AVIAN CONSERVATION & ECOLOGY

VOLUME 20, ISSUE 1, ARTICLE 16

Çakmak, E., Ç. Akın Pekşen, B. Boran, T. Hatipoğlu, and C. Can Bilgin. 2025. High nuclear genetic diversity of Birecik semi-wild population of endangered Bald Ibis (*Geronticus eremita*) from Turkey. Avian Conservation and Ecology 20(1):16. https://doi.org/10.5751/ACE-02835-200116

Research Paper

High nuclear genetic diversity of Birecik semi-wild population of endangered Bald Ibis (*Geronticus eremita*) from Turkey

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ABSTRACT. The Northern Bald Ibis (*Geronticus eremita*) is an endangered species. The western population is estimated to be approximately 700 individuals, located in the Souss Massa region of Morocco. In contrast, the eastern population, now semi-wild, is approximately 300 birds, situated in Birecik, Turkey. Recent reintroduction attempts in Europe have relied exclusively on captive populations derived from the western population and have been accompanied by research on their genetic structure and diversity. However, to date there has been no comprehensive study of a similar nature on the eastern population. In this study, we used five polymorphic microsatellite markers that are specific to the Bald Ibis to investigate the genetic diversity in 100 individuals of the Birecik population. We further sequenced the mitochondrial ND5 fragment in 46 adult individuals in order to reveal genetic differences between the eastern and western populations. Despite the limited number of founder individuals, the nuclear diversity of the Birecik population exhibited a high level of diversity, as measured by allelic richness and expected heterozygosity. However, mtDNA ND5 sequencing revealed a single haplotype (eastern haplotype 1) in all individuals of the eastern population, which differs from the haplotype (western haplotype 1) found in the western population by a single nucleotide. A specific mutation in mtDNA haplotypes, different migratory behavior, and highly restricted gene flow resulting from a long period of breeding as a semi-wild population, combined with geographical isolation, suggest that the eastern population (now only represented by the Birecik semi-wild population) is distinct from the western population. This unexpectedly high genetic diversity indicates the Birecik semi-wild population could act as the source population for reintroduction elsewhere.

À Birecik, la grande diversité génétique nucléaire de la population semi-sauvage d'Ibis chauve (*Geronticus eremita*), une espèce menacée d'extinction en Turquie

RÉSUMÉ. L'Ibis chauve (*Geronticus eremita*) est une espèce menacée. Sa population occidentale est estimée à environ 700 individus, situés dans la région de Souss Massa au Maroc. Sa population orientale, aujourd'hui semi-sauvage, compte environ 300 individus concentrés à Birecik, en Turquie. Les récentes tentatives de réintroduction en Europe se sont appuyées exclusivement sur des populations captives dérivées de la population occidentale et ont été accompagnées de recherches sur leur structure et leur diversité génétiques. Toutefois, il n'existe à ce jour aucune étude complète de nature similaire sur la population orientale. Dans cette étude, nous avons utilisé cinq marqueurs microsatellites polymorphes spécifiques à l'Ibis chauve pour étudier la diversité génétique de 100 individus de la population de Birecik. Nous avons également séquencé le fragment ND5 mitochondrial de 46 individus adultes afin de révéler les différences génétiques entre les populations orientale et occidentale. Malgré le nombre limité d'individus fondateurs, la population de Birecik présentait un niveau élevé de diversité nucléaire, mesuré par la richesse allélique et l'hétérozygotie attendue. Cependant, le séquençage de l'ADNmt ND5 a révélé un haplotype unique (haplotype oriental 1) chez tous les individus de la population orientale, qui diffère par un seul nucléotide de l'haplotype trouvé dans la population occidentale (haplotype occidental 1). Une mutation spécifique dans les haplotypes de l'ADNmt, un comportement migratoire différent et un flux génétique très restreint résultant d'une longue période de reproduction sous forme de population semi-sauvage, combinés à l'isolement géographique, suggèrent que la population orientale (aujourd'hui représentée uniquement par la population semi-sauvage de Birecik) est distincte de la population occidentale. Cette diversité génétique élevée et inattendue indique que la population semi-sauvage de Birecik pourrait servir de population source pour sa réintroduction ailleurs.

Key Words: *Bald Ibis, genetic diversity, microsatellites, mtDNA*

INTRODUCTION

The Northern Bald Ibis, *Geronticus eremita* (Linnaeus 1758), is a migratory bird belonging to the Threskiornithidae family. This species had a fragmented distribution with populations in Central Europe, North Africa, and the Middle East until the seventeenth century (Bowden et al. 2008, Böhm and Pegoraro 2011, Böhm et al. 2021). However, it was eradicated from Central Europe nearly 400 years ago due to habitat loss, climate change, and direct persecution (Böhm and Pegoraro 2011), which makes it one of the threatened bird species (Böhm et al. 2021). The remaining

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wild or semi-wild populations outside Europe now occur in the western (Morocco) and the eastern Mediterranean (Syria and Turkey). Since 2018, the Northern Bald Ibis has been classified as Endangered on the IUCN Red List of Threatened Species (BirdLife International 2018). The last known wild population in Morocco comprises about 708 relict free-flying adult birds (Oubrou and El Bekkay 2018, Böhm et al. 2021). There are many captive populations, with total number reaching more than 1700 individuals including non-breeding birds in 2018. All captive populations since 1950s originate from the Moroccan population (Böhm et al. 2018). Although monitoring in the field has been extremely difficult since 2015 due to security issues, the Syrian population now appears to be functionally depleted (Böhm et al. 2021).

The Birecik colony in SE Turkey is the main extant population in the East (Fig. 1). The rapid decline of the population in this colony since 1950 was due to excessive use of organochlorines such as DDT (Akçakaya et al. 1992, Hatipoğlu 2016). By 1973, only 23 breeding adults had remained with ongoing high mortality and reproductive failure (Arihan 1999). In 1977, the General Directorate of Nature Conservation and National Parks of Turkey and the Society for Protection of Nature (DHKD) started a project to save the Northern Bald Ibis population in Birecik. Therefore, 41 birds were caught and held in semi-captivity between 1977–1989 whereas a similar number of birds remained in the wild. However, in the 1990s no wild individuals were left that could breed in nature without human support (Akçakaya 1990, Yeniyurt et al. 2016). Captive birds are released in early spring and they forage in the surrounding habitats as free-flying birds until July, when they are moved back into captivity to prevent migration and increase survival rates (Akçakaya 1990, Yeniyurt et al. 2016). By 2021, there were 83 breeding pairs and 101 fledglings in Birecik (unpublished report of DKMP). The distinct migratory behavior of the Birecik population which historically moved - and when given the chance, also now moves - south to winter in southern Arabia and Ethiopia (e.g., Serra et al. 2015) while the western population does not migrate but rather is resident or lives a nomadic existence (Cramp and Simmons 1998, Matheu et al. 2020).

Understanding the genetic background of wild or captive populations of threatened species is essential to increase the success of any conservation approach (Attard et al. 2016). High genetic diversity helps populations cope with environmental fluctuations such as diseases, habitat alteration, or climate change (Tracy et al. 2011). Ensuring adequate genetic diversity is particularly important when using displaced or captive-bred individuals to establish a new population (Johnson et al. 2021). Additionally, selection of the most suitable individuals from the *ex-situ* pool is an important factor that often determines the success of reintroduction projects (Saura et al. 2008, Wirtz et al. 2018).

Genetic analyzes are especially relevant during any reintroductions as it is fundamental to analyze the genetic variability of founders to prevent inbreeding and to ensure a good probability of persistence (Witzenberger and Hochkirch 2011). With this scope and by using both nuclear and mitochondrial markers, we aimed to analyze the genetic diversity of Northern Bald Ibis (*Geronticus eremita*) within the Birecik population and compare it to European captive and Moroccan wild populations.

Fig. 1. Map of the location study population. Thin lines depict country boundaries.

METHODS

Sampling and DNA extraction

No field survey or specimen collecting was performed for this study. Blood samples of 100 *Geronticus eremita* semi-wild individuals (51 adults and 49 chicks) were collected by the veterinarians in Birecik (37.04 N, 37.99 E).

Genomic DNA was isolated using Qiagen Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) by following the manufacturer's protocol.

Microsatellite analysis

Samples were genotyped at five microsatellite loci developed for the Northern Bald Ibis (Wirtz et al. 2016). Locus-specific forward primers fluorescently labeled (HEX or FAM) at 5′-end were used in multiplex reaction. PCR was conducted using 5x HOT FIREPol® Blend Master Mix (HOT FIREPol DNA polymerase, 5x blend master mix, 2.5 mM MgCl2, 2 mM dNTP, BSA, blue and yellow dyes; Solis Biodyne) in a total reaction volume of 25 µl, containing 1 µl of genomic DNA, 0.3 µM of each primer pair and, 1× Master Mix (Solis Biodyne). Touch-down PCR was implemented under the following conditions: 15 mins 95 °C heat activation followed by 30 cycles of 20 sec at 95 °C, 1 min at TA °C (optimum annealing temperature for multiplex set, Table 1), 1 min at 72 °C, and a final extension of 10 mins at 72 °C. Two-percent agarose gel electrophoresis was used to qualify PCR amplifications. Genotyping was repeated three times per sample if the low peak signal was detected. An ABI-PRISM 3100 sequencer (Applied Biosystems) was used to determine allele sizes.

Mitochondrial ND5 analysis

The NADH hydrogenase subunit 5 (ND5) gene 732 bp in length was amplified in 49 adult individuals using the primers Av12976tSerF 5′-CAA GAA CTG CTA ACT CTT GTA TCT G-3′and Av13734ND5R 5′-AAT CCA AAT TGG GCT GAT TTT CC-3′ designed by Wirtz et al. (2018). 5x Hot FIREPol® Blend Master Mix (Solis Biodyne) was used for PCR amplification of this marker. The total reaction volume of 25 µl contained 1x Blend Master Mix, 0.3 µM of each primer, and 1 µl of genomic DNA. Amplification for ND5 was conducted as follows: initial denaturation at 95°C for 15 mins, 38 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 1 min and

Table 1. Characterization of 5 polymorphic microsatellite primers for Bald Ibis (*Geronticus eremita*) with locus name; primer sequence; repeat motif; fluorescence dye name (Tag); annealing temperature; expected and observed allele size range.

Locus	Primer Sequence (5' - 3')	Repeat motif	Tag	TA (^{0}C)	Expected allele size	Observed allele size
A02	F:GCTTGAAGTGAAGGTCCTATGG R:TCAGTCTAGGTGAAGGTGCC	$(GATAGA)_{12}$	FAM	59	292-322	293-311
A06	F:CTGCAACTGGAAACTGGTAGG R:GTTCAAAGCTGCACCAGGG	(TAGAA)13	FAM	61	338-358	335-360
A09	F:ACTTCCCTGGTGAACTGC R:CATGCAGGACAGGAAAACAAATAG	(TAAGGG)10	HEX	59	228-252	232-362
B05	F:TTTGGTCAGCCCTGAAGCG R:ACTCAGGATCGCATTTCACC	(CTATT)9	HEX	61	189-204	191-201
B06	F:TGCCATGTCCCTACCTTGG R:TCCGGCAGTTGGACTAGATG	(AATAG)9	FAM	59	209-234	211-231

elongation at 72°C for 4 mins. The final elongation was done for 10 mins at 72°C. Two-percent agarose gel electrophoresis was used to qualify PCR amplification. All sequencing reactions were performed by using ABI terminator 3.1. Kit (Applied Biosystems Inc., Foster City, CA, USA). PCR products were sequenced in both directions to increase accuracy of reading. An ABI 3730x1 Genetic Analyzer (Applied Biosystems) was used for electrophoresis and determination of fluorescently labeled nucleotides.

Data analysis

Microsatellite analysis

Raw sequence data was examined with the PEAK SCANNER 2.0 (Applied Biosystems) software to identify peaks and fragment sizing with the standard. FreeNA (Chapuis and Estoup 2007) was used to test for null alleles. Genotyping errors, allelic dropout, and scoring of stutter peaks were assessed statistically using MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004). In order to determine linkage disequilibrium, gene diversity and allelic richness were calculated by FSTAT 2.9.3 (Goudet 2001). Deviations from Hardy-Weinberg Equilibrium was tested using GenAlEx 6.5 (Peakall and Smouse 2012). The software ML-RELATE (Kalinowski et al. 2006) was used to estimate relatedness and relationships between pairs of individuals. This method calculates the maximum likelihood estimates of relatedness (r) using a downhill simplex routine. The software assigns pairs of samples into four common pedigree relationships: unrelated (U), half-siblings (HS), full-siblings (FS), and parent-offspring (PO). However, there is no class for lower-level relatedness (e.g., cousins and nieces) in these analyzes. It is, therefore, reasonable to assume that these may be misclassified as HS, at least more frequently than unrelated individuals. In addition, polymorphic information content (PIC), indicating the possible utility of markers in identifying individuals, was estimated with CERVUS 3.0.7 (Marshall et al. 1998, Kalinowski et al. 2007); where PIC > 0.5 is highly informative, 0.5 > PIC > 0.25 is moderate informative, and PIC < 0.25 is somewhat informative (Botstein et al. 1980). To reveal any recent bottlenecks, the software BOTTLENECK 1.2.02 (Cornuet and Luikart 1996, Piry et al. 1999) was used. Under the two-phase mutation model (TPM), the statistical significance of heterozygosity excess was measured by means of Wilcoxon's sign rank test; the mode-shift graphical method was applied to estimate allele frequency shift after a bottleneck event (Luikart et al. 1998). It is expected that alleles carrying intermediate frequencies (about 0.1–0.2) are more widespread than alleles carrying low frequencies (< 0.1; Ganapathi et al. 2012).

Mitochondrial ND5 analysis

The alignment of all ND5 sequences was performed using the Clustal W algorithm (Thompson et al. 1997) in MEGA X (Kumar et al. 2018). Alignments were checked by eye for accuracy and corrected manually. Haplotype diversity (Hd) was calculated using DnaSP program v.6 (Rozas et al. 2017). Mitochondrial DNA sequences representing distinct haplotypes were deposited in GenBank.

RESULTS

Relatedness of study individuals

DNA isolation from blood samples of 100 individuals was successfully performed. All microsatellite loci were amplified successfully in all individuals. No loci were detected to contain null alleles (r < 0.2) when tested with FreeNa (10,000 replicates; Table 2). The probable presence of linkage disequilibrium in the population of 100 individuals was calculated using FSTAT version 2.9.4. Looking at possible comparisons between pairs of loci in the population, no significant linkage disequilibrium was detected after Bonferroni correction and each locus was evaluated independently. When MICROCHECKER was used to statistically assess the presence of genotypic errors, allelic dropouts, and artifacts (such as stutters), none of the five polymorphic loci proved to have any genetic errors in 100 individuals. Therefore, all five loci were used in further analysis.

Of 4950 possible pairwise combinations among 100 individuals, ML-RELATE predicted 1545 family relationships. These were classified as 294 full-siblings, 552 half-siblings, and 699 parent-offspring relationships. Unrelated (U) pairs of samples were 3405. None of the individual sample pairs assigned to the three relatedness classes were significant on their own.

Nuclear genetic diversity, inbreeding, and bottleneck

Five nuclear loci were successfully genotyped for all samples (n = 100). A total of 23 alleles were detected in all loci throughout the population. These five loci exhibited high genetic diversity based on allelic richness (A_R), gene diversity, and observed heterozygosity (H_o; Table 2). The allelic richness was highest in loci A06 and A09 (Table 2). All loci were determined to be in

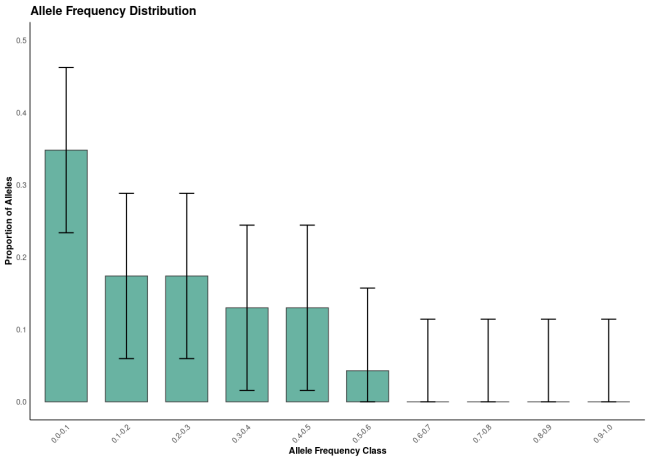
Table 2. Genetic diversity at 5 loci studied. Allelic Richness (AR), Observed Heterozygosity (Ho), Expected Heterozygosity (HE), Polymorphic information content (PIC), Hardy-Weinberg Equilibrium (HWE) and Fixation Index (Fis) values.

Pop	Locus	N	A_R	H_o	H_E	Gene Diversity	Null allele freq.	PIC	HWE	F_{is}
Birecik	A02	100	4.000	0.760	0.676	0.679	0.00000	0.613	NS	-0.119
	A06	100	6.000	0.700	0.687	0.690	0.00009	0.634	NS	-0.014
	A09	100	6.000	0.680	0.665	0.668	0.02069	0.611	NS	-0.018
	B05	100	3.000	0.500	0.569	0.573	0.04872	0.587	NS	0.127
	B06	100	4.000	0.640	0.677	0.680	0.03003	0.622	NS	0.059
Mean		100	4.600	0.656	0.655	0.658		0.593		

NS: non-significant.

Hardy-Weinberg Equilibrium (HWE). No signs of inbreeding were detected (Table 2). The 5 loci used were found to be highly informative loci (PIC > 0.5) in the *G. eremita* population (Table 2). According to the Wilcoxon test, the observed proportion of heterozygotes showed no deviation from expectation under mutation-drift equilibrium using a TPM (one-tailed for H excess: P = 0.5) in the pooled sample, which indicates that the Northern Bald Ibis population was not going through a recent bottleneck. The proportion of alleles in different allele frequency classes (0–0.1 low; 0.9–1 high allele frequency class) showed the normal L-shaped distribution rather than the mode-shifting distribution which would be expected for bottleneck populations (Fig. 2).

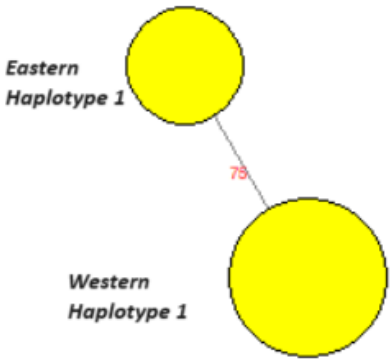
Fig. 2. Mode shift of allele frequencies indicating no recent genetic bottleneck.



Mitochondrial genetic diversity

The NADH hydrogenase subunit 5 (ND5) gene was subjected to sequencing in 49 adults from the Birecik population, resulting in the identification of one substitution when compared to the western population. The same nucleotide (A) at position 75 was identified in all individuals of the Birecik population and also previously documented in Wirtz et al. (2018) as eastern haplotype 1 (Accession no: MF682061.1; Wirtz et al. 2018) in five individuals of Turkish and Syrian origin. Consequently, the Birecik population exhibits a single, fixed haplotype (Fig. 3).

Fig. 3. The haplotype network was constructed for all western and eastern populations.



DISCUSSION

Loss of genetic diversity due to high rates of inbreeding are both important issues in captive breeding as they often lead to reduced population growth and increased risk of extinction (Willoughby et al. 2015, Choi et al. 2020). Our results show that despite a relatively small founder base, the captive population of Northern Bald Ibis is not strongly affected by a loss of genetic diversity. Although Wirtz et al. (2018) studied more loci than we did, they identified nearly the same level of allelic richness (A_R, range 3.53–4.09) for their study populations: captive individuals (zoo), wild birds (WB), the "Waldrappteam" population (WRT), and the "Proyecto Eremita" population (PE). Similarly, using the same set of microsatellite loci, a comparable level of average heterozygosity (H_o) of 0.575 was observed in Brazilian Roseate Spoonbill populations (Miño and Del Lama 2007). The level of heterozygosity (H_o, range 0.500-0.760) we report is also comparable to other Bald Ibis populations (Wirtz et al. 2016, 2018). Similar to our findings, Wirtz et al. (2018) showed that although only a small sample of eight individuals originated from the eastern wild population, they had higher allelic richness than any *ex-situ* group studied or the western wild population.

Despite the founder population being no more than 20 pairs, no statistically significant inbreeding coefficient (F_{IS} varied from -0.119 to 0.127) was determined for the Birecik population. This may be explained by an already very diverse gene pool to start with and/or individuals preferring to mate with individuals they

are more distantly related to (Smith et al. 1997, Hoffman et al. 2007). Negative inbreeding coefficients (F_{IS}) were found even for populations with as few as two founders as in the case of the Crested Ibis population in South Korea (Choi et al. 2020), implying it is possible for some populations to overcome adverse effects of low founder size. Although, Wirtz et al. (2018) also detected no sign of inbreeding, the relatively higher F_{IS} value for the European zoo population may be explained by deviations from the HWE for this population due to the Wahlund effect. Selecting mates from unrelated individuals is the dominant pattern in birds and mammals (Smith et al. 1997, Hoffman et al. 2007). However, a full understanding of the consequences of inbreeding in wild populations requires not only the detection of relatedness but also long-term measures of reproductive success and survival (Brzeski et al. 2014).

mtDNA ND5 sequencing revealed an only one haplotype (eastern haplotype 1) in the eastern population. This was found to be a single nucleotide (eastern haplotype 1: Accession no: MF682061.1) different from the haplotype (western haplotype 1: MF682062.1) found in the western population. Although substitution at position 75 of the gene was not linked to the origin of the sample by Wirtz et al. (2018), this mutation was found to be present in all Birecik individuals and absent in western birds (Wirtz et al. 2018). Despite the presence of a single mutation observed between the eastern and western populations, this specific haplotype is exclusively observed in the eastern population of the Northern Bald Ibis (Birecik, Turkey).

There have been massive efforts to reintroduce the Northern Bald Ibis into Austria, Switzerland, and southern Germany, using captive-bred individuals of the western origin, which have succeeded in establishing a population of about 200 free-ranging birds (Fritz 2021). For the geographically isolated eastern colony in Birecik, on the other hand, efforts until now have mainly focused on growing its numbers (Arihan 1999, Hatipoğlu 2016). However, in order to reduce the risk of disease or other catastrophes wiping out the only colony, it is proposed that new colonies need to be set up at suitable sites elsewhere (e.g., Schenker and Erhardt 2023). Our results suggest such new breeding colonies are unlikely to suffer due to inbreeding since the semi-captive population appears to have enough diversity to select founders with different enough genetic backgrounds.

Overall, the documented geographic, genetic, and behavioral differences confirm that the Birecik semi-wild population constitutes the last representative of the eastern population and is considered as an important separate piece of its western relatives. The presence of a high nuclear genetic diversity indicates that the Birecik semi-wild population is a critical candidate for reintroduction to release of the species to locations other than Birecik.

Author Contributions:

Study design: E.Ç., C.C.B.; sample collection: T.H.; data analysis: E.Ç., Ç.A.P., B.B.; writing: E.Ç., Ç.A.P., C.C.B.

Acknowledgments:

This study was supported by Aksaray University Scientific Research Projects Fund under the 2020-030 grant.

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Editor-in-Chief: Alexander L. Bond
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