Assessing genotyping errors in mammalian museum study skins using high-throughput genotyping-by-sequencing

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Abstract

The use of museum specimens held in natural history repositories for population and conservation genetic research is increasing in tandem with the use of massively parallel sequencing technologies. Short Tandem Repeats (STRs), or microsatellite loci, are commonly used genetic markers in wildlife and population genetic studies. However, they traditionally suffered from a host of issues including length homoplasy, high costs, low throughput, and difficulties in reproducibility across laboratories. Massively parallel sequencing technologies can address these problems, but the incorporation of museum specimen derived DNA suffers from significant fragmentation and exogenous DNA contamination. Combatting these issues requires extra measures of stringency in the lab and during data analysis, yet there have not been any high-throughput sequencing studies evaluating microsatellite allelic dropout from museum specimen extracted DNA. In this study, we evaluate genotyping errors derived from mammalian museum skin DNA extracts for previously characterized microsatellites across PCR replicates utilizing high-throughput sequencing. We found it useful to classify samples based on DNA concentration, which determined the rate by which genotypes were accurately recovered. Longer microsatellites performed worse in all museum specimens. Allelic dropout rates across loci were dependent on sample quantity, with high concentration museum specimens performing as well and recovering quality metrics nearly as high as the frozen tissue sample. Based on our results, we provide a set of best practices for quality assurance and incorporation of reliable genotypes from museum specimens.

Keywords Microsatellite · Museum specimens · Degraded DNA · Population genetics · SSRseq

Introduction

Natural history repositories contain invaluable specimen collections for scientific use across diverse fields (Lane 1996; Williams 1999; Lister and Group 2011; Blagoderov et al. 2012). Many of these specimens represent populations that no longer exist due to land-use change and anthropogenic landscape alterations over the past century (Smith et al.

2013). Additionally, museum specimens often represent the few or only representatives of endangered or rare species, provide important vouchers for comparison with modern samples, and provide genetic resources for species which may be difficult to sample in wild habitats (Miller et al. 2009; White et al. 2018). Consequently, usage of museum specimens for research incorporating DNA analysis is increasing.

The degraded DNA associated with museum specimens is known to require extra measures of stringency in order to combat issues with exogenous DNA contaminants (Paabo et al. 2004; Rizzi et al. 2012) and highly fragmented endogenous DNA (Campana et al. 2012; Hawkins et al. 2016a; McDonough et al. 2018). Studies which reliably sequence DNA from museum specimens undergo stringent protocols (e.g., processing in appropriate lab spaces) to prevent contamination and to combat the low quantity and fragmented nature of the extracts. Downstream from wet lab procedures, additional bioinformatic steps must be taken to ensure the resulting genetic sequence data represent the target taxa.

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Truly ancient samples (archaeological and permafrost specimens) have characteristic degradation patterns associated with misincorporation of nucleotides—namely cytosine to uracil deamination—(Hofreiter et al. 2001; Jónsson et al. 2013; Kistler et al. 2017) but degradation patterns are only beginning to be understood and vary by sample type (Shapiro and Hofreiter 2012; Weiß et al. 2016), with museum specimens lacking the characteristic cytosine–uracil deamination (McDonough et al. 2018).

Short tandem repeats (STRs), or microsatellite loci, are useful markers for numerous applications and widely implemented in the field of conservation genetics to evaluate genetic diversity and population structure (e.g., Bilska and Szczecińska 2016; Arbogast et al. 2017). Capillary electrophoresis (CE), a fragment size genotyping technique, was previously standard practice for microsatellite genotyping, but the advent of high-throughput sequencing (HTS) introduced new possibilities (Curto et al. 2019; Tibihika et al. 2019). Degraded DNA present unique obstacles for HTS methodology, yet as more studies incorporate low quality samples, advances in laboratory and bioinformatic processes are making these samples more accessible (Andrews et al. 2018). HTS technology has allowed for rapid identification of microsatellite loci in non-model organisms on a genomic scale (Miller et al. 2013; Silva et al. 2013; Duan et al. 2014; Griffiths et al. 2016), and the simultaneous sequencing of thousands of putative loci as compared to traditional cloning methods (Glenn and Schable 2005). In addition to the cost reduction of microsatellite isolation (as low as \$24 USD per locus as detailed in: (Abdelkrim et al. 2009), some of the issues known to occur when genotyping microsatellites via CE can be alleviated using HTS technologies (Vartia et al. 2016). For example, fragment size analysis via CE has been known to provide (albeit sometimes predictably) shifted sizes when samples are run on different machines (Morin et al. 2009), but access to raw sequences from HTS allows precise allele sizing (Darby et al. 2016).

A number of studies have evaluated how to transform raw sequences into microsatellite genotypes (Darby et al. 2016; Vartia et al. 2016; De Barba et al. 2017; Zhan et al. 2017; Barbian et al. 2018; Pimentel et al. 2018; Šarhanová et al. 2018). Each of these genotyping-by-sequencing (GBS) studies has evaluated some aspects of the biases induced when comparing sequences from HTS platforms to CE genotyping. Commonly addressed issues include evaluation of stutter and PCR artifacts (e.g. off-target amplification, PCR product adenylation) as these still occur in HTS-based methods, but can be mitigated using emerging bioinformatic analyses (Barbian et al. 2018; Tibihika et al. 2019). Length homoplasy occurs when alleles of the same length have different nucleotide sequences (Darby et al. 2016), and GBS studies have also shown recovery of additional alleles based on direct sequence analysis, which increases genetic

resolution (Curto et al. 2019; Lepais et al. 2020). Although challenges exist for direct comparison of HTS based microsatellite genotypes with those via CE, the ability to generate comparable datasets is paramount in order to build off previous research and inform larger, potentially landscape-based conservation plans.

Despite the wide range of studies already published on GBS, none have specifically evaluated genotyping errors that occur from mammalian study skin sourced DNA (hereafter ‘museum specimen DNA’). Genotyping errors arise when the observed genotype of a sample differs from a consensus genotype (Pompanon et al. 2005), and previous studies have estimated error rates from fecal (De Barba et al. 2017; Barbian et al. 2018), tissue (Vartia et al. 2016; Lepais et al. 2020), and hide and hair samples (Donaldson et al. 2020). Here we invoke HTS GBS to assess genotyping errors derived from *Glaucomys oregonensis* (Humboldt’s flying squirrel) museum specimen DNA extracts, for five previously characterized microsatellites across PCR replicates of individual samples. More specifically, we analyzed the allelic dropout rate, a type of genotyping error where a true allele fails to amplify in PCR (Broquet and Petit 2004), across three datasets:

- (1) Replicate dataset: For every sample, each microsatellite and mitochondrial PCR replicate underwent library preparation.
- (2) Pooled dataset: For every sample, all microsatellite and mitochondrial PCR replicates were combined together prior to library preparation.
- (3) Bioinformatic dataset: For every sample, bash scripting ‘cat’ commands were used to combine all reads from the replicate dataset prior to genotyping.

The error rates described here will serve as the first for GBS of dry museum study skins, and provide best practices for subsequent studies on museum specimen samples.

Methods

Samples

Total genomic DNA from 147 samples was extracted for a population genetic study on *Glaucomys oregonensis* (Yuan et al. in prep.). A subset of seven samples was included in this study to establish baseline data for quantifying allelic dropout in degraded source material. From the screened samples, DNA concentrations were measured with a NanoDrop One Spectrophotometer (ThermoScientific) and a Qubit 2.0 (Life Technologies) using a high sensitivity DNA kit. Based on instrument sensitivity, different raw values were used to bin samples as ‘high concentration’ museum

specimens (HCMS) or ‘low concentration’ museum specimens (LCMS) (O’Neill et al. 2011). Additional quantification details are provided in the Supplemental Materials. One frozen tissue sourced specimen was also included to observe if similar rates of allelic dropout would be detected from a non-degraded sample (Table 1).

Microsatellite selection

We tested three sample types: tissue, ‘high’, and ‘low’ concentration museum samples to evaluate amplification across microsatellites of varying lengths. Previously published microsatellites from *Glaucomys sabrinus* (northern flying squirrel)—a species which historically included *G. oregonensis*—were used in this study (GS-2, GS-4; Zittlau et al. 2000, and GLSA-12, GLSA-22, GLSA-52; Kiesow et al. 2011, Table 2). Two ‘short’ microsatellites were selected (GS-2 and GS-4), which included any marker under 150 bp in length according to published allele sizes, two ‘medium’ (GLSA-12 and GLSA-22, 150–200 bp), and one ‘long’ microsatellite (GLSA-52, > 200 bp).

DNA extraction

Total genomic DNA from museum specimens was isolated using Qiagen QIAamp DNA Mini Kits (Qiagen, Valencia, CA) in a lab designated for degraded DNA, while DNA from tissue samples was processed in a standard DNA facility

using Qiagen DNeasy Blood and Tissue Kits following standard animal tissue protocols. DNA was eluted in 100 µl of buffer AE for all museum specimens and 200 µl for tissue samples. The degraded DNA lab protocol included placing a blank extraction control minimally every 12 samples, extensive bleaching, exposing consumables and pipettes to ultraviolet radiation, routine changing of gloves, and processing samples under a PCR workstation (AirClean Systems model AC600). Raw DNA concentrations were evaluated using 1 µl of DNA on a NanoDrop One Spectrophotometer (ThermoScientific).

Polymerase chain reaction

Multiplexed PCR attempts failed repeatedly, so singleplexed PCR was performed in 16 µl reactions containing 2.0 µl of DNA template (~0.01–127.4 ng/µl), 4.5 µl of ddH₂O, 0.5 µl of each primer, and 8.5 µl of DreamTaq Green PCR Master Mix. PCR negative controls were added minimally every 47 samples with each PCR. When amplifying microsatellites with the GS-2 and GS-4 primers, 0.3 µl of ddH₂O was replaced with bovine serum albumin (BSA, New England Biolabs, Inc., 12 mg) to reduce PCR inhibition. For all samples a touchdown PCR profile was used: 95 °C for 1 min; 2 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 45 s; 2 cycles changing 60 °C to 58 °C; 2 cycles changing 58 °C to 54 °C; 2 cycles changing 54 °C to 52 °C; 35 cycles of 95 °C for 15 s, 50 °C for 30 s, 72 °C for 45 s; 72 °C for 5 min.

Table 1 Summary of samples used in this study

Sample:	DNA Quality:	Subspecies:	Sex:	Collection Locality:	Year Collected:	Storage Conditions:	Sample Type:	DNA Concentration: NanoDrop/Qubit in ng/ul	Haplotype: (Yuan et al. in prep)
HSU1836	HCMS	<i>G. o. stephensi</i>	M	Humboldt County	1975	Skin and skull (room temperature)	Skin clip, cartilage, toepad	30.0 / 20.6	3
UMMZ79755	HCMS	<i>G. o. californicus</i>	F	San Bernardino County	1926	Skin and skull (room temperature)	Skin clip	5.1/0.702	6
UMMZ79760	HCMS	<i>G. o. californicus</i>	F	San Bernardino County	1926	Skin and skull (room temperature)	Skin clip	28.6/ 7.5	6
MVZ5211	LCMS	<i>G. o. californicus</i>	F	San Bernardino County	1905	Skin and skull (room temperature)	Skin clip	-2.2 / <0.005	6
MVZ2088	LCMS	<i>G. o. californicus</i>	F	Riverside County	1908	Skin and skull (room temperature)	Skin clip	1.8/0.284	6
LACM95619	LCMS	<i>G. o. californicus</i>	M	Riverside County	1919	Skin (room temperature)	Skin clip	1.4/0.184	6
HSU8180	Tissue	<i>G. o. laszivus</i>	M	Plumas County	1992	Tissue (frozen at -80°C)	Tissue subsample	63.7 ng/µl	12

HCMS= high concentration museum specimen

LCMS= low concentration museum specimen

HSU: Humboldt State University Vertebrate Museum

UMMZ: University of Michigan Museum of Zoology

MVZ: Museum of Vertebrate Zoology, University of California Berkeley

LACM: Los Angeles County Museum

Table 2 Primers used for microsatellite and cytochrome-*b* amplification

Microsatellites					
Name	Sequence	Repeat motif	Size range	Anneal temp. (°C)	Source:
GS-2	F: AACATTCTCGCCACATCTAA	(GT)16	96–124 (S)	48.6	Zittlau et al. (2001)
	R: CTACACCCCCAGCCCTACAA			55.8	
GS-4	F: CTTCTTGAGTTGCTGGGGTGAC	(GT)15	104–112 (S)	55.1	Zittlau et al. (2001)
	R: ATCTAAACCATAACACACACACA			49.3	
GLSA-12 ^a	F: A G CATATGGAACCCCATATCAG	(GT)5A(TG)3TTT(GT)5	154–170 (M)	54.8	Kiesow et al. (2011)
	R: GGCAAGATTTGTATCCTTGG				
GLSA-22	F: CCTGAAAATGATGCATGTGG	(CA)15	168–188 (M)	54.8	Kiesow et al. (2011)
	R: AGAGTAGGCTGTTTCCTTGAGG				
GLSA-52	F: TCCATCCACAGTGTGTGAGC	(CA)16	210–254 (L)	57.5	Kiesow et al. (2011)
	R: CCTGGAGTCCACTCAAGCAT				
Cytochrome- <i>b</i>					
L14724	5'-GATATGAAAAACCATCGTTG-3'				Irwin et al. (1991)
H15910	5'-GATTTTGGTTTACAAGACCGAG-3'				Oshida et al. (2000)
GOR_R1	5'-CTCGGCCTACATGGAGAAAA-3'				This study

Microsatellites were characterized by length (S = short, < 150 bp; M = medium, 150–200 bp; and L = long, > 200 bp) and repeat motif (see Supplemental Materials for details). The GOR_R1 reverse primer for cytochrome-*b* was newly designed for this study as previously published cytochrome-*b* primers did not amplify in *Glaucomys oregonensis*

S short

M medium

L long

^aGLSA-12 Forward primer was ordered with an error- our primer was lacking the 'G' in the second position; however, the locus still amplified in PCR despite this error

Successful PCR amplifications were replicated twice in the tissue sample and three times in museum samples. Following amplification, 1.5% agarose gels were run with a 100 bp size standard (Invitrogen) and stained with GelRed (Biotium) or SYBR Green (Invitrogen). To remove residual primers, dNTPs, and nontarget molecules, solid phase reversible immobilization (SPRI) cleaning via magnetic beads was performed (following Rohland and Reich 2012) in a ratio of 1 part PCR product to 1.5X magnetic beads (KAPA beads, Roche). Cleaned PCR products were eluted in 20 µl ddH₂O.

A fragment of the mitochondrial cytochrome-*b* gene was also amplified in PCR and sequenced to ascertain whether levels of mtDNA would be indicative of nDNA. Mitochondrial DNA methods and analysis can be found in the Supplemental Materials.

Library preparation

For the replicate dataset, each microsatellite and mitochondrial PCR replicate underwent library preparation (resulting in 114 libraries). For the pooled dataset, all PCR replicates were combined together prior to library preparation. Two µl of each PCR product for both cytochrome-*b* and microsatellites were pooled for museum specimens, while 2 µl of each cytochrome-*b* replicate and 3 µl of each microsatellite replicate were pooled for the tissue sample. For the bioinformatic dataset, we combined all reads from the replicate dataset together using bash scripting 'cat' commands prior to genotyping to evaluate if genotypes would vary based on PCR product coverage.

Individual dual iTru style indices (Glenn et al. 2019) were ligated using KAPA Illumina Library Preparation Kits (Roche, # KK8232) following Hawkins et al. (2016b). Libraries were amplified in 25 µl reactions containing 1.25 µl of each iTru adapter, 2.5 µl ddH₂O, 7.5 µl of stubby

adapter-ligated DNA, and 12.5 µl of KAPA HiFi HotStart ReadyMix. The thermocycler conditions were 98 °C for 45 s; 10 cycles (tissues) or 14 cycles (museum samples) of 98 °C for 15 s, 60 °C for 30 s, 72 °C for 1 min; 72 °C for 5 min. A library preparation control (ddH₂O) was included in

Table 3 Summary of recovered genotypes from CHIIMP v 0.3.1 for all datasets, ConGenR, and the TapeStation

				Accuracy Across Amplification				Bioinformatic Dataset				Pooled Dataset		Final Genotypes		ConGenR Consensus Genotypes		TapeStation Electropherogram Allele Calls	
								Average Accuracy per Micro											
Sample	Replicate	Micro	Source	Allele 1	Allele 2	Allele 1	Allele 2	Micro		Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
HSU 8180	1	GS-2	Tissue	84*	84*	100%	100%	86%		84*	84*	84*	84*	84	84	84	84		
HSU 8180	2			84*	84*														
HSU 1836	1		HCMS	98❖	104❖	66%	66%	Among 'high quality' 89%		96❖	98❖	98❖	104❖	96	98	96	98		
HSU 1836	2			96❖	98❖														
HSU 1836	3			96❖	98❖														
UMMZ 79755	1		HCMS	84* Δ	84* Δ	100%	100%			84*	84*	84	84	84	84	84	84		
UMMZ 79755	2			84* Δ	84* Δ														
UMMZ 79755	3			84* Δ	84* Δ														
UMMZ 79760	1		HCMS	84*	84*	100%	100%			84*	84*	84	84	84	84	84	84		
UMMZ 79760	2			84* Δ	84* Δ														
UMMZ 79760	3			84* Δ	84* Δ														
MVZ 2088	1		LCMS	84	84	100%	100%	Among 'poor quality' 75%		84* Δ	84* Δ	84❖	84❖	84	84	84	84	88	88
MVZ 2088	2			84*	84*														
MVZ 2088	3			84*	84*														
MVZ 5211	1		LCMS	-	-	-	-			-	-	82❖	84❖	-	-	-	-		
MVZ 5211	2			-	-														
MVZ 5211	3			-	-														
LACM 95619	1		LCMS	82*	82*	50%	50%			82	84	82	84	82	84	82	84	88	88
LACM 95619	2			-	-														
LACM 95619	3			84*	84*														
HSU 8180	1	GS-4	Tissue	92	96	50%	100%	68%		96*	96*	96*	96*	96	96	96	0		
HSU 8180	2			94	96														
HSU 1836	1		HCMS	92	96	100%	100%	Among 'high quality' 100%		92	96	94	96	92	96	92	96	99	97
HSU 1836	2			92	96														
HSU 1836	3			92	96														
UMMZ 79755	1		HCMS	-	-	100%	100%			96	98	92	92	92	96	92	96	112	112
UMMZ 79755	2			92	96														

Table 3 (continued)

UMMZ 79755	3			92 ♠ 96 ♠									
UMMZ 79760	1		HCMS	92 ♠ 96 ♠	100%	100%		92 ♠ 96 ♠	92 94	92 96	92 96		
UMMZ 79760	2			92 ♠ 96 ♠									
UMMZ 79760	3			92 ♠ 96 ♠									
MVZ 2088	1		LCMS	92 96	100%	100%	Among 'poor quality' 33%	96* 96*	96* 96*	96 96	92 96		
MVZ 2088	2			92 96									
MVZ 2088	3			92 96									
MVZ 5211	1		LCMS	104* 104*	0%	0%		100 104	- -	- -	104 0		
MVZ 5211	2			97 ♠ 105 ♠									
MVZ 5211	3			- -									
LACM 95619	1		LCMS	120 124	0%	0%		105 ♠ 109 ♠	109 ♠ 124 ♠	- -	0 0		
LACM 95619	2			105 109									
LACM 95619	3			87 ♠ 98 ♠									
HSU 8180	1	GLSA-12	Tissue	156* 156*	100%	100%	83%	156 156	156* 156*	156 156	156 156		
HSU 8180	2			156* 156*									
HSU 1836	1		HCMS	160 ♠ 162 ♠	100%	100%	Among 'high quality' 77%	160 162	158 ♠ 160 ♠	160 162	160 162	177 205	
HSU 1836	2			160 ♠ 162 ♠									
HSU 1836	3			160 ♠ 162 ♠									
UMMZ 79755	1		HCMS	158 ♠ 158 ♠	66%	100%		156 158	158* 158*	158 158	158 158	177 203	
UMMZ 79755	2			156 158									
UMMZ 79755	3			158* 158*									
UMMZ 79760	1		HCMS	156 158	66%	100%		156 158	158* 158*	156 158	156 158	173 198	
UMMZ 79760	2			158* 158*									
UMMZ 79760	3			156 156									
MVZ 2088	1		LCMS	158* ^ 158* ^	-	-	Among 'poor quality' NA	158 158	158 158	- -	158 0		
MVZ 2088	2			- -									
MVZ 2088	3			- -									
MVZ 5211	1		LCMS	- -	-	-		- -	158 160	- -	- -		
MVZ 5211	2			- -									
MVZ 5211	3			- -									
LACM 95619	1		LCMS	- -	-	-		- -	143 ^ 158 ^	- -	- -		
LACM 95619	2			- -									
LACM 95619	3			- -									

Table 3 (continued)

HSU 8180	1	GLSA-22	Tissue	169❖	171❖	100%	100%	100%	169❖	171❖	169❖	171❖	169	171	169	171	
HSU 8180	2			169❖	171❖												
HSU 1836	1		HCMS	171	173	100%	100%	Among 'high quality'	171❖	173❖	171❖	173❖	171	173	171	173	
HSU 1836	2			171	173			100%									
HSU 1836	3			171	173												
UMMZ 79755	1		HCMS	177❖	179❖	100%	100%		177	179	177	179	177	179	177	179	
UMMZ 79755	2			177	179												
UMMZ7 9755	3			177❖	179❖												
UMMZ 79760	1		HCMS	177❖	179❖	100%	100%		177	179	177	179	177	179	177	179	214 214
UMMZ 79760	2			177	179												
UMMZ 79760	3			177	179												
MVZ 2088	1		LCMS	-	-	50%	100%	Among 'poor quality'	179*	179*	179*	179*	179	179	179	0	
MVZ 2088	2			177	179												
MVZ 2088	3			179*	179*												
MVZ 5211	1		LCMS	-	-	-	-	50% two with no data	-	-	171❖	177❖	-	-	-	-	
MVZ 5211	2			-	-												
MVZ 5211	3			-	-												
LACM 95619	1		LCMS	-	-	-	-		-	-	175❖	177❖	-	-	-	-	
LACM 95619	2			-	-												
LACM 95619	3			-	-												
HSU 8180	1	GLSA-52	Tissue	257	257	100%	100%	100%	257	257	251	257	257	257	257	257	
HSU 8180	2			257	257												
HSU 1836	1		HCMS	255	255	100%	100%	Among 'high quality'	255	255	255	255	255	255	255	255	
HSU 1836	2			255	255			100%									
HSU 1836	3			255	255												
UMMZ 79755	1		HCMS	255	255	100%	100%		-	-	251	251	255	255	255	255	258 258
UMMZ 79755	2			-	-												
UMMZ7 9755	3			255	255												
UMMZ 79760	1		HCMS	255	255	100%	100%		255	255	250	251	255	255	255	0	255 255
UMMZ 79760	2			-	-												
UMMZ 79760	3			253	255												
MVZ 2088	1		LCMS	-	-	-	-	Among 'poor quality'	-	-	-	-	-	-	-	-	
MVZ 2088	2			-	-												

Table 3 (continued)

MVZ 2088	3			-	-			NA	-	-	-	-	-	-	-	-	
MVZ 5211	1		LCMS	-	-	-	-		-	-	-	-	-	-	-	-	
MVZ 5211	2			-	-												
MVZ 5211	3			-	-												
LACM 95619	1		LCMS	-	-	-	-		-	-	-	-	-	-	-	-	
LACM 95619	2			-	-												
LACM 95619	3			-	-												
						83%	89%										

SAMPLE Tissue

SAMPLE High concentration Museum Specimen (HCMS)

SAMPLE Low concentration Museum Specimen (LCMS)

* Possible PCR Stutter removed by CHIIMP

^ PCR Artifacts removed by CHIIMP

❖ More than two prominent sequences as determined by CHIIMP

Indicates less than 500 raw reads and genotypes should not be used

Fig. 1 Scatter plot of average quality of PCR replicates following prinseq-lite. Replicates for each specimen are shown across the x-axis, and the percentage of ‘good’ reads are shown on the y-axis. Samples are sorted by type: tissue, high concentration museum specimens, and low concentration museum specimens. Individuals of the same sample type are separated by a dashed line

all steps. After library prep, an agarose gel was run to ensure successful adapter ligation. Products were purified via SPRI as detailed above.

Sequencing

Sequencing occurred on an Illumina MiSeq using a 2 × 300 PE v.3 kit at the Center for Conservation Genomics, Smithsonian Conservation Biology Institute, Washington DC or using a 2 × 250 PE v.2 Nano kit at the Laboratory of Analytical Biology, National Museum of Natural History, Smithsonian Institution, Washington DC. Reads were demultiplexed and downloaded from the BaseSpace Server (Table S1).

Quality filtering

Samples were run through FastQC v 0.11.9 (Andrews 2010) and CutAdapt v 1.18 (Martin 2011) for quality filtering and adapter removal. Phred scores were required to be ≥ 20 averaged across each read. Prinseq v 0.20.4 (Schmieder and Edwards 2011) was run on each library to determine the proportion of low quality reads (Table S1). Commands are provided in the Supplementary Materials.

Genotyping

CHIIMP v 0.3.1 (Barbian et al. 2018) was used to generate genotypes for all datasets. At each locus, a genotype was called if there were minimally 5 reads (counts.min = 5), sequences were considered alleles only if the read count constituted minimally 5% of the total reads for that locus (fraction.min = 0.05), and all loci were given a 20 bp length buffer. CHIIMP identified the most likely alleles and

provided data on whether or not the sample had PCR stutter, PCR artifacts, and more than two possible allele sequences. ConGenR (Lonsinger and Waits 2015) was also run to generate consensus genotypes for the replicate dataset. To determine the ‘final genotypes’ (Table 3) that would be used for population genetic analyses, any genotype with a prinseq quality score < 70% and any called with only one successful PCR replicate were discarded. Then, the genotypes generated from CHIIMP and ConGenR were compared across all datasets, and if there were any incongruent alleles we manually evaluated the sequences to discern the cause, whether it was due to actual differences in repeat motif number, primer site mismatches, or another reason (Online Appendix 1).

Fragment analysis

As the primers used for PCR were not fluorescently labelled for CE, we generated electropherograms for a subset of samples across all loci to evaluate allele size utilizing a TapeStation 2200 (Agilent) with High Sensitivity tapes. PCR products were diluted 1:4–1:10 depending on DNA quantification determined by a Qubit 2.0 using a High Sensitivity kit.

Statistical analyses

Descriptive statistics, a single factor analysis of variance (ANOVA), and a linear regression were calculated in Microsoft Excel v16.16.20 using the data analysis add in. The ANOVA compared the percentage of reads passing prinseq-lite quality filters against sample types. MicroDrop v1.01 (Wang and Rosenberg 2012) was run on the pooled dataset using default parameters to evaluate allelic dropout rates across samples and loci. We did not enforce

Table 4 MicroDrop v1.01 and ConGenR results for the pooled and replicate dataset with a comparison of the initial raw CHIIMP output to the final processed data

Microdrop				ConGenR			
Pooled dataset		Final genotypes		Replicate dataset		Final genotypes	
	N = 7	“Best practices”	N = 7		N = 7	“Best practices”	N = 7
Raw CHIIMP output		Processed CHIIMP output		Raw CHIIMP output		Processed CHIIMP Output:	
Locus specific dropout rate	%	Locus specific dropout rate	%	Locus specific dropout rate	%	Locus specific dropout rate	%
GS-2	0.00	GS-2	0.01	GS-2	18.75	GS-2	18.75
GS-4	15.08	GS-4	0.00	GS-4	8.33	GS-4	0.00
GLSA-12	0.07	GLSA-12	12.73	GLSA-12	9.09	GLSA-12	9.09
GLSA-22	0.00	GLSA-22	0.00	GLSA-22	0.00	GLSA-22	0.00
GLSA-52	48.02	GLSA-52	36.33	GLSA-52	0.00	GLSA-52	0.00
Average:	12.63	Average:	9.81	Average:	7.23	Average:	5.57
Individual dropout rate	%	Individual dropout rate	%	Sample bin dropout rate	%	Sample bin dropout rate	%
HSU 8180	14.73	HSU 8180	8.47	Tissue	0.00	Tissue	0.00
HSU 1836	0.00	HSU 1836	0.00	HCMS	5.00	HCMS	4.76
UMMZ 79755	7.30	UMMZ 79755	0.00	LCMS	33.33	LCMS	20.00
UMMZ 79760	0.00	UMMZ 79760	0.00	Overall dropout rate	8.77	Overall dropout rate	6.45
MVZ 2088	40.58	MVZ 2088	59.61				
MVZ 5211	18.31	MVZ 5211	1.00				
LACM 95619	0.00	LACM 95619	77.36				
Average:	11.56	Average:	20.92				
		Average w/o MVZ 5211:	24.24				

For MicroDrop, both locus specific and individual rates of estimated allelic dropout are provided. For ConGenR, locus specific and sample bin rates are provided

Hardy–Weinberg Equilibrium on our data due to the low number of alleles and samples. The program was run once on the raw CHIIMP genotypes and once on the final genotypes (Table 3). MicroDrop calculates allelic dropout rates using an expectation–maximization algorithm in a maximum-likelihood method, but it is not designed for replicated PCR samples, so ConGenR (Lonsinger and Waits 2015) was used to evaluate allelic dropout in the replicate dataset across loci and DNA concentration bins. ConGenR calculates allelic dropout rates by comparing all PCR replicate genotypes to a consensus genotype. It is primarily based on Broquet and Petit (2004) but will calculate allelic dropout for homozygous individuals when a false allele is present. Additionally, ConGenR was run once on the raw CHIIMP genotypes and once on the final genotypes.

Results

Effects of sample quality

A total of 387,810 reads were sequenced across all samples. Sample quality was determined by prinseq-lite, which

uses standard quality control measures including length distribution, GC content, and ambiguous bases (Table S1). Mean quality across all samples was 85.6%, with a range of 57.43–99.48% (median = 92.6%, mode = 96.82%). Separated by sample type, the mean quality was as follows: 95.99% (SE ± 0.99%), 95.06% (SE ± 0.68%), and 73.84% (SE ± 2.10%) for tissue, HCMS, and LCMS respectively (Table S2). The ANOVA was significant ($P = < 0.001$) and the regression resulted in an R^2 of 0.44 ($P = < 0.001$), indicating our DNA quantification bins were predictive of sequencing quality.

CHIIMP genotypes were accurate for the tissue sample, especially in the pooled dataset. Mismatched alleles were recovered most frequently in LCMS, which routinely appeared to fail PCR. Mismatches were often associated with PCR stutter, PCR artifacts, or more than two prominent sequences, as identified by CHIIMP (Table 3). Individual samples did not appear to recover specific CHIIMP flags across all replicates, neither did specific microsatellites; however, the locus GS-2 recovered frequent flags for all three metrics (Table 3).

The HCMS recovered consistently high-quality sequences as determined by prinseq-lite. Only a single PCR replicate

Fig. 2 Best practices flowchart, with emphasis on low concentration samples. By following these practices, we reduced our allelic dropout by about 3% across loci according to MicroDrop v1.01, though we also had to remove many of the LCMS genotypes as we could not confirm authenticity

from UMMZ 79755 had < 85% pass quality metrics. All other replicates were over 85%, and most had over 95% pass quality filters. The quality of LCMS replicates ranged from 69.3 to 81.3%, with a high amount of variation among replicates (Fig. 1). For example, LACM 95619 sequence quality ranged from 67.7 to 92.05% for GS-4. In this instance, three completely different sets of alleles were recovered, providing no confidence in those genotypes despite one replicate recovering a 92.05% quality score.

Effects of microsatellite length

We recovered genotypes more frequently for shorter microsatellites across all samples. As expected, the tissue sample (HSU 8180) had consistent genotypes called across all replicates, except in one instance where a 2 bp difference was detected between PCR replicates in GS-4 (Table 3). In this case, both the bioinformatic dataset and pooled dataset recovered a homozygous genotype. Based on read depth, it is possible the minor alleles (92 and 94 in replicates 1 and 2

respectively) were sequencing errors or PCR stutter related to high depth of coverage.

The HCMS performed as well and occasionally better than the tissue sample, but often had more than two prominent sequences flagged (Table 3) and still had some missing allele calls (e.g., GS-4 in UMMZ 79755). Overall, HCMS resulting genotypes were reliable, and only appeared to lack confirmation in GLSA-52, the longest microsatellite evaluated.

The LCMS sometimes recovered accurate genotypes, however there was more variation in data quality (Fig. 1), so their genotypes required stringent evaluation. It was clear that as microsatellite length increased, genotype reliability decreased, but even the shortest marker GS-2 lacked four out of nine genotypes, of which two did not match across replicates. GS-4 had one missing and five mismatched genotypes, GLSA-12 had eight missing and one mismatch, GLSA-22 had seven missing, and GLSA-52 was missing all genotypes.

Allelic dropout rates

MicroDrop was run on the raw CHIIMP genotypes called from the pooled dataset and again on the final genotypes. In the pooled dataset, locus specific dropout rates ranged from 0 (GLSA-22) to 48% (GLSA-52) and rates across loci for each sample ranged from 0 (HSU 1836, UMMZ 79760, and LACM 95619) to 40.58% (MVZ 2088) (Table 4). Following manual genotype evaluation, locus specific dropout rates ranged from 0 (GLSA-22 and GS-4) to 36.3% (GLSA-52) and 0 (HSU 1836, UMMZ 79760) to 100% (MVZ 5211) for individual samples. When MVZ 5211 was removed, the highest rate recovered was in LACM 95619 (77.3%). The LCMS had higher dropout rates after manually evaluating genotypes due to the removal of low confidence genotypes.

Allelic dropout rates from ConGenR differed from MicroDrop which was unsurprising given their different algorithmic implementations. In the replicate dataset, locus specific dropout rates ranged from 0 (GLSA-22 and GLSA-52) to 18.75% (GS-2) and stayed the same after manual genotype evaluation (Table 4). The average rate across loci decreased, however, because the rate for GS-4 decreased from 8.33 to 0%. ConGenR also calculated overall allelic dropout rates for HCMS, LCMS, and the tissue sample (Table 4). As expected, dropout rates were highest in LCMS and lowest in the tissue sample.

Electropherogram comparisons

Genotypes from the TapeStation 2200 were either exact matches or 1–20 bp larger than the CHIIMP and ConGenR calls (GS-2, GS-4, and GLSA-52). Some alleles appeared to be larger on the TapeStation (GLSA-12 and GLSA-22) and some replicates also repeatedly failed on the TapeStation

and during GBS (MVZ 5211 at GLSA-22 and MVZ 2088 at GLSA-12, Fig. S1).

Discussion

Museum collections are increasingly being used for molecular sequencing, yet comparative studies on the retrieval and reliability of microsatellite genotypes from these data sources are not readily available. Here we show that while museum specimens can recover reliable and important genotypes for elusive species, additional precautions must be made prior to acceptance of HTS generated genotypes, particularly for LCMS.

Best practices

As depicted in Fig. 2, first bin samples based on DNA concentration. Second, we suggest performing minimally three successful PCR replicates per locus prior to genotyping and including mtDNA to ensure endogenous DNA presence. Samples deemed LCMS may require additional replication compared to HCMS. Visualization of PCR products on a TapeStation/Bioanalyzer can inform the need for additional replicates prior to library preparation and sequencing. After successful amplification, perform library preparation on PCR products and sequence to a minimum depth of 1000 reads per sample per microsatellite marker on an Illumina platform with adequate insert length. Samples should be re-sequenced if the minimum read depth is not met.

Next, run CHIIMP and prinseq-lite in parallel to generate genotypes and evaluate sequence quality. Samples with low quality scores from prinseq should be noted as they may be prone to erroneous genotypes. Low concentration samples should be evaluated for mismatched genotypes to determine where the differences occur (e.g., primer region or repeat elements). If the primer sequence varies, manually correct the length as if the entire primer sequence was included, and ignore primer site size mismatches as this is likely an artifact of sequencing or amplification errors. If an allele does not have a priming site error, it is important to evaluate if the size shifts follow evolutionary patterns. For example, if a dinucleotide sequence shifts by two base pairs that makes evolutionary sense. However, stutter sequences are often frame shifted by the repeat motif size, and stutter evaluation in dinucleotide microsatellites is challenging due to the short difference between true alleles and stutter peaks (O'reilly et al. 2000; Barbian et al. 2018). Thus, we also recommend designing tetranucleotide assays whenever possible. CHIIMP calculates the proportion of reads associated with various genotypes, and based on the proportion of sequences with one repeat motif difference it can be flagged as stutter (a modifiable parameter).

Across the replicate dataset we evaluated differences in individual PCR replicate genotypes from samples of varying DNA concentrations. We found HCMS were generally in agreement across the three datasets and provide justification for pooling PCR replicates prior to library preparation, significantly reducing cost. The LCMS were variable, but still often yielded the same allele calls from all three datasets. If a critical sample yields low DNA concentrations, individual library preparation can alleviate dropout concerns in those samples. Otherwise, LCMS individuals should be excluded from population genetic studies.

Microsatellite recovery

Our data separated samples into three categories before genotyping: tissue, HCMS, and LCMS, based on DNA concentration. Genotypes were recovered at a higher rate than expected based on failed PCRs from LCMS. The LCMS recovered a PCR amplification success rate of 21.4% (Table S3) yet recovered 30 genotypes out of 72 possible (42%, Table 3). This did not include removal of problematic genotypes. When only agreeable genotypes were included, this reduced the 30 genotypes to 6 or 8.3%. Despite this low rate of confirmation among the LCMS, this study quantifies rates for genotyping success via GBS on degraded museum specimens for the first time. Alternatively, for HCMS the rate of recovered genotypes was 91.7% (66 out of 72), and 86.1% (62 out of 72) for agreeable genotypes. For the tissue sample, 100% (16 out of 16) of the replicates resulted in a genotype, with 87.5% (14 out of 16) confirmed by the second genotype recovered. This data provides robust support that the rate of disagreement in the HCMS is negligible, only 1.4% lower than the tissue sample, and has shown that samples which do not reliably amplify in PCR are prone to inaccurate genotypes.

Again, the LCMS genotypes recovered required fine scale evaluation to ensure accuracy and repeatability for downstream analyses, as inaccurate allele calls could affect population genetic inferences. Variable genotypes were much more prevalent in LCMS (16 instances in the LCMS versus only two in the HCMS), but may not be specifically due to allelic dropout as in fecal samples (Piggott et al. 2004; Regnaut et al. 2006), since alleles outside the expected bin sizes were recovered (see GS-4 for the LCMS) and only rarely was potential allelic dropout recovered (see GS-2 for LACM 95619). Further optimization of the size buffer setting in CHIIMP may eliminate these alleles, as CE analysis would traditionally ignore peaks outside of the expected size range in programs like GeneMapper™ (Applied Biosystems).

We were not able to compare the final genotypes recovered here to other population genetics studies on *G. oregonensis*, as this is the first time such a study has occurred in this species. The study performed by Barbian et al. (2018)

used GBS to re-genotype chimpanzees with known life-history data and previous CE genotypes, and noted a shift of 1–3 bp in genotype results between their CE and GBS data, which we also recovered with certain loci across the TapeStation electropherograms. We noticed larger fragments (~ 17–45 bp) from the TapeStation than the GBS data at locus GLSA-12 and GLSA-22, which may result from bioinformatic adapter removal steps. Many studies have reported shifted alleles of the same PCR products on different runs of an automated capillary sequencer or with a different size standard (Haberl and Tautz 1999; Ellis et al. 2011).

In concordance with Campana et al. (2012), there was no direct correlation between mtDNA and nDNA amplification success rates in our data. All samples recovered reliable mtDNA signatures, even though many (particularly the LCMS) lacked nDNA at microsatellite loci (Supplemental Materials). Additionally, the HCMS recovered reliable genotypes across all markers, but the longer the microsatellite, the worse the locus amplified. This was especially apparent for the LCMS which failed to recover genotypes for the longest locus. Preferential amplification of short microsatellites was also observed in Rizzi et al. (2012) and Wandeler et al. (2003).

Most genotypes generated by ConGenR matched our final genotypes (Table 3), though in some cases ConGenR indicated uncertainty (denoted with a '0'). ConGenR also assigned genotypes to MVZ 5211 at GS-4 and MVZ 2088 at GLSA-12, but we did not as MVZ 5211 had variable allele calls and MVZ 2088 had only one amplified PCR replicate at that locus. For MVZ 2088 at GS-4, the 92/96 genotype ConGenR assigned and the 96/96 genotype we assigned were actually the same sequence with the same number of repeats, but the 96 bp allele contained four more reverse primer base pairs (Online Appendix 1).

The CHIIMP pipeline (Barbian et al. 2018) worked well for our samples, and allowed for optimization and customization of commands for recovering more strict or lenient genotypes. The combination of multiple replicates of PCR, quality filtering, and manual evaluation of CHIIMP results increased our confidence in the genotypes recovered by museum specimens in this study. However, it also highlighted various methods microsatellites could be genotyped which stresses the need to standardize genotyping procedures.

Allelic dropout rates

According to MicroDrop, we recovered high rates of allelic dropout averaging 12.6% in the pooled dataset across all five microsatellite loci. After manual editing, the average rate of allelic dropout decreased to 9.8% across all loci. The average rate of allelic dropout across all loci according to ConGenR, however, was 7.23%, and this rate was reduced to 5.57%

after manual evaluation. ConGenR calculates dropout rates based completely on PCR success, so the difference between MicroDrop and ConGenR was within expectations.

MicroDrop allelic dropout rates in the LCMS (average of 19.6%) conformed to rates reported for various studies of avian fecal allelic dropout (mean of 21%, Regnaut et al. 2006) but was much higher than rates reported from chimpanzee fecal samples via GBS (7%, Barbian et al. 2018). This may be due to the fact that Barbian et al. (2018) only genotyped samples at loci that had over 500 reads (counts.min = 500). ConGenR also reported very high LCMS dropout rates (33.3%), but the rate did decrease to 20% after manual evaluation which would conform to rates reported in Regnaut et al. (2006). Results from Donaldson et al. (2020) corroborated our findings, and noted that allelic dropout and false alleles increased while PCR success decreased following sample dilutions.

The HCMS all recovered very low rates of allelic dropout, 2.4% in MicroDrop and 5% in ConGenR, which decreased to < 0.001% and 4.76% respectively after manual evaluation. These three samples performed on par with GBS studies derived from tissue samples (Darby et al. 2016) and provide robust evidence for the utility of museum specimens in recovery of microsatellite genotypes.

In our tissue sample, raw results from CHIIMP generated a high rate of allelic dropout in MicroDrop (14.7%) compared to 0.4% in Darby et al. (2016). Following manual evaluation, the rate was reduced to 8.5%, which was still higher, but this was partially due to one instance of primer trimming (GS-4).

Dropout rates for the pooled dataset separated by length were 7.5% for short, 0.35% for medium, and 48% for long loci. The higher rate observed in short loci is likely attributed to non-specific amplification or higher amounts of stutter and PCR artifacts. After manual evaluation, we recovered a 0.0025%, 6.3%, and 36% dropout rate for short, medium, and long loci respectively.

Number of alleles

We found that the number of alleles recovered by CHIIMP in all datasets was higher than in our final genotypes due to removal of inaccurate alleles from the raw data. The number of alleles per locus remained the same at two loci (GS-2 and GLSA-12, although a different allele was present in the final genotypes for both loci; allele '96' in GS-2 and '162' in GLSA-12), and decreased in the other three loci. Compared to the number of alleles recovered from *G. sabrinus*, we recovered fewer alleles in GS-2, GS-4, GLSA-22, and GLSA-52, and more alleles in GLSA-12. However, previous research in *G. sabrinus* included more individuals and a wider geographic distribution. Therefore, we do not expect the alleles recovered here to be exhaustive for

G. oregonensis, and our counts seem reasonable for seven individuals.

Furthermore, multiple studies have noted recovery of additional allelic diversity from GBS, traditionally lost in CE, as a result of direct access to allele sequences and the ability to evaluate homoplasy. For example, Darby et al. (2016) found a 44% increase in alleles after accounting for homoplasy in their dataset (164 to 294 alleles). We also recovered homoplastic events in the replicate dataset (Online Appendix 2), and recovered 28 unique alleles based on fragment length but 39 unique alleles based on whole sequence polymorphisms (28.2% increase). The increase in alleles was most prominent in GLSA-52 (3–9 alleles).

Conclusion

GBS is an effective way to generate affordable genotype results for degraded specimens when stringent protocols and deep sequencing is performed. Our costs were under ~\$22 per sample (Online Appendix 3), which is higher than other GBS studies (Darby et al. 2016), however, we only performed singleplex PCR, and if multiplex PCRs were performed the cost could be significantly reduced.

Several bioinformatic pipelines have already been developed to generate microsatellite genotypes from HTS data (De Barba et al. 2017; Barbian et al. 2018; Pimentel et al. 2018; Tibihika et al. 2019), and have screened a variety of starting template types including tissue, hair, and fecal samples. However, this is the first time GBS methods have been applied to evaluate allelic dropout rates from mammalian study skin derived DNA samples. Our results show that when binning samples by DNA concentration, robust genotyping can be recovered from museum specimens, especially for samples deemed HCMS. On the other hand, repeated PCR is necessary for LCMS, and this does not completely eliminate the opportunity for false genotypes to be incorporated into the dataset.

Museum specimens are important as they provide temporal perspective and inclusion of rare species, but appropriate QC measures need to be undertaken to ensure accurate genotypes. Our data demonstrate the ability to reliably incorporate microsatellite genotypes from early twentieth century museum study skins, in combination with modern surveys, to evaluate spatial and temporal shifts in population genetics.

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Author contributions MTRH and SCY conceived of the study and performed laboratory work. SCY and EM performed bioinformatics. MTRH and SCY wrote the manuscript and performed statistical analyses. All authors analyzed the data and approved of the final manuscript.

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Data availability Cytochrome-*b* sequences can be found on GenBank accessions MT498442–MT498448. Raw data from all microsatellite replicates have been uploaded to GenBank SRA under the following Bioproject: PRJNA721448 and Biosamples: SAMN18718222–8. Microsatellite output files from the CHIIMP pipeline can be found at Figshare: 10.25573/data.13491642; 10.25573/data.13491792; 10.25573/data.13492041.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Animal research Not applicable.

Consent to participate Not applicable.

Consent to publish All authors are aware and consent to publish.

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