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Tools and Technology

Bringing Back the Dead: Genetic Data from Avian Carcasses

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ABSTRACT Advances in laboratory methods and DNA sequencing technology have enabled genetic analyses of noninvasive samples, museum specimens, fossil material, and more recently, carcasses. We extracted DNA from decayed avian carcasses that were salvaged from a marine environment in southern Texas, USA, during 2010–2014, at 2 weeks to 3 months postmortem. Extracts of DNA from toepad, bone, and feather yielded usable DNA for molecular sexing and amplification of a 223–base pair portion of the mtDNA control region. Most samples displayed signs of degradation, including small fragment sizes and heteroplasmy in mtDNA sequences consistent with deamination during the decay process. The ability to extract usable DNA from avian carcasses salvaged in a marine environment has implications for the ecology and management of waterbirds, many of which are rare, sexually monomorphic, and poorly understood.

KEY WORDS avian carcass, genetic analysis, mitochondrial DNA, molecular sexing.

Extraction of genetic material from noninvasive or fossil specimens is a tedious and nontrivial procedure because of low quantity and quality of DNA. Degradation of DNA begins soon after the death of the organism or deposition of material into the environment. Once cellular repair and maintenance of DNA ceases, nucleic acids are exposed to enzymatic decay, microorganisms, and environmental damage via breakage, lesions, oxidation, and reaction with organic molecules (Pääbo et al. 2004). The most obvious result is fragmentation of DNA strands into pieces <500 base pairs (bp) in size. However, lesions or oxidation can interfere with polymerase chain reaction (PCR) amplification of intact DNA sequences. Furthermore, sequencing errors become more common, attributable to deamination of DNA bases that results in C→G to T→A transitions (Stiller et al. 2006). Eventually, DNA is degraded to the point that all information is lost.

Advanced methods for the extraction, preservation, and amplification of DNA have revolutionized use of genetic data in studies of wildlife (DeYoung and Honeycutt 2005). Noninvasive sampling of genetic material derived from hair, feces, saliva, or diet items has become commonplace (Waits and Paetkau 2005). Museum specimens are increasingly used as sources of genetic material, decades to centuries after collection (Wandeler et al. 2007, Bantock et al. 2008). The ability to analyze ancient DNA from fossil materials has provided additional insights into the phylogeographic and demographic history of both extant and extirpated taxa (Bunce et al. 2003, Huynen et al. 2003, Allentoft et al. 2010).

Despite the technological advances in amplification of low-quality DNA, use of wildlife carcasses for DNA sampling is not common; this may be due to the condition of the carcass, stage of decay, and associated perceptions of sample degradation. Several recent studies have reported successful genetic analyses on samples obtained from mammalian and fish carcasses (Takahashi et al. 1998, Merkes et al. 2014, Korstian et al. 2016). The use of decayed avian carcasses in genetic analyses is not well-documented, but would be a valuable tool for studying species that are rare, elusive, or difficult to capture, including shorebirds, wading birds, and seabirds. However, many species of waterbirds live in warm, humid climates, where environmental conditions are not conducive to the preservation of DNA. In 2010–2013, we captured and tagged 22 reddish egrets (*Egretta rufescens*) with satellite transmitters (Koczur 2017). Feather samples were obtained at capture, but attempts at molecular sexing were unsuccessful. Like many species of waterbirds, reddish egrets are sexually monomorphic, making molecular sexing the most accurate technique for determining gender. Seven of the marked reddish egrets died during the study period, and carcasses were recovered. Our objectives were to 1) determine whether usable DNA could be obtained from tissues taken from carcasses in advanced stages of decay, 2) identify the sex of individual

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reddish egrets, and 3) amplify and sequence a portion of the mitochondrial DNA control region.

STUDY AREA

The Laguna Madre, Texas, USA, was a shallow, hypersaline lagoon consisting of large expanses of wind tidal flats. It extended approximately 185 km from north to south and averaged 7 km in width. The climate was subhumid-to-semiarid east-coast subtropical (Fulbright et al. 1990); precipitation was highly variable and average temperatures ranged from 8.3° C to 33.3° C (Brown et al. 1976). Carcasses were recovered from the Laguna Madre (26°52′17.10″N, 97°26′33.95″W) and adjacent Baffin Bay (27°16′2.89″N, 97°34′54.39″W), and found in a range of conditions from on islands in dry vegetation to wet tidal flats.

METHODS

We collected 7 adult reddish egrets postmortem from islands and mud flats within the Laguna Madre and Baffin Bay, Texas, during 2010 to 2014 (Fig. 1). Duration between death and retrieval ranged from about 2 weeks to 3 months based on satellite telemetry history. Carcasses were frozen after collection. In 2014, we captured 8 additional egrets for an ongoing study, and collected blood samples (1.5 mL), which were stored at 2° to 8° C (Texas A&M University-Kingsville, Institutional Animal Care and Use Committee #2013-05-23). Molecular sexing and sequencing mtDNA from living and decayed egrets allowed us to use DNA extracted from the living birds to assess the quality of genetic material obtained from carcasses.

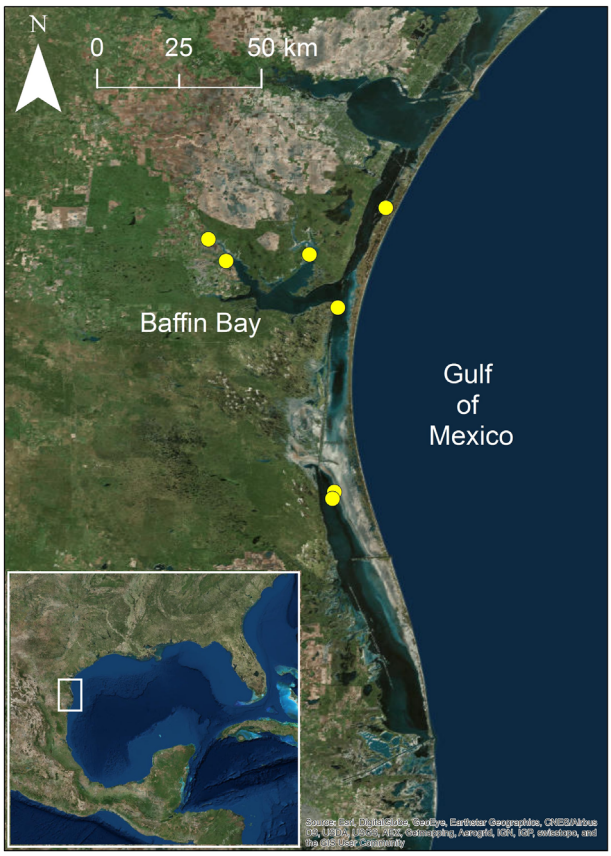

Figure 1. The Laguna Madre, a hypersaline estuary in southern Texas, USA, showing collection locations of reddish egret carcasses during 2010–2014.

We targeted bone and footpads as tissues most likely to preserve DNA in carcasses because of their prior use with museum specimens and fossil remains. We also attempted to extract from flight feathers because DNA might be preserved in the calmus. Tissues obtained from carcasses are vulnerable to contamination from scavengers, as well as microbial and fungal DNA from the process of decay (Mulligan 2005). We developed an anticontamination protocol based on Yang et al. (1998), Hummel (2003), Pääbo et al. (2004), and Mulligan (2005) for handling samples derived from carcasses. Bleach (sodium hypochlorite) and ultraviolet (UV) light are often used to remove contaminating contemporary DNA from the surface of fossils and archaeological and museum specimens (Kalmár et al. 2000, O'Rourke et al. 2000, Gilbert et al. 2007, Hekkala et al. 2011, Lacan et al. 2011). The hydroxyapatite, which comprises most of the bone matrix, is resistant to bleach and protects endogenous ancient DNA from the effects of bleach (Kemp and Smith 2005). Ancient DNA has also been extracted successfully from feathers decontaminated with bleach (Rawlence et al. 2009, Olsen et al. 2012). We also hypothesized that the thick, heavily keratinized skin of the legs and feet of reddish egrets would provide protection to endogenous DNA. Exposure to UV light will degrade exposed DNA on the surface of the tissue, but will not harm endogenous DNA located within the tissue. We soaked bones, pieces of footpad skin, and feathers in 100% bleach for 5 min, then in deionized water for 5 min. We then placed all samples under UV light for 1 hr. We conducted DNA extractions and preamplification procedures in a separate laboratory, where no previous genetic work on herons and egrets had occurred. We used separate equipment, supplies, and reagents for carcass-derived and contemporary samples. Prior to working with carcass-derived samples, we sterilized equipment and benchtops with 20% bleach solution; we exposed smaller equipment and supplies to UV light for 1 hr. We prohibited re-entry into the laboratory designated for carcass-derived samples on the same day that we worked in the "contemporary" DNA lab.

We prepared bones for DNA extraction by exposing them to liquid nitrogen until they became brittle. We then crushed the bones into powder using mortar and pestle. We used separate mortars and pestles for each bone and processed samples one at a time. We extracted genomic DNA from blood ($n = 8$), footpad skin ($n = 3$), feathers ($n = 4$), and powdered bone ($n = 6$) using a commercial kit (Puregene DNA Purification Kit, Qiagen, Valencia, CA, USA). We used each type of sample from individual carcasses that was available; some carcasses did not have the legs and feet intact upon collection as a result of scavenging or decay. We performed duplicate extractions from bone and footpad skin and single extraction from feathers; negative controls were done for every tissue extraction. We assessed the quality of

the DNA extracted from carcasses via gel electrophoresis on a 1% agarose gel containing ethidium bromide, viewed under UV light. High-quality genomic DNA will appear as bright, compact bands indicative of high molecular weight, whereas degraded genomic DNA will produce smears (Reynolds et al. 1991).

Molecular sexing was accomplished by amplifying portions of the chromo-helicase-DNA (CHD) binding protein genes located on the W and Z sex chromosomes using the primers WZ-common (5′-CCCTTCACTTCCATTAAAGC-3′), W-specific (5′-CCCAACCCAAAAG TACAAG-3′), and P8 (5′-CTCCCAAGGATGAGRAAYTG-3′) to amplify portions of the CHD-W and CHD-Z genes (Griffiths et al. 1998, Jensen et al. 2003, Wang et al. 2011). We fluorescently labeled the P8 primer (5′ HEX) to enable detection and sizing of fragments on an automated DNA sequencer. We amplified CHD genes using the PCR in 20-μL reaction volumes containing 1.0 μL of genomic DNA extract, 10 μL of AmpliTaq® Gold PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 10 pmol of each primer, 1.0 μL of bovine serum albumin (2 mg/mL, Thermo Fisher Scientific), and sufficient double-deionized water to reach the final volume. We performed all PCR reactions using an ABI 2720 thermal cycler (Thermo Fisher Scientific) with the following protocol: initial denaturation at 94° C for 10 min; 45 cycles of denaturation at 94° C for 30 s, annealing at 46° C for 45 s, extension at 72° C for 25 s, and a final extension at 72° C for 5 min. We included 2 negative PCR controls and negative extraction controls in each round of amplification of CHD genes from carcasses to detect contamination of samples or reagents, or pipetting errors. The DNA extracted from blood samples served as positive controls.

The primer pair P8/WZ-common amplified 250 bp of CHD-Z and the P8/W-specific pair amplified 140 bp from CHD-W (Wang et al. 2011). Females are the heterogametic sex in birds (WZ), and males are homogametic (ZZ). Therefore, the electropherograms from females should exhibit 2 peaks, the 250-bp fragment from CHD-Z and the 140-bp fragment from CHD-W; males will display a single peak at 250 bp. We combined 1.0 μL of PCR product from each reaction with a denaturing mixture of 11.0 μL of Hi-Di Formamide and 0.5 μL of ROX 500 size standard (Thermo Fisher Scientific). We then loaded PCR product mixtures on to an ABI Prism 3130xl Genetic Analyzer (Thermo Fisher Scientific) for separation and detection. We quantified fragment sizes and resolved them into male or female genotypes using GeneMapper version 4.0 (Thermo Fisher Scientific), followed by visual inspection and verification.

To further assess the quality of DNA obtained from carcasses, we amplified a portion of the mitochondrial control region and compared our results to those found by Bates et al. (2009). We amplified a 223-bp portion of the mitochondrial control region using forward (5′-CCTGTACTAGAACCAT-3′) and reverse (5′-TGTGAAGAGCGAGT-3′) primers developed by Bates et al. (2009) following the protocol therein. We included 2 negative PCR controls and the negative extraction controls in each round of control-region amplification involving tissues from carcasses to guard against contamination of samples or regents, or pipetting error.

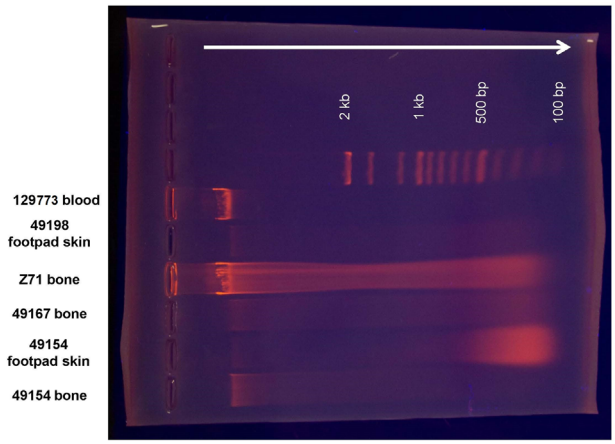

Figure 2. Electrophoresis of total DNA extracted from bone and foot pad skin samples obtained from salvaged carcasses of reddish egrets in southern Texas, USA, during 2010–2014 compared with total DNA extracted from a blood sample. Note the streaks produced by extracts obtained from carcasses, which indicates low quality DNA due to postmortem degradation.

We ran all PCR products on a 1% agarose gel containing ethidium bromide to verify successful amplification. We purified products from successful amplifications via an enzymatic method (ExoSAP-IT™, Affymetrix, Inc., Santa Clara, CA, USA) and sequenced them in both directions using the BigDye Terminator v1.1 Cycle Sequencing Kit

Table 1. Results of sexing by genotyping for blood- and carcass-derived samples of reddish egrets sampled in southern Texas, USA, during 2010–2014.

Sample	Tissue	Sex
49148	Bone	M
	Bone	M
49154	Bone	M
	Bone	M
	Foot pad skin	M
49167	Bone	M
	Bone	M
	Foot pad skin	M
49198	Bone	F
	Bone	F
	Primary feather	F
49422	Primary feather	M
49422b	Bone	M
	Bone	M
	Primary feather	M
Z71	Bone	M
	Bone	M
	Foot pad skin	M
	Foot pad skin	M
49166	Blood	F
49167	Blood	F
49196	Blood	F
129770	Blood	F
129771	Blood	M
129772	Blood	M
129773	Blood	F
129774	Blood	F

(Thermo Fisher Scientific). We removed dye terminators using the DyeEx 2.0 Spin Kit (Qiagen), and loaded sequencing reaction products on an ABI Prism 3130xl Genetic Analyzer for separation and detection. We visually inspected the electropherograms of each sequence and constructed contigs from the forward and reverse sequences using the computer programs Sequencher, version 4.6 (Gene Codes, Ann Arbor, MI, USA) and Geneious, version 10.0.2 (Kearse et al. 2012). We verified species identity of each control-region sequence using the Basic Local Alignment Search Tool (BLAST) algorithm (Zhang et al. 2000, Morgulis et al. 2008) in the GenBank database (www.ncbi.nlm.nih.gov, accessed 2 Dec 2014). We checked for evidence of heteroplasmy, the presence of >1 distinct mitochondrial haplotypes in the same individual sample, which may indicate degradation (Avise 2000). We inspected electropherograms of each contig for the presence of mismatched nucleotide bases in forward and reverse strands and marked heteroplasmic positions with the appropriate ambiguity code (Bowden-Cornish 1985). We further assessed the extent of postmortem degradation in carcass-derived control region sequenced 2 ways. First, we examined nucleotide composition among samples and replicates using the computer program MEGA7 (Kumar et al. 2016). Postmortem degradation may cause DNA sequences to vary in nucleotide composition among tissues types and replicates from the same sample. Second, we aligned carcass- and blood-derived control-region sequences with control-region sequences from Bates et al. (2009) using the computer program Clustal X (Larkin et al. 2007). We then examined the relationships among control-region sequences from our samples, including all possible haplotypes from heteroplasmic samples, and those of Bates et al. (2009) by constructing a median joining network (Brandelt et al. 1999) using the computer program SplitsTree4, version 4.14.4 (Huson and Bryant 2006). We also estimated haplotype (*Hd*) and nucleotide diversity (π) of the entire data set using the computer program DnaSP, version 5 (Librado and Rozas 2009) for comparison with results obtained by Bates et al. (2009). We resequenced heteroplasmic samples if novel haplotypes were observed and performed the analysis again with the new sequences. Postmortem degradation that causes a limited number of nucleotide single base changes may result in "novel" haplotypes that are closely related (separated by 1–3 mutational steps) to previously observed haplotypes, whereas severe degradation may result in sequences that do not align properly or "novel" haplotypes that are separated from most other haplotypes by >3 mutational steps.

RESULTS

We successfully extracted DNA from all samples obtained from reddish egret carcasses. Gel electrophoresis indicated that genomic DNA extracted from carcasses was of lower quality, as evidenced by smears produced by carcass-derived genomic DNA in comparison with bright, compact bands from blood-derived genetic material indicative of high molecular weight DNA (Fig. 2). We amplified DNA in all toe pad, bone, and feather samples. We were able to

Table 2. Carcass-derived mitochondrial control-region sequences of reddish egrets sampled in southern Texas, USA, during 2010–2014 showing the variable sites at positions 1–9, 29, and 78. A blood-derived control-region sequence is included for comparison. The letters R and Y represent ambiguous nucleotides (R = A or G, Y = C or T).

Sample	Tissue	Replicate	1	2	3	4	5	6	7	8	9	29	78
129770	Blood	1	T	T	T	G	G	A	G	C	G	A	T
49148	Bone	1	.	.	.	.	.	.	.	.	.	G	Y
49148	Bone[a]	1	.	.	.	A	.	.	.	.	.	.	.
49148	Bone	2	.	.	.	A	.	.	.	.	.	.	.
49154	Bone	1	.	.	.	.	.	.	.	.	A	.	.
49154	Bone	2	.	.	.	.	.	.	.	.	A	.	.
49154	Skin	1	.	.	.	.	.	.	.	.	A	.	.
49154	Skin	2	.	.	.	.	.	.	.	.	A	.	.
49167	Bone	1	C	.	.	.	.	.	.	.	.	.	.
49167	Bone	2	.	.	.	.	.	.	.	.	.	.	.
49167	Skin	1	C	.	.	.	.	.	.	.	.	.	.
49167	Skin	2	C	.	.	.	.	.	.	.	.	.	.
49198	Bone	1	.	.	.	A	.	.	.	.	.	.	.
49198	Bone[a]	1	.	.	.	.	.	.	.	.	.	G	R
49198	Bone	2	.	.	.	.	.	.	.	.	.	G	R
49198	Bone[a]	2	.	.	.	.	.	.	.	.	.	G	Y
49198	Feather	1	.	.	.	.	.	.	.	T	.	G	R
49198	Feather[a]	1	.	.	.	.	.	.	.	T	.	G	Y
49422b	Bone	1	.	.	.	.	.	.	.	.	.	.	.
49422b	Bone	2	.	.	.	.	.	.	.	.	.	.	.
49422b	Feather	1	.	.	.	.	.	.	.	.	.	.	.
49422b	Feather[a]	1	.	.	.	.	.	.	.	.	.	.	.
Z71	Bone	1	.	.	.	.	.	.	.	.	.	.	.
Z71	Bone	2	.	.	.	.	.	.	.	.	.	.	.
Z71	Skin	1	.	.	.	.	.	.	.	.	.	.	.
Z71	Skin	2	.	.	.	.	.	.	.	.	.	.	.

[a] Resequenced heteroplasmic sample replicates.

Table 3. Mitochondrial DNA control-region haplotypes observed in blood and carcass-derived samples from reddish egrets marked in southern Texas, USA, during 2010–2014. We present for each individual the number of haplotypes observed (*H*, including all potential haplotypes from heteroplasmic sequences), number of novel haplotypes, number of haplotypes previously observed (known) by Bates et al. (2009), and sequencing quality score or range of scores (HQ%) of contigs.

Sample	Tissue	*H*	Novel haplotypes?	Known haplotypes	HQ%
49148	Bone	3	Yes (2)	G	84.0–93.4
49154	Bone, foot pad skin	1	No	H	90.0–95.0
49167	Bone, foot pad skin	2	No	A, B	84.2–98.4
49198	Bone, feather	9	Yes (6)	D, E, G	83.1–96.8
49422b	Bone, feather	1	No	A	74.3–96.6
Z71	Bone, foot pad skin	1	No	A	86.1–98.1
49166	Blood	1	No	A	96.9
49167	Blood	1	No	A	97.8
49196	Blood	1	No	E	97.8
129770	Blood	1	No	A	98.1
129771	Blood	1	No	D	97.2
129772	Blood	1	No	F	96.9
129773	Blood	1	No	B	99.1
129774	Blood	1	No	A	100.0

determine sex of all carcasses (1 F, 6 M) and all blood extracts (6 F, 2 M). Results of sexing were identical among duplicated extracts and replicated PCR runs (Table 1).

We obtained control-region sequences from 8 blood, 3 footpad, 6 bone, and 2 of 3 feather samples (6 of 7 carcasses). We deposited control-region sequences of blood- and carcass-derived sequences in GenBank as accession numbers KY499839-KY499871. We observed no mismatches among forward and reverse strands for any of the blood samples. We detected mismatches in carcass-derived replicates from samples 49148 and 49198 at position 78; however, the presence of mismatches among forward and reverse strands was not consistent among replicates for either sample (Table 2). We detected no additional mismatches among forward and reverse strands. We observed within-sample sequence variation among replicates at positions 1, 4, 9, and 29 for samples 49148, 49167, and 49198. However, within-sample sequence variation among replicates was limited to 1–2 base changes. Most of the heteroplasmic sites involved C→T and G→A transitions; evidence of transversions was apparent in sample 49198. As a result of within-sample sequence variation, >1 haplotypes, including 6 "novel" variants, were observed among samples 49148, 49167, and 49198 (Table 3). These 6 "novel" variants were closely related to previously observed haplotypes, separated by 1–3 mutational steps (Fig. 3). All blood samples and carcass-derived samples from 49154, 49422b, and Z71 were identical to previously observed haplotypes. Haplotype and nucleotide diversity of the entire data set was $Hd = 0.746$ (SD = 0.029) and $\pi = 0.006$ (SD = 0.0004), respectively. Haplotype ($Hd = 0.700$, SD = 0.033) and nucleotide diversity ($\pi = 0.005$, SD = 0.0004) was slightly lower after the removal of the 3 heteroplasmic samples. Control-region sequences from blood samples had higher quality scores (HQ% = 97–100%) than most of the carcass-derived samples (HQ% = 74–98).

DISCUSSION

Our results show that small fragments of nuclear and mitochondrial DNA can be successfully amplified from avian carcasses, despite advanced degradation in a marine environment. Previous studies have demonstrated that bone generally yields greater amounts of, and higher quality, ancient DNA than is yielded by soft tissues (Lassen et al. 1994). In addition, ancient DNA is more likely to be obtained from the heavily keratinized skin of the foot than feathers (Mundy et al. 1997). In general, we were successful

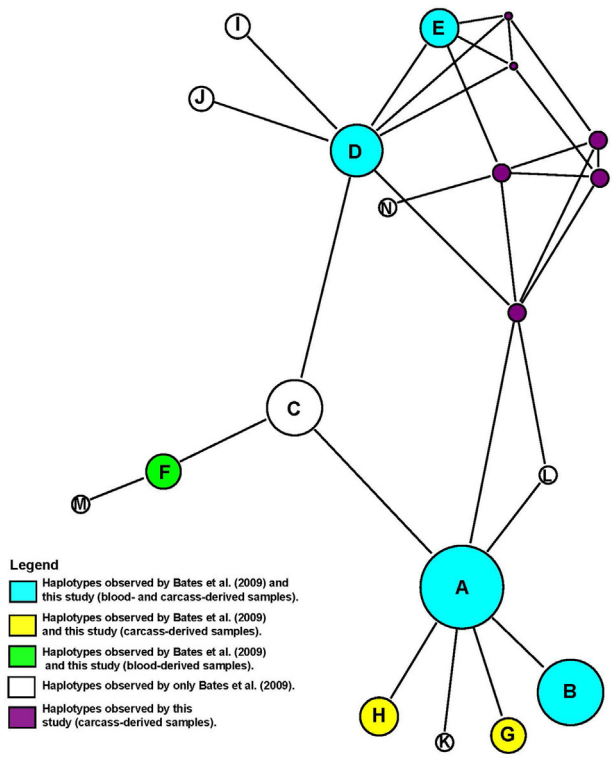

Figure 3. Median joining network of mitochondrial control-region haplotypes (223 bp) from reddish egrets marked in southern Texas, USA, during 2010–2014. Each circle represents a unique haplotype. The size of each circle proportional to haplotype frequency. Boldface letters (A, B, C, etc.) designate individual haplotypes previously observed by Bates et al. (2009). Colors of haplotypes reflect whether a blood- or carcass-derived control-region sequence or both was identical to these haplotypes. Line segments connecting haplotypes represent a single base substitution.

in obtaining useful DNA from all tissue types and most mitochondrial control-region sequences matched previously identified haplotypes.

Although we were able to assign sex to all of the carcass samples, we observed a skew in sex ratio between the samples derived from live birds versus carcasses. Allelic dropout is a common problem for low-quality samples, including many studies that employ noninvasive samples (Burger et al. 1999, Mills et al. 2000, Sefc et al. 2003). The frequency of allelic dropout is often positively correlated with the size of the fragment targeted by amplification, where large fragments are more likely to drop out (Sefc et al. 2003). If allelic dropout had occurred, we would expect more samples genotyped as females because the CHD-Z fragment, which occurs in both sexes, is larger (250 bp) than the female-specific fragment, CHD-W (140 bp). Only 1 of the 7 carcass samples was determined to be female. Furthermore, we performed 2–3 replicate amplifications for each sample, and results were consistent among tissues from the same individual. Previous studies of reddish egrets have reported skewed sex ratios in small samples of hatch-year and adult birds (Geary et al. 2015, Koczur et al. 2015), which may be due in part to the timing or methods of capture. In addition, these carcasses represent mortalities, which may indicate sex-bias in survival. Overall, we have no indication that contamination or allelic dropout influenced sex ratio in the carcass or live bird samples.

Vertebrate heteroplasmy can result from somatic mutations in individuals, the origin and survival of variants within a female lineage (Avise 2000), or (rarely) "paternal leakage" or transmission of mitochondria from sperm to zygote (Bromham et al. 2003, Kvist et al. 2003, Zhao et al. 2004). Bates et al. (2009) observed no evidence of heteroplasmy in 149 mitochondrial control-region sequences from reddish egrets. The occurrence and distribution of heteroplasmy among tissue replicates that we observed was most likely caused by postmortem DNA damage. Hydrolytic and oxidative damage to DNA molecules occurs rapidly after death as a result of the lysosomal nucleases, activities of bacteria, fungi, and scavenging insects, and abiotic factors, such as high temperatures and extremes of pH (Hummel 2003, Pääbo et al. 2004). Deamination is a common form of molecular damage observed in DNA extracted from the remains of dead organisms and is especially rapid for cytosine (Lindahl 1996, Hofreiter et al. 2001, Stiller et al. 2006, Sefc et al. 2007). Deamination converts cytosine to uracil and adenine to hypoxanthine, which results in cytosine-to-thymine (C→T) and guanine-to-adenine (G→A) transitions during PCR (Lindahl 1996, Hofreiter et al. 2001, Gilbert et al. 2003). The oxidation of cytosine, thymine, and guanine will produce transversions (C→A, G→T, C→G, and G→C; Pääbo 1989, Lamers et al. 2009). Transitions are more common in postmortem DNA than transversions (Pääbo 1989). Our results are consistent with previous observations in that most of the heteroplasmy was due to transitions rather than transversions. Within-sample heteroplasmy suggests that postmortem degradation has affected sequence composition and quality of mitochondrial DNA in bone, foot pad skin, and feather replicates. This conclusion is further supported by the fact that control-region contigs derived from carcasses had overall lower quality scores than those from blood samples.

Our results show that usable DNA can be derived from decayed avian carcasses collected in a marine environment 2 weeks to 3 months postmortem. Many species of waterbirds are sexually monomorphic, and researchers must rely on molecular sexing. Therefore, little is known about sex-specific behavior or population demographic parameters. The ability to amplify genetic markers in extracts from waterbird carcasses could provide valuable information on longevity and mortality in males and females, migratory strategies, habitat use, site fidelity, and sex-biased dispersal. Although carcasses may be a valuable source of mitochondrial DNA, researchers should evaluate sequences for signs of degradation, including heteroplasmy and consistency among replicate extracts from different tissues. Damaged DNA obtained from fossils or museum specimens may lead to inaccurate or misleading estimates of phylogenetic relationships, genetic diversity, and population sizes (Ho et al. 2007, Axelsson et al. 2008). Spurious or novel control-region haplotypes of reddish egrets attributable to deamination or other postmortem damage were 1–3 bp from previously observed haplotypes. The inclusion of DNA sequences from heteroplasmic samples resulted in greater haplotype and nucleotide diversity, whereas the exclusion of these samples resulted in estimates of genetic diversity similar to those obtained by Bates et al. (2009; $Hd = 0.705$, $\pi = 0.005$). Although in this case, the inclusion of spurious haplotypes would have had a minor effect on phylogeographic structure and genetic diversity, our results reinforce the previous suggestion that studies based on DNA derived from fossils, carcasses, and archaeological and museum specimens should estimate the extent of postmortem molecular damage and consider how it may have impacted estimates of genetic relatedness and diversity (Axelsson et al. 2008).

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