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Using Non-Invasive Sampling Techniques to Test Genetic Markers for the Little Blue Heron (*Egretta caerulea*)

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ABSTRACT

As with most wading birds in North America, the true conservation status of the Little Blue Heron, (*Egretta caerulea*) is unknown. Even though wading bird census is still improving, it has been evident that populations have been declining for decades. Environmental stressors, both natural and anthropogenic, are the main contributing factor to the decline observed in heron populations. Microsatellite genetic markers can be used to evaluate the genetic diversity and overall health of a population. Microsatellite markers were chosen for this study to demonstrate that population genetic studies can be conducted with non-invasive DNA sampling because microsatellites are PCR based and only require a minimal amount of DNA. We determined that the markers created for other herons from the same genus (Egretta) along with other closely related herons had a successful cross amplification in the Little Blue Heron. We found that usable quantities of DNA can be extracted from salvaged heron artifacts including carcasses, eggshells and molted feathers. This study provides a resource for future conservation genetic studies and population genetic studies on herons and other birds as well.

1 INTRODUCTION

The Little Blue Heron, or *Egretta caerulea*, is a common resident of the marshes and estuaries of Florida. All herons are considered threatened wading birds in the state of Florida and is a species of high concern in the North American Waterbird Conservation Plan (Kushlan et al., 2002). The Little Blue Heron is listed on the 2016 State of North America's Birds' Watch List. This list includes bird species that are continually declining and most at risk of extinction. The Little Blue Heron is also listed on the Migratory Bird Treaty Act of 1918.

The decline of many herons, including the Little Blue Heron, could be related to environmental stressors such as human development, that leads to loss of wetland habitat and an increased exposure to pesticides and chemical runoff (Kushlan, 1997). Because of these pressures, food abundance and quality and habitat availability are decreased creating limitations on nesting wading birds (Kushlan, 1997). Population genetic theory predicts that these stressors and dsiturbances within nesting locations, such as competition with other species for space, result in reduced breeding success and lowered genetic diversity over time (Barrowclough, 1980). Genetic diversity can be measured by estimating heterozygosity and number of alleles. Thus, we predict that heterozygosity and allele number would be reduced in stressed populations compared to more robust or optimal-nesting populations.

A stressful environment includes conditions which lead to a sharp reduction in the fitness of a population. This was the case with White Ibis population numbers decreasing with habitat destruction and pesticide exposure (Kushlan, 1997). When environmental conditions are changed causing a decrease in the overall reproductive output of a population, this could lead to inbreeding, or even crashes, and ultimately, permanent damage to that population(Barrowclough, 1980).When the fitness of a population is reduced, fewer individuals successfully rear young in a season, leading to fewer alleles passed to the next generations and therefore lowered genotypic and phenotypic variation can be observed at the genetic level. Alternatively, any shift in the environment to which an organism is normally adapted, if not fatal, will lead to adaptations to the new conditions(Barrowclough, 1980). In this case, certain genotypes or alleles would be favored, reducing the frequency of others(Barrowclough, 1980).

Little Blue Herons are susceptible to several stressors. Wading birds, including the Little Blue Heron, that consume prey from flooded agricultural fields risk contamination by heavy metals from agricultural runoff (Kushlan, 1997). The exposure to the excess nutrients and heavy metals from pollution can internally stress Little Blue Heron populations foraging in them. This internal stress is sublethal and can lead to genotoxicity, eggshell thinning and reduced reproduction (Kushlan, 1997). The availability or quantity of prey can also influence the number of Little Blue Heron nests as well as the overall size of the clutches in a season. According to Klassen et al. (2016), the number of Little Blue Heron nests was influenced by the availability of large marsh fish across the estuary; larger prey availability promotes nesting within that area. When larger prey was abundant the nest size and number of nests increased. Direct human disturbances also harm breeding Little Blue Heron populations by causing adults to abandon nests, and eggs and chicks to die (Rodgers & Smith, 1997). Rodgers & Smith (1997) determined that even the smallest disturbances increased energy expenditure from unwarranted flight and alertness. From the literature, it is evident that Little Blue herons are very susceptible to stress even if it is from their own species, competing for space or prey.

We hypothesized a loss of alleles in Little Blue Heron populations is associated with reduced reproductive success, or selection, as a response to environmental stressors. The purpose of this study was to use population genetic markers to compare levels of genetic variability in multiple Little Blue Heron populations to assess how stress may be affecting birds nesting in highly disturbed environments. Microsatellite markers are often used to investigate genetic structuring and the genetic diversity of populations. Microsatellite markers are co-dominant, which allows heterozygotes to be distinguished from homozygotes, as well as hypervariable, containing many alleles per locus. The variation observed at these loci are generally independent of natural selection because microsatellites, themselves, are non-coding. They can however, reflect a reduction in overall genetic diversity in a population even if it is a result of selection at other loci.

It is important to test and optimize microsatellite loci available in the literature on our target species, as highly polymorphic microsatellites will be useful for answering population genetics

questions. Dai et al. (2013), published 23 microsatellite markers developed from the Chinese Egret, *Egretta eulophoetes*, the same genus as Little Blue Heron. Dai et al. (2013) also tested them on other species of the same genus; however *Egretta caerulea* was excluded. Huang et al. (2009) also published microsatellite markers developed from the Chinese Egret. Campanini et al. (2012) published microsatellite markers developed from the Cattle Egret, *Bubulcus ibis*. We selected a subset of microsatellite markers from each publication that seemed promising for our study. We included loci that were amplified in Little Blue Herons, loci that amplified in the genus Egretta, and some loci that met neither of these criteria but which were tetranucleotide repeats that worked on multiple species. Microsatellite markers are PCR-based and only require a minimal amount of DNA; thus, we were able to sample birds non-invasively by collecting feathers, abandoned eggshells and carcasses.

One of the main challenges we faced was determining the most effective way to extract DNA from salvaged artifacts. These challenges that we encountered included extracting DNA from degraded carcass tissue, extracting DNA from the keratinized feather calamus and determining from which part of the eggshell should DNA be extracted. The methods presented in this research demonstrate an effective protocol for extracting DNA from these three sample types, and a set of microsatellite markers that show promise in Little Blue Herons.

2 MATERIALS AND METHODS

Sample Collection

Samples were collected from Little Blue Herons nesting at Zoo Tampa at Lowry Park (Lowry Park Zoological Society, Tampa FL) and from a roosting site located in Brandon, Florida. The samples were stored in $-80\,^{\circ}\mathrm{C}$. In total, 63 presumed Little Blue Heron samples were collected as salvage specimens. Three were carcasses; 18 were abandoned eggshells, and 42 were feathers. Eleven feathers were of unknown origin and could be identified to species by barcode sequencing in the future.

DNA Extraction

DNA was extracted from feathers, carcasses or eggshells, involving these basic steps: lysis of cells and degradation of proteins, degradation of RNA, isolation of DNA from proteins, desalting the DNA, and resuspension of DNA in storage buffer solution. The calamus or quill of the feather was cut with a razor blade into fourths. The most basal portion of the calamus does not contain any veins but was the portion embedded into to the skin follicle of the organism. Several genomic DNA extraction kits were tried on multiple feather samples to evaluate efficacy. We tried the following kits: Wizard Genomic DNA Purification Kit (Promega , Madison WI), Purelink Genomic DNA Kit (Thermo fisher Scientific, Waltham, MA) and Nucleospin DNA Rapid Lyse tissue and Nucleospin DNA Insect kits (Macherey Nagel, Düren, Germany).

Considering none of the conventional genomic DNA extraction kits had steps designed to break down keratin, we also tested several approaches to accomplish this. First, we tried manually breaking down the keratin by grinding the calamus in liquid nitrogen; this resulted in a loss of samples. Second, we tried repeated freeze, thaw cycles using liquid nitrogen. Some of these samples were extracted using Nucleospin DNA Rapid Lyse tissue kit (Macherey Nagel, Düren, Germany) but yielded low quantities of DNA. Third we tested a buffer solution that was added during the digestion phase for its efficacy at breaking down keratin. Specifically we added TNca buffer as described by Hellmann et al. (2001) containing freshly mixed dithiotheritol (DTT).

Muscle and tissue from carcasses and eggshells were extracted and digested in the same manner using the TNca buffer with DTT. The outer shell was detached from the protein membranes and was kept separated throughout the extraction process. It is still unknown if both the outer shell and protein membranes contain only the chick's DNA or also the mother's DNA.

TNca/DTT Digestion Protocol and DNA Isolation

For samples treated with the TNca/DTT protocol, we carried out the following steps. The cut calamus was digested in 100 ul of TNca buffer as described by Hellmann et al. (2001) containing: 10 mM Tris-HCL, 100 mM NaCl, 1 mM CaCl2, 2% SDS, 39 mM DTT and $250\,\mu\mathrm{g/mL}$ proteinase K. The calamus was incubated at $56\,^{\circ}\mathrm{C}$ with gentle agitation overnight. After digestion, samples were carried through a standard phenol/chloroform/isoamyl alcohol (PCI) extraction as described by Sambrook et al. (1989). Purified DNA samples were quantified using a NanoDrop 1.0 spectrophotometer (Thermofisher Scientific, Waltham, MA). DNA concentration was estimated by measuring the absorbance at 260nm. To evaluate the purity of each sample, we observed the 260/280 ratio for protein contamination and 230/260 ratio for salt contamination. Isolated DNA samples were stored in the freezer at $-20\,^{\circ}\mathrm{C}$.

PCR

The DNA extract was used for microsatellite amplification with traditional polymerase chain reaction (PCR) methods. Fifteen microsatellite markers were tested (see Table 1) using DNA extracted from fresh and degraded carcass tissue types. Eight of these 15 markers were additionally tested for successful amplification on eggshells and feathers (Table 1). A gradient of six annealing temperatures from $46\,^{\circ}\mathrm{C}$ to $56\,^{\circ}\mathrm{C}$ separated by two degrees celsius for each locus was tested to find the optimal temperature (Table 1). PCR products were visualized with the inter-callating flourescent dye, GelStar (Lonza, Basel, Switzerland) that was mixed into agarose gel while it was liquid. A 100 base pair ladder (Azura, Raynham, MA) was run alongside the PCR products in the gel to allow estimation of the size of the products. The gels were photographed using ChemiDoc MP imager (BioRad, Hercules CA). The loci were scored: good, faint or poor based on amplification success rates (Table 1). We considered PCR products that were bright, without alternate bands, and that appeared to be polymorphic, to be good. We recorded the PCR product size estimate (Table 1).

3 RESULTS AND DISSCUSION

The common DNA extraction by column method using the Nucleospin DNA Rapid Lyse tissue kit was successful for the tissue samples from carcasses and yielded adequate quantities of isolated DNA; however, yields were low from the calamus of the feathers.

Locus Name	Species Developed in	LBH Sample Type Tested	Optimal Ta (°C)	Score	Approx. PCR Product Size (bp)	Individuals Tested
Ae 04[1]	E. eulophotes	Carcass	56	Good	250	ECA 2
Ae 09[1]	E. eulophotes	Carcass	54	Good	460	ECA 2
Ae 24[1]	E. eulophotes	Eggshell	50	Good	450	ECA 1es, ECA 2es
Ae 24[1]	E. eulophotes	Carcass	48-50	Good	300	ECA 1, ECA 2
Ae 28[1]	E. eulophotes	Carcass	56	Good	400	ECA 1, ECA 2
Ae 28[1]	E. eulophotes	Eggshell	56	Good	400	ECA 1es, ECA 2es
Ae 28[1]	E. eulophotes	Feather	56	Good	450	ECA 6
Ae 36[1]	E. eulophotes	Carcass	56	Good	300	ECA 1, ECA 2
Ae 36[1]	E. eulophotes	Eggshell	56	Good	400	ECA 1es, ECA 2es
Ae 36[1]	E. eulophotes	Feather	54	Faint	400	ECA 6
Ae 37[1]	E. eulophotes	Carcass	48	Good	400	ECA 1, ECA 2
Bi 15[2]	B. ibis	Carcass	50	Faint	500	ECA 2
Bi 20[2]	B. ibis	Carcass	48	Faint	300	ECA 1, ECA 2
Bi 26[2]	B. ibis	Carcass	54-56	Good	300	ECA 1 , ECA 2
Bi 26[2]	B. ibis	Eggshell	54	Good	300	ECA 1es, ECA 2es
Ee 22[3]	E. eulophotes	Carcass	48	Good	300	ECA 1, ECA 2
Ee 22[3]	E. eulophotes	Eggshell	48	Good	450	ECA 1es, ECA 2es
Ee 28[3]	E. eulophotes	Carcass	N/A	Poor	N/A	N/A
Ee 30[3]	E. eulophotes	Carcass	56	Good	500	ECA 1, ECA 2
Ee 43[3]	E. eulophotes	Carcass	48	Good	400	ECA 1, ECA 2
Ee 43[3]	E. eulophotes	Eggshell	56	Good	400	ECA 1es, ECA 2es
Ee 45[3]	E. eulophotes	Carcass	N/A	Poor	N/A	N/A
Ee 46[3]	E. eulophotes	Carcass	50	Good	400	ECA 2

Table 1. Cross amplification of 15 microsatellite loci on *E. caerulea* from tissue samples. Eight of these 15 microsatellites were additionally tested on feather and eggshell sample types. Loci name from original characterization publications (Dai et al., 2013)[1] (Campanini et al., 2012)[2] (Huang et al., 2009)[3] Score band brightness. N/A indicates locus that did not amplify.

Since DNA is only located in the calamus of the feather, the β-keratins needed to be broken down to access more nucleated cells. With the use of PCI, a more traditional extraction protocol, the addition of the TNca buffer and the freshly dissolved DTT during the digestion process, yielded much higher quantities of DNA in ng/uL from both the feathers and the eggshell samples. Adequate DNA was isolated from both the shell and membranes using this protocol; however, column extraction methods were not attempted.

Microsatellite markers developed on Cattle Egrets successfully cross amplified in Little Blue Herons, but resulted only in faint bands (Table 1). It might be possible to optimize some or all of these in Little Blue Herons, rendering them useful in population studies. Of the markers created for the Chinese Egret, 10 of the 12 had successful cross amplification rates with "good" scores (Table 1). Two of these markers did not amplify and scored as "poor"; they are not recommended to use to assess Little Blue Heron population diversity. Of the 10 successful markers six additional tests were completed using the following markers: Ae 24, Ae 28, Ae 36, Bi 26, Ee 22, Ee 43 and two additional tests using Ae 28 and Ae 36 (Table 1). These tests concluded that amplification was successful using DNA isolated from eggshells and feathers.

We demonstrated that we can get usable genomic DNA from salvaged Little Blue Heron specimens including feathers, degraded carcasses, and eggshells. We also demonstrated that there are sufficient high-quality polymorphic markers in the literature that work on this species to go forward with the study. Thirteen of the loci that we tested worked and had successful cross amplification rates. We amassed a reasonable sample size from the zoo population. Sampling should be continued within regional Florida marshes in order to complete genetic comparisons to determine the effects of stress on the genetic diversity of Little Blue Herons.

These preliminary results set up future research studies to carry on with sample collection to acquire the necessary population data in order to make genetic comparisons. This research will provide data as a major resource for future conservation genetic studies not just on the Little Blue Heron, but other species of herons and birds as well. The results from this study provide a groundwork for future studies on the genetic structure, molecular evolution and conservation management of this vulnerable species and the other Ardeid species. Other studies that could be conducted incorporating these methods include the relationship between reduced food quality because of contamination of waters by chemical runoff and the quantity of nests, or stress-hormone expression by Little Blue Herons during mating season in stressful environments versus optimal nesting environments using a qPCR analysis.

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