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Conservation

Genetic diversity of the Common Black Hawk *(Buteogallus anthracinus)* population in Los Tuxtlas, Mexico, based on microsatellite markers

Diversidad genética de la población de la aguililla negra menor (Buteogallus anthracinus) *en Los Tuxtlas, México, basada en marcadores microsatelitales*

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Abstract

The Common Black Hawk (*Buteogallus anthracinus*) is a raptor associated with wetlands. Many of its populations are in decline as a consequence of habitat loss and degradation. However, there are no published studies on the genetic variation of their populations. We characterize for the first time the genetic variation of a population of *B. anthracinus* in southern Veracruz, Mexico. We used feathers from 19 individuals to extract DNA and amplify and genotype 9 microsatellite loci. Samples were collected from nests (n = 4), at feeding sites (n = 5), and directly from chicks (n = 2), young individuals (n = 7), and 1 adult. Six out of 9 microsatellites were polymorphic and 3 monomorphic for this population. The highest number of alleles (n = 7) was observed at the BswD107w, with an average of 4.2 alleles per polymorphic locus. Mean observed and expected heterozygosity were 0.41 and 0.53, respectively. These values are on the lower end of those found for other birds of prey, but there is no evidence of high levels of inbreeding in the population. The polymorphic microsatellite loci analyzed in this study provide a useful tool to continue characterizing the genetic variation of *B. anthracinus* populations and evaluate possible inter-population differences.

Keywords: DNA; Population genetics; Mangrove; Bird of prey; Veracruz

Resumen

El Aguililla negra (*Buteogallus anthracinus*) es una rapaz asociada con humedales. Muchas de sus poblaciones están en declive por pérdida y transformación del hábitat. Sin embargo, no existen estudios que analicen los niveles

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de su variación genética. Reportamos la primera caracterización de la variabilidad genética en una población de *B. anthracinus* de Veracruz, México, mediante el uso de marcadores moleculares. Utilizamos plumas de 19 individuos para extraer ADN, amplificar y genotipificar 9 loci de microsatélites. Las plumas fueron recolectadas en nidos (n = 4), en sitios de alimentación (n = 5), directamente de los pollos (n = 2), de individuos inmaduros (n = 7) y de 1 individuo adulto. Seis de los 9 microsatélites resultaron polimórficos y 3 monomórficos para la población. El número más alto de alelos (n = 7) se observó en el marcador BswD107w, con un promedio de 4.2 alelos por locus polimórfico. Los promedios de heterocigosidad observada y esperada fueron 0.41 y 0.53, respectivamente. Aunque estos valores son relativamente bajos comparados con otras especies de rapaces, no se encontró evidencia de altos niveles de endogamia. Los microsatélites polimórficos analizados proveen una herramienta para continuar caracterizando genéticamente poblaciones de *B. anthracinus* y analizar posibles diferencias interpoblaciones.

Palabras clave: ADN; Genética de la población; Manglar; Rapaz; Veracruz.

Introduction

The development of genetic molecular tools is considered crucial for analyzing genetic diversity and to inform species management and conservation strategies. For example, genetic markers have been used to define the taxonomic boundaries of species, subspecies, and populations that require conservation attention (Haig et al., 2011), determine population structure and gene flow (Funk et al., 2007), and discern the temporal and spatial movement of migratory birds and its implications for population management (Bounas et al., 2018). Genetic data have also been used to evaluate demographic, behavioral, and ecological aspects important to design management strategies, such as the relatedness among individuals in a population (e.g., Müller et al., 2001), philopatry (Rudnick et al., 2005), extra-pair paternity and genetic mating system (Griffith et al., 2002; Kraukauer, 2008), and to measure the impact of anthropogenic disturbance and climate change on wildlife (Gebhardt et al., 2009; Martínez-Cruz et al., 2004). Particularly, microsatellite markers have been widely used in conservation genetics because they are selectively neutral, hypervariable, and possess codominant alleles (Ellegren, 1992; Nesje et al., 2000), which make them useful to evaluate levels of genetic diversity within populations and assess population structure with strong statistical power (Busch et al., 2005; Kalinowski, 2002; Sarasola et al., 2012).

Molecular markers have been used to characterize genetic diversity and structure in a number of endangered raptor species to aid in their management and conservation (e.g., Gebhardt et al., 2009; Hailer et al., 2005; Hull et al., 2007; Martínez-Cruz et al., 2002, 2004; Rudnick et al., 2005, 2008). In general, raptors are more affected by anthropogenic activities than other birds. These activities include habitat alteration and destruction, intentional killing, intentional and unintentional poisoning, electrocution, and climate change (reviewed in McClure et al., 2018). Understanding how these threats affect the genetic variation of a population is fundamental to the design of conservation and management strategies (Allendorf et al., 2013). The Common Black Hawk (*Buteogallus anthracinus)* is a bird of prey in the family Accipitridae that is protected under the Migratory Bird Treaty Act of 1918 in the United States of America (USFWS, 2013). Although *B. anthracinus* is globally considered as of "Least Concern", many of its populations are in decline (BirdLife International, 2017, 2019). It is classified as a "Threatened" species in Arizona, New Mexico, Utah, and Texas (NatureServe 2019; Texas Parks & Wildlife, 2019), and it is subject of special protection in the Norma Oficial Mexicana (NOM-059-SEMARNAT-2010) for the protection of native wildlife in Mexico (Semarnat, 2010).

Buteogallus anthracinus inhabits mangroves and riparian vegetation, and can be found in woods on mud banks, and on beaches and swamps (Howell & Webb, 1995; Peterson & Chalif, 1989; Schennel, 1994; Sibley, 2000). It is widely distributed in the Americas, ranging from southwestern United States through Mexico and Central America, Colombia, northern Venezuela, northeastern Peru and on the Island of San Vicente in the Lesser Antilles. In Mexico, it ranges from the states of Sonora, Chihuahua, and Tamaulipas, extending southwards through the Gulf of Mexico and the Pacific coast and reaching the Yucatan peninsula (González-Salazar, 2010). Due to their obligate reliance on mangrove and riparian vegetation, *B. anthracinus* is strongly affected by habitat disturbance. Mexico is one of the countries that have the most mangrove vegetation in the world (5% of the world's total), but the rate of deforestation of this ecosystem is extremely high (Aburto & Rojo, 2015), affecting the survival of all species that depend on it. In particular, the mangroves of Sontecomapan, Veracruz, Mexico have experienced high rates of destruction and fragmentation as a consequence of anthropogenic activities associated with the expansion of cattle ranching in the region (Mendoza et al., 2005).

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In this study, we used microsatellite markers to characterize the genetic diversity of a resident population of *B. anthracinus* that lives in the mangrove of Sontecomapan, Veracruz, Mexico (Fig. 1). To our knowledge, no previous studies have been published reporting genetic diversity in *B. anthracinus*, so this study contributes to testing heterospecific molecular markers for this species, evaluates the levels of genetic variation found in this population, and serve as an initial step for the long-term monitoring of the *B. anthracinus* population in Sontecomapan. This is particularly important considering the strong anthropogenic pressures on the species in the area, which not only include habitat destruction, but also poaching and disturbance by tourism (Carmona-Díaz et al., 2004).

Materials and methods

We opportunistically collected *B. anthracinus* feathers during the nesting (February to July) and post-nesting (August to November) periods of 2012, 2013 and 2014, in Sontecomapan, Catemaco, Veracruz, and surrounding areas (Fig. 1). Sampling occurred as part of a larger project on ecology, nesting behavior and morphology for the species (Barradas-García, 2016). We categorized sampled individuals as chicks, inmatures and adults. Chicks were individuals found in the nest and unable to fly and leave the nest (Schennel, 1994). Adults were differentiated from inmmatures based on morphological (e.g., feather color patterns) and morphometric (e.g., tail length) data (Howell & Webb, 1995; Schennel, 1994; Sibley 2000). Here we present results from 19 feathers collected from captured individuals (2 chicks, 3 immatures, and 1 adult), from nests (2 adults and 2 unknown), found in areas near nesting sites (5 adults), or those that fell from individuals during capturing attempts (4 immatures around feeding sites) (Table 1). To capture immature and adult birds we used Bal-chatri traps with the land crab *Cardisoma huangumi* and the blue crab *Callinectes sapidus* as bait. After sample collection, we hydrated the birds, placed an

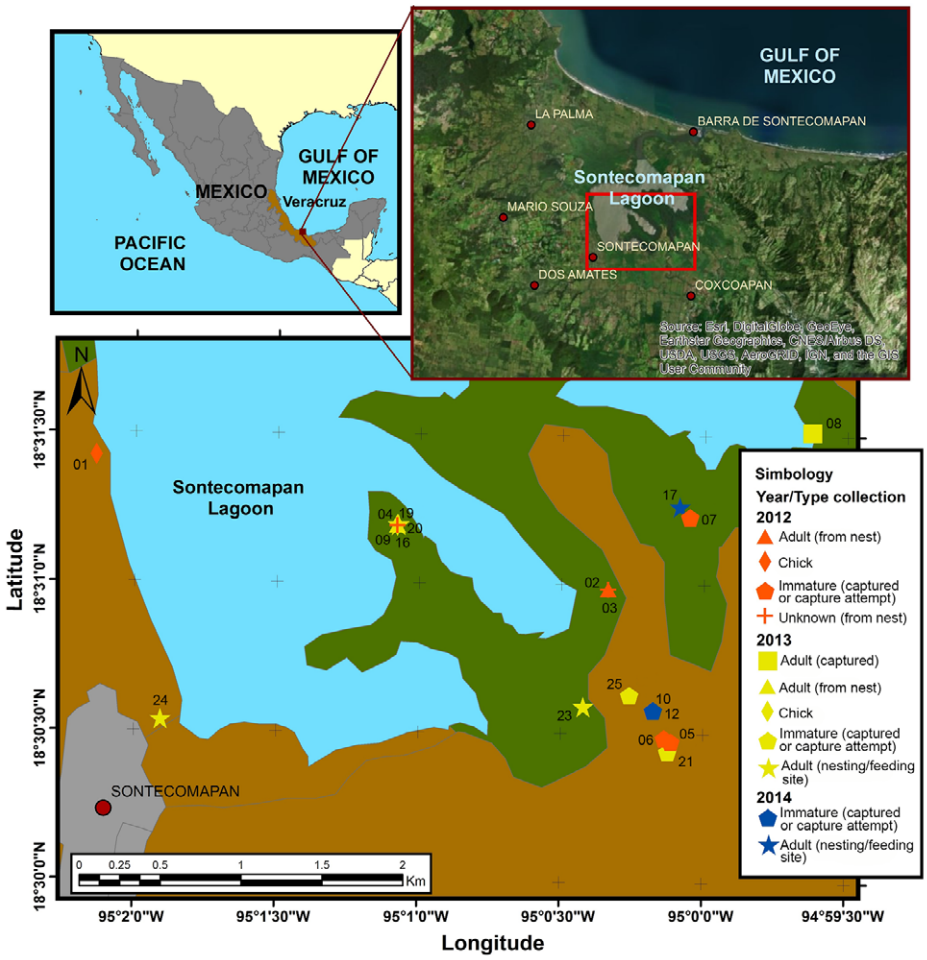

Figure 1. Map of the study site showing the location of the study area

Table 1
List of samples used in this study with coordinates of collection site. Collection type refers to whether samples were collected directly (from chicks, immature individuals, or adults) or indirectly (from nests, or from nesting/feeding sites).

ID	Collection type	Year of collection	Latitude	Longitude
BAST-01	Chick	2012	-95.0356	18.5237
BAST-02	Nest	2012	-95.0056	18.5164
BAST-03	Nest	2012	-95.0056	18.5164
BAST-04	Nest	2012	-95.0180	18.5199
BAST-05	Immature	2012	-95.0018	18.5079
BAST-06	Immature	2012	-95.0022	18.5081
BAST-07	Immature	2012	-95.0008	18.5205
BAST-08	Adult	2013	-94.9937	18.5253
BAST-09	Chick	2013	-95.0180	18.5199
BAST-10	Immature	2014	-95.0029	18.5096
BAST-12	Immature	2014	-95.0029	18.5096
BAST-16	Adult in nest	2013	-95.0180	18.5199
BAST-17	Nesting site. Adult	2014	-95.0014	18.5210
BAST-19	Nesting site. Adult	2013	-95.0179	18.5199
BAST-20	Nesting site. Adult	2013	-95.0179	18.5199
BAST-21	Immature	2013	-95.0020	18.5073
BAST-23	Nesting site. Adult	2013	-95.0070	18.5098
BAST-24	Nesting site. Adult	2013	-95.0317	18.5089
BAST-25	Immature	2013	-95.0043	18.5105

ID leg band, and released them at the site where they were captured. We preserved the feathers in 70% ethanol using Ziploc bags (modified from Taberlet and Bouvet [1991], and Gaur et al. [2017]). All collections were carried out under the scientific collector's license number SGPA/DGVS/02826/12 to HHBG. Samples were transported to the University of Michigan following all legal requirements from Mexico (CITES exportation permit # 70144) and the US (USDA importation permit # 124948).

Molecular work was carried out at the Genomic Diversity Laboratory, of the Department of Ecology and Evolutionary Biology, University of Michigan. DNA was extracted from the tip of the calamus of each feather with the DNeasy Blood & Tissue Kit (Qiagen, Inc.) following the manufacturer's protocols. DNA was stored at -20 ºC. In order to verify that the feathers belonged to *B. anthracinus* individuals, we amplified and sequenced a fragment of ~ 350 bp of the cytochrome b mitochondrial gen (cyt b) using primers L14996 (Sorenson et al., 1999) and CB2 (Palumbi, 1996) and compared these sequences with those publicly available on the GenBank database of the National Center for Biotechnology Information.

Amplification of the cyt b fragment was carried out through PCR in a final reaction volume of 10 µL, including 1 µL 10X Buffer, 1 µL dTNPs (2mM, of each dNTP), 0.8 µL $MgCl_2$ (50 mM) 0.25 µL of each primer (10 mM each), 5.7 µL ddH_20, 0.045 µL of Platinum Taq (Invitrogen) and 1 µL of DNA. The cycling conditions for the PCR profile were 2 min at 94 ºC, followed by 35 cycles of 45 seconds at 94 ºC, 45 seconds at 50 ºC, 1 min at 72 ºC and ending with a final extension of 2 min at 72 ºC. PCR products were visualized in 2% agarose gels using GelRed (Biotium, Inc.). Products with single bands were sent to the Sequencing Core Facilities of the University of Michigan, where they were sequenced using an ABI 3730xl DNA Analyzer.

A panel of 11 microsatellite loci previously isolated from other birds of prey phylogenetically proximate to the *B. anthracinus* was tested. After PCR optimization, only 9 loci successfully amplified single products (Table 2). PCR reactions were conducted using fluorescently labeled primers, following similar conditions as described above for cyt b, but with an increase in the final extension time (72 ºC for 10 minutes). Optimization of annealing temperatures (Ta) for each locus was carried out using temperature gradients before genotyping and selecting an optimal temperature at which bright single bands were observed (Table 2).

PCR products were also genotyped at the Sequencing Core Facilities of the University of Michigan using fragment analysis in an ABI 3730xl DNA Analyzer. Alleles sizes were identified using GeneMarker version 1.97 (SoftGenetics, State College, PA). Potential genotype errors such as the presence of null alleles, and errors due to stuttering and exclusion of large alleles (i.e., large allele dropout) were evaluated using a Micro-Checker version 2.3.3 (Van Oosterhout et al., 2004). GenAlEx 6.41 (Peakall & Smouse, 2006) was used to calculate the number of alleles per locus (Na), fixation index (F_{IS}), the probability of Identity (PI), and the Queller and Goodnight's relatedness coefficient (r) per pair of individuals. Arlequin 3.5.1.3 (Excoffier & Lischer, 2010) was used to analyze Hardy-Weinberg equilibrium (HWE), Linkage Disequilibrium (LD) and observed (H_o) and expected heterozygosity (H_e). Levels of statistical significance were corrected for multiple tests using the Bonferroni correction (Rice, 1989).

Table 2

Microsatellite loci successfully amplified in this study for *Buteogallus anthracinus*. Ta °C = annealing temperature, N = number of individuals genotyped, Na = number of alleles found in our sample.

Locus	Ta ºC	N	Na	Allele size range (this study)	Species from which it was originally isolated	Original reference
IEAAAG04	57	20	4	212-223	*Aquila heliaca*	Busch et al. (2005)
IEAAAG15	57	20	3	113-121	*A. heliaca*	Busch et al. (2005)
IEAAAG14	60	20	4	177-189	*A. heliaca*	Busch et al. (2005)
HaL 04	57	19	1	153	*Haliaeetus albicilla*	Hailer et al. (2005)
Bbu42	61	20	5	151-161	*Buteo buteo*	Johnson et al. (2005)
BswD107w	61	17	7	160-195	*B. swainsoni*	Hull et al. (2007)
Hf-C3F2	57	19	1	169	*Hieraaetus fasciatus*	Mira et al. (2005)
Hf-C1E8	57	17	2	231-240	*H. fasciatus*	Mira et al. (2005)
Hf-C5D4	59	20	1	169	*H. fasciatus*	Mira et al. (2005)

Results

We recovered a small fragment of mitochondrial *cyt b* sequence (233-270 bp) for all sampled individuals. Sequences of all 19 feathers matched those publicly available in GeneBank for *B. anthracinus* specimens with 99.6-100% sequence identity. Seventeen samples had sequences that were identical to each other and to all available sequences in GeneBank, whereas 2 samples (BAST-09 and BAST-16) showed a single non-synonymous nucleotide difference in this region that changed a Tyrosine into a Histidine, representing a new cyt b haplotype for the species. The lack of variation in our samples for this mitochondrial region is not surprising, given that the amplified region is invariable across specimens from Arizona (GeneBank accession number GQ264779), Panama (AY987327, GQ264777, GQ264778), and Costa Rica (EU583331), but sequencing this fragment allowed us to verify that the analyzed feathers belonged to *B. anthracinus* individuals.

Of the 9 microsatellite loci analyzed, 6 were polymorphic and 3 monomorphic for the population of *B. anthracinus* in Sontecomapan, Veracruz (Table 2). The number of alleles per polymorphic locus varied from 2 to 7, with an average of 4.2 alleles per locus. There was no evidence of linkage disequilibrium for any of the loci. After the Bonferroni correction locus, BswD107w showed evidence of deviation from HWE (Table 2). Results from Micro-Checker detected signals of null alleles in this locus, which may explain the deviation from HWE. However, the exclusion of this locus did not qualitatively affect the results. Thus the results for all analyses we present included all 6 loci. Observed heterozygosity (H_o) per locus varied from 0.06 to 0.75, with an average of 0.41, whereas expected heterozygosity (He) per locus varied from 0.26 to 0.81, with an average of 0.53 (Table 3).

Our estimate of the probability of identity (PI) using these markers in the population was low ($PI = 3.1\times10^{-3}$, $PI_{sib} = 3.0\times10^{-2}$), meaning that these loci have high power to differentiate individuals. Furthermore, no individuals had identical genotypes for all loci, confirming that all of them represented unique individuals. Inbreeding coefficient (F_{IS}) was 0.19, and fourteen individuals (70%) showed high coefficients of relatedness ($r > 0.5$) with at least another individual, suggesting that some individuals in our sample may be related.

Table 3

Observed and expected heterozygosity (H_o and H_e, respectively), deviation from Hardy-Weinberg equilibrium (*P*) and Fixation Index (F_{IS}; Coefficient of Inbreeding) for 6 polymorphic microsatellite loci in the population of *B. anthracinus* in Sontecomapan, Veracruz.

Locus	H_o	H_e	*p*	F_{IS}
IEAAAG04	0.60	0.58	ns	-0.06
IEAAAG15	0.40	0.34	ns	-0.19
IEAAAG14	0.50	0.54	ns	0.05
Bbu42	0.75	0.64	ns	-0.21
BswD107w	0.18	0.81	***	0.77
HF-C1E8	0.06	0.26	ns	0.77
Mean	0.41	0.53		0.19

*** ($p < 0.001$); ns = no statistical significance.

Discussion

The level of heterozygosity found in our study population was on the lower end of those reported using the same microsatellite loci in eagles and hawks (e.g., *Aquila heliacal*, *A. nipalensis* and *Haliaeetus albicilla*, Busch et al., 2005; *Buteo buteo*, Johnson et al., 2005; *Hieraaetus fasciatus*, Mira et al., 2005; *Buteo swainsoni*, Hull et al., 2007), and for different microsatellite loci in populations of other raptor species (e.g., *Aquila adalberti*, Martínez-Cruz et al., 2004; *Haliaeetus albicilla*, Hailer et al., 2005; *Harpia harpyja*, Banhos et al., 2008; *Buteo ridgwayi*, Woolaver et al., 2013). However, given that there is no reference data of other populations of *B. anthracinus* it is impossible to make appropriate interpretations on the levels of genetic diversity for the study population relative to other populations of the species.

None of the samples analyzed showed the same genotype (i.e., no sample had exactly the same alleles on each locus); therefore, we assume that they represent different individuals. However, we suggest that there is a high number of related individuals in the population, at the level of parent-offspring or siblings. Considering that our sampling method included 3 consecutive nesting seasons, and that *B. anthracinus* is reported to be socially monogamous (Schennel, 1994) with breeding pairs being faithful to their nesting sites in the Sontecomapan mangrove (Barradas-García & Morales-Mávil, 2007; Barradas-García et al., 2004), it is likely that our sample includes parents and offspring, or siblings, which could cause the low levels of diversity that we observed in our results. For example, in 2012 we collected a feather (BAST-04) from a nest, and in 2013 we collected a feather from an adult (BAST-16) and a feather from a chick (BAST-09) in the same nest. It is likely that all these individuals are a parent and offspring and/or siblings. Similarly, in 2012 we collected 2 feathers from the same nest, 1 from an adult (BAST-02), and another (BAST-03) presumably from its offsprings (although it could potentially be from its mate). Our inbreeding coefficient estimate shows some (although not high) level of inbreeding among our samples, which is consistent with our sampling of potentially related individuals. However, to accurately determine the actual level of relatedness among the different individuals sampled in this population, genotyping a larger number of polymorphic loci would be required to ensure reasonable statistical power.

In Mexico, *B. anthracinus* is a resident species that lives in different habitats, but is an obligate riparian nester with relatively low abundance and depends on the resources of the mangrove ecosystem (Arizmendi et al., 1990; Bojorges-Baños, 2011; De Labra & Escalante, 2013; Ortiz-Pulido et al., 1995; Rodríguez-Estrella & Brown, 1990; Vázquez-Pérez et al., 2009). The mangrove of Sontecomapan, Veracruz, harbors a breeding population of *B. anthracinus* (Barradas-García & Morales-Mávil, 2007; Barradas-García et al., 2004). However, it is unknown whether *B. anthracinus* individuals are phylopatric (i.e., remain in the same population where they were born) or disperse to breed in other populations in the region, so the extent at which this population is isolated from other neighboring populations remains unknown. All but one the captured birds in this study were banded with coded metal bands and the long-term monitoring and genetic characterization of individually recognized young and adult individuals in this population will allow a better understanding of the general dispersal patterns of this species. Furthermore, phylogeographic studies have found that Los Tuxtlas region harbors unique lineages for several bird species (e.g., Ornelas et al., 2013). This study sets the foundation to compare the population of *B. anthracinus* in Los Tuxtlas to other populations across its distribution range to understand their evolutionary history and evaluate the genetic structure among populations that could be used to ensure proper management and conservation strategies for this species.

The 6 polymorphic microsatellite loci analyzed in this study for *B. anthracinus* provide a useful tool that can help to characterize genetic differences among populations throughout the geographical distribution of the species. The results presented here represent an initial attempt at understanding the extent of genetic diversity in this species and could be the basis for comparative studies to determine if the genetic diversity of *B. anthracinus* is sub-divided into different populations that may require particular management actions.

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