable effects on stress levels and behavior (Moore et al. 2000; Schuett et al. 2004). Increases in stress hormones can negatively impact wildlife by depressing immune function that may result in the animal being more susceptible to disease (Lind et al. 2018). Trauma experienced during capture can also change behavior of wildlife. After a traumatic experience, an animal may alter habitat use in the area of capture to avoid future conflict (Germano 2007). The concerns of handling wildlife are amplified when studying threatened and endangered species where the loss or change of behavior of a small number of individuals could have disproportionate impacts on the population. Due to these concerns, the use of non-invasive genetic sampling that eliminates or minimizes handling time is often preferred in conservation genetic research.

Blood is a standard and reliable source of high-quality DNA (Lanci et al. 2012), but its collection can be prohibitive depending on the organism. Previously, conservation genetic projects have successfully used DNA collected from a variety of non-invasive sources. Such non-invasive methods include buccal swabs, cloacal swabs, hair or feathers, fecal samples and environmental DNA (Taberlet et al. 1999; Beja-Pereira et al. 2009). However, if the objective is the identification of fine-scale population genetic patterns, higher quality DNA may be required for genotyping than can be obtained via techniques considered non-invasive (Taberlet et al.

1999). If the quantity of DNA is small, fragmented, or contaminated, genotyping may be hindered due to amplification of false alleles or to allelic drop-out (Pereira et al. 2009). Fine-scale genetic studies of threatened and endangered wildlife populations require both high-quality samples and minimally invasive techniques.

Reptiles represent a particular challenge to conservation genetic studies that require high-quality samples and minimally invasive techniques. Due to minimal access to the main blood vessels in some reptiles, blood extraction methods can require the involvement of multiple technicians or specially trained personnel. This is restrictive when field work is conducted in remote locations or by technicians working independently. Buccal swabs are often used as a way of obtaining DNA from reptiles (Miller 2006; Broquet 2007; Beebee 2008; Schulte et al. 2011; Mucci 2014); however, this method is not practical when dealing with venomous species. Scale clipping is a faster and easier alternative technique to drawing blood, but it is not considered non-invasive. Scale clipping can result in an open wound, leaving the animal susceptible to infection (Weary 1969). DNA can also be successfully extracted from reptile feces (Jones et al. 2008) and shed skin (Fetzner 1999) but the ability to collect these sources from wild individuals is limited. In order to minimize the amount of handling time per individual and number of technicians needed to collect samples in the field, a reliable source of DNA other than blood needs to be identified.

Cloacal swabs have previously been used as a less invasive alternative for providing high-quality DNA for both venomous and nonvenomous squamate reptiles (Ford et al. 2016). The collection of cloacal swabs is comparable to the ease in which buccal swabs can be collected

but the method is applicable to a wider range of species since it can safely be conducted on venomous animals. Although cloacal swabs provide an alternative source of DNA in reptiles, DNA contamination and degradation is of concern (Miller 2006). DNA extracted from mucosal swabs, such as cloacal and buccal swabs, is likely unsuitable for use in genomics research that requires long, continuous reads of DNA due to potential contamination and degradation. Despite these limitations for genomic studies, researchers have successfully amplified microsatellite loci from DNA extracted from reptile buccal and cloacal swabs (Miller 2006; Lanci et al. 2012; Mucci 2014). Because microsatellite loci are short fragments of DNA (< 600 base pairs), successful amplification is less dependent on high-quality, contiguous template DNA.

To meet the need for a DNA collection method that eliminates the invasive and technically challenging process of extracting blood, we compared the performance of DNA extracted from blood and cloacal swabs in a fragment analysis genotyping experiment in an endangered population of Timber Rattlesnakes (*Crotalus horridus*) in Ohio. While historically widespread throughout Ohio, Timber Rattlesnake populations have declined and disappeared throughout the state due to habitat loss, fragmentation, and human persecution. They are now only known from small, isolated populations in southeastern Ohio (Ohio Department of Natural Resources [ODNR] Division of Wildlife 2012). We currently know nothing about the genetic diversity of the few remaining Timber Rattlesnake populations in Ohio. Given that Timber Rattlesnakes are both dangerous to handle and locally endangered, a non-invasive DNA collection procedure is vital to the future conservation and management of this state endangered species.

MATERIALS AND METHODS

***Sample Collection.*—**We collected DNA samples from 23 Timber Rattlesnakes captured in Vinton-Furnace State Experimental Forest in Vinton County, Ohio. For each individual, we collected both a blood and cloacal swab, and duplicate cloacal and blood swabs were taken from four snakes for a total of 54 genetic samples. We used sterile wooden swabs with cotton buds (Hardy Diagnostics, Santa Maria, California, USA) to collect both cloacal and blood samples. Cloacal swabs were collected at the time of capture by inserting the entirety of the cotton bud into the cloaca, swabbing the internal mucosal tissue for approximately three seconds, and then immediately placing the cotton bud in 95% ethanol. We collected blood on swabs from surgical wounds during radio-transmitter implantation or through phlebotomy. Samples were stored at -20°C until processing.

***DNA Extraction, and Quantity and Quality.*—**We extracted DNA using a Chelex 100 InstaGene Matrix (BioRad Laboratories, LLC, Hercules, California, USA) following protocols detailed in Peterman et al. (2012). Using a sterile razor blade, we removed approximately five mm of the cotton bud and further cut it into smaller pieces, approximately 0.1 mm in size. We then placed the pieces in a 1.7 mL centrifuge tube in 200 µl of Chelex 100, vortexed for 15 seconds, and incubated overnight at 60°C. We then removed the cotton pieces and vortexed again for 15 seconds before a final incubation for 20 minutes at 95°C. We spun the samples for two minutes at 10,000 RPM and removed and stored the supernatant in a -20°C freezer. We estimated the quantity and quality (A260/A280) of the DNA using a NanoDrop 2000c and then standardized all DNA extractions to 30µg/µl. To compare the quantity and quality of DNA obtained from

blood and cloacal samples, we performed a Mann-Whitney U test ($\alpha = 0.05$) because of moderate non-normality of measurements (Shapiro-Wilk: blood concentration $W = 0.929$, $p = 0.084$, cloacal concentration $W = 0.901$, $p = 0.020$; blood purity $W = 0.937$, $p = 0.105$, cloacal purity $W = 0.905$, $p = 0.024$). Two high-concentration outliers (>800 ng/µl) were present in the cloacal DNA measurements, and these observations were omitted from statistical analyses. To test for associations between quantity and quality of each DNA as a function of source we performed simple linear regressions. Diagnostic checks of fitted linear models indicated suitability of use with our data. All statistical analyses were conducted with R 3.5.2 (R Core Team 2019).

Microsatellite Genotyping. **—** Polymerase chain reaction (PCR) were used to amplify four loci using previously identified primers specific for population genetic analysis of Timber Rattlesnakes (Villarreal et al. 1996). We fluorescently tagged the four forward primers (7_87, 5A, 5_183, and 7_144) (Villarreal et al. 1996) and ran 10 µl PCR reactions using AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific, Inc, Waltham, Massachusetts, USA) under the following conditions: initial denaturation of 95°C for 10 minutes followed by 25 cycles of 95°C for 15 seconds, 58°C for 30 seconds and 72°C for 30 seconds. We then ran a final extension at 72°C for five minutes. We genotyped PCR products on an Applied Biosystems Genetic Analyzer (3730xl) at the Plant Microbe Genomics Core Facility at The Ohio State University. We then analyzed the genetic information using GeneMarker (SoftGenetics, LLC, State College, Pennsylvania, USA). From these data, we assessed the allelic dropout and peak height for each sample at each locus**.** To classify peak height we designated three classes: high quality sequences had heights > 1000, fair-quality sequences had heights 500–1000 and poor-quality sequences had

a height < 500. Because of non-normality, we compared mean peak heights between blood and cloacal DNA sources using a Mann-Whitney U test ($\alpha = 0.05$).

RESULTS

Overall, there was a positive relationship between DNA quality and concentration in blood DNA ($F_{1, 25} = 9.42$, $P = 0.005$, $R^2 = 0.245$), but a negative relationship with cloacal DNA ($F_{1, 23} = 16.88$, $P < 0.001$, $R^2 = 0.398$; Fig. 1). The average quality of blood samples was 1.89 (± 0.344 SD), while the average quality of cloacal samples was 0.94 (± 0.334). The average concentration of blood samples was 273.1 ng / µl (± 157.6), while the average concentration of cloacal samples was 153.8 ng / µl (± 100.1). Quality ($U = 668.5$, $P < 0.001$) and concentration ($U = 513$, $P = 0.001$) of blood and cloacal samples differed significantly.

We obtained complete genotypes for all 27 of our blood samples and 16/27 (59%) of our cloacal samples. When comparing cloacal genotypes to blood genotypes, we observed allelic dropout at 18 cloacal loci including two cloacal samples that did not amplify at all. The allele peak height, as measured by GeneMarker, of cloacal samples was significantly lower (mean = 8,364 ± 9,398) than the peak height of blood (mean = 15,581 ± 11,979) ($U = 7653$, $P < 0.001$). For blood swab samples, the vast majority of peaks (97%) were of high-quality with few exceptions (3% fair, 2% poor, and 0% failed to amplify). Cloacal swab samples also yielded many high-quality peaks (75%); however, there was a greater prevalence of fair (46%) and low quality (12%) peaks, as well as a small percentage that failed to amplify at all (8%). The fragment lengths of the four microsatellite loci included in this study are all less than 200 base pairs (bp), with a range of 94 – 162 bp (Table 2). There is no relationship between the length of the amplified fragment and the

frequency of amplification failure. The locus with the shortest fragment length, 94 bp, and the locus with the longest fragment length, 162 bp, have the same failed amplification rate of 22.2%.

DISCUSSION

Our results show that our Chelex extraction protocols yielded significantly less DNA of a poorer quality from cloacal samples than blood samples. We also observed a negative relationship between quality and quantity in the cloacal samples; as the DNA quantity of cloacal samples increased, the DNA quality decreased. We found the reverse pattern in blood samples, with DNA quality increasing at higher DNA concentrations. Cloacal swab samples are susceptible to contamination, such as the snake's microbiome, defensive musk secretions, or fecal material, which may contain DNA from consumed prey and high concentrations of uric acid. Such contamination may contribute to the negative relationship between quality and concertation in cloacal samples.

The cloacal outliers indicated in Fig. 1 both had high concentration of DNA (outlier A = 875.4 and outlier B = 831.1 ng/ µl) and low quality (outlier A = 0.36 and outlier B = 0.57 A260/280). The outlier samples differed in amplification across the screened loci. Outlier B amplified well at three loci with peak heights > 2,000, but exhibited poor amplification at locus 7-144 (peak height = 421). Outlier A had poor amplification at three of the four screened loci. The peak heights of the alleles for locus 7-144 were below 200 and therefore could not be scored reliably. Two of the other three loci, 7-087 and 5A, had weak signals of less than 600. The third locus, 5-183, had considerable amplification with sample peaks exceeding 3,000. These outlier samples may be examples of snakes that recently defecated prior to capture or that released musk as a defensive response during handling. Field notes were not recorded on snake response to

capture, but this documentation is recommended in future studies that seek to use cloacal swabs. Additional, more focused research on the effects of feces and musk on cloacal swab DNA would help resolve these outstanding questions.

Another potential source of DNA contamination when using cloacal swabs is the presence of DNA from multiple snakes. As snakes have internal copulation as well multiple paternity (Uller and Olsson 2008), there is potential for DNA from other snakes to be present in the cloaca of a female. To avoid this risk, it may be prudent to only collect blood samples during mating season. However, this may be impractical because many field seasons overlap with snake breeding. We recommend careful examination of microsatellite genotypes during scoring to possibly detect individuals with more than two alleles per locus that might indicate the presence of foreign DNA as a result of copulation.

A known source of amplification failure or allelic dropout when using non-invasively collected DNA is the length of the microsatellite (Broquet et al. 2008); longer fragments are more susceptible to degradation and tend to exhibit higher rates of allelic dropout or failure in non-invasive samples. There is no evidence that the amplification issues observed in this study are related to the microsatellite fragment length. All loci in this study were of relatively small length (<170 bp) and both the longest and shortest fragments had equivalent amplification issues.

Despite the decreasing quality of cloacal DNA samples as concentration increased, the results of this study indicate that cloacal DNA can be used for microsatellite genotyping. Overall, alleles amplified from DNA extracted from blood samples had greater peak heights and therefore

blood samples were easier to assign genotypes. Due to decreased peak height from cloacal DNA, genotyping results from cloacal samples had to be carefully evaluated locus by locus to generate accurate genotypes. Without manual inspection of electropherograms, automated scoring, even with reduced peak height thresholds, could possibly produce errors in genotyping calls or no calls at all. Our study contained a small sample size of individuals and loci, so manually scoring alleles was tractable, but consideration should be given to genotyping large populations if manual scoring is required, as this increases the potential for human error or bias.

Future studies should evaluate the use of cloacal DNA standardized to higher quantities. In our study, we standardized blood and cloacal genetic material to the same concentration based on NanoDrop measurements. Because the cloacal DNA contained more impurities than blood DNA, this likely resulted in standardized cloacal genetic samples that contained much less template DNA than blood samples. If cloacal samples were standardized to higher quantities, there potentially would have been more template DNA present, which could ultimately improve microsatellite genotyping. Previous studies that did not standardize cloacal swab DNA samples to a given quantity had promising results indicating that increased quantity of cloacal swab DNA in a sample may lead to less allelic dropout and PCR products that are more comparable to those obtained from blood samples (Miller 2006 and Ford et al. 2017).

Another consideration when using cloacal swabs as a source of DNA is the extraction method. The DNA extraction protocols used in this study can be conducted at a lower cost per sample than many other techniques, but may result in lower quality DNA. Chelex DNA extractions are a reliable method for extracting DNA for use in microsatellite genotyping, but are

not recommended for use with projects that require high quality DNA (Eggert et al. 2005). It is reasonable to assume that applying other methods to cloacal swab DNA extractions may increase yield and decrease PCR inhibition from proteins and nitrogenous waste. For example, modified phenol-cholorform extractions (Renshaw et al., 2015) or PCR inhibitor removal kits may decrease the amount of impurities in extracted DNA, and therefore increasing the success rate of microsatellite amplification. Additional research comparing various DNA extraction and inhibitor removal methods would be helpful in the verification of cloacal swabs as an alternative sampling method for DNA.

In conclusion, our findings demonstrate that cloacal swabs can be an alternative when taking blood samples is not feasible; however, care should be taken in data analysis when using cloacal swabs for microsatellite genotyping. Standardizing the samples using higher quantities and quality of cloacal DNA and minimizing stress to the organism is most likely to achieve the best results with the least amount of contamination.

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TABLE 1. Summary table describing the range, mean, and standard deviation of DNA concentration and quality in relation to DNA extracted from blood or cloacal swabs. Mean and standard deviation of peak heights, measured in relative fluorescence units (RFU) describe the amplification of alleles on an electropherogram.

Sample Type	Sample Size	Range of DNA Quantities (ng / µl)	DNA Quantity Mean (ng / µl) ± SD	Range of Quality (260/280)	Quality (260/280) Mean ± SD	Peak Height Mean (RFU) ± SD
Cloacal	27	40.9 – 875.4	153.8 ± 100.1	0.64 – 1.35	0.94 ± 0.33	8,364 ± 9,398
Blood	27	71.5 – 662.4	273.1 ± 157.6	1.25 – 2.94	1.89 ± 0.34	15,581 ± 11,979

TABLE 2. Summary of microsatellite locus fragment length and amplification failure of blood and cloacal DNA samples. Allele size range is presented in base pairs (bp) and the percentage of samples that failed to amplify was calculated for each locus out of the 27 DNA samples screened with each source of DNA.

Locus	Allele Size Range (bp)	Number of Blood / Cloacal Samples that Failed to Amplify	Percentage of Blood / Cloacal Samples That Failed to Amplify
7-144	94–115	0 / 6	0% / 22.2%
5-183	120–127	0 / 2	0% / 7.4%
5A	137–162	0 / 6	0% / 22.2%
7-87	140–151	0 / 4	0% / 14.8%

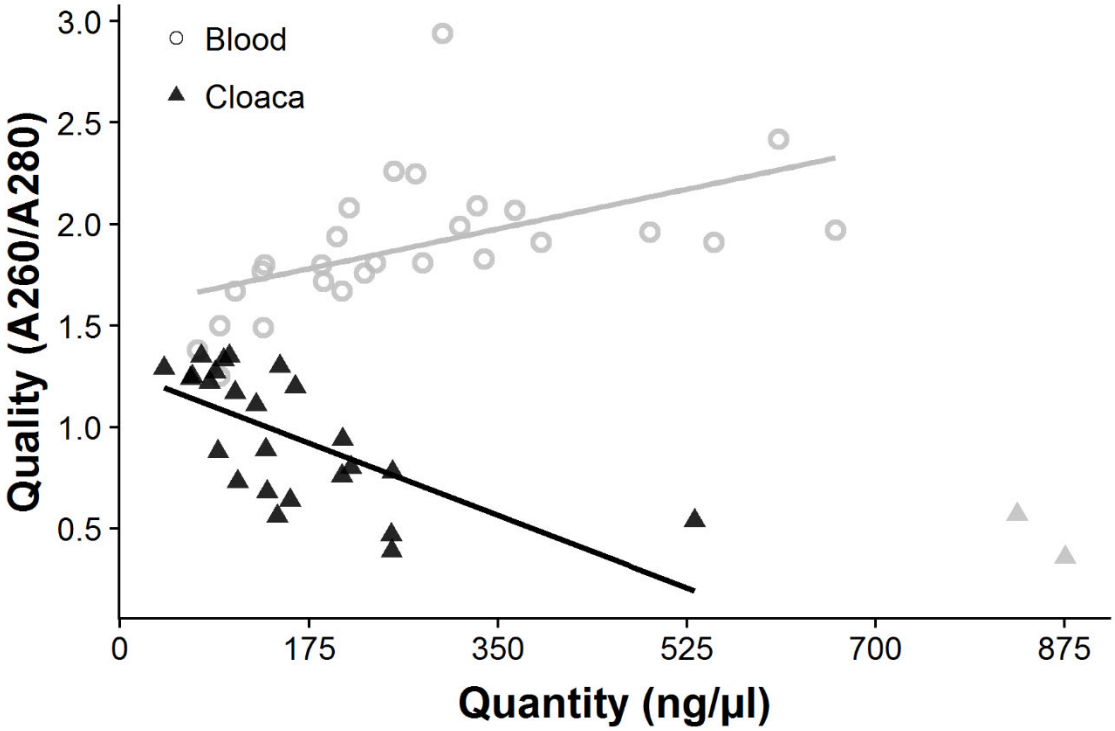

FIGURE 1. Relationship between quantity and quality of cloaca (solid triangles) and blood (hollow circles) DNA samples. Quality refers to the purity of a DNA sample and is assessed by the ratio of absorption at 260 nm and 280 nm (A260/A280), while quantity is the yield (concentration) of DNA (ng / µl). An A260/A280 ratio close to 1.8 is considered pure DNA, values less than 1.8 indicate protein contamination, and values greater than 1.8 indicate RNA contamination. The gray-filled triangle symbols are indicate outlier cloaca samples that were withheld from statistical analyses.