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RESEARCH ARTICLE

Optimizing the genetic management of reintroduction projects: genetic population structure of the captive Northern Bald Ibis population

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Abstract

Many threatened species are bred in captivity for conservation purposes and some of these programmes aim at future reintroduction. The Northern Bald Ibis, *Geronticus eremita*, is a Critically Endangered bird species, with recently only one population remaining in the wild (Morocco, Souss Massa region). During the last two decades, two breeding programs for reintroduction have been started (in Austria and Spain). As the genetic constitution of the founding population can have strong effects on reintroduction success, we studied the genetic diversity of the two source populations for reintroduction ('Waldrappteam' and 'Proyecto eremita') as well as the European zoo population (all individuals held ex situ) by genotyping 642 individuals at 15 microsatellite loci. To test the hypothesis that the wild population in Morocco and the extinct wild population in the Middle East belong to different evolutionary significant units, we sequenced two mitochondrial DNA fragments. Our results show that the European zoo population is genetically highly structured, reflecting separate breeding lines. Genetic diversity was highest in the historic samples from the wild eastern population. DNA sequencing revealed only a single substitution distinguishing the wild eastern and wild western population. Contrary to that, the microsatellite analysis showed a clear differentiation between them. This suggests that genetic differentiation between the two populations is recent and does not confirm the existence of two evolutionary significant units. The European zoo population appears to be vital and suitable for reintroduction, but the management of the European zoo population and the two source populations for reintroductions can be optimized to reach a higher level of admixture.

Keywords Captive breeding · Conservation genetics · Genetic lineages · Microsatellites · Reintroduction

Introduction

A large number of species is severely threatened in their natural habitats and many of them are in need of captive breeding to avoid extinction (Conde et al. 2011; Conway 2011). Ex situ conservation (i.e. the conservation of species outside their natural habitat) and the subsequent reintroduction of species have become increasingly important tools in conservation (Armstrong and Seddon 2008; Bowkett 2009; Dolman et al. 2015; Seddon et al. 2007). However, both captive breeding and reintroductions are highly sophisticated approaches, requiring the consideration of multiple aspects of the species' biology, such as for example reproductive behaviour, feeding habitats, habitat affiliation and potential threats, to name just a few (Armstrong and Seddon 2008; Kirkwood 2003). Captive breeding should aim at maintaining a maximum genetic diversity within the ex situ population and avoiding inbreeding depression, which means

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reduced fitness due to mating of related parents (Hedrick and Garcia-Dorado 2016), or outbreeding depression due to breeding of too distantly related individuals (Lynch 1991; Willoughby et al. 2015). To avoid outbreeding, it is advisable to use individuals belonging to a single evolutionary significant unit in source populations (Grueber et al. 2015; Lacy 1994; van Dyke et al. 2008; Willoughby et al. 2015; Witzenberger and Hochkirch 2011). High genetic diversity supports populations to cope with environmental fluctuations, such as diseases, habitat alterations or climate change (Tracy et al. 2011). Problems as for instance unknown founder individuals, small effective population sizes of founders or biased exchange between zoos (e.g. individuals are only exchanged within national borders or between geographically nearby institutions) can lead to failure of reintroduction projects (Ballou et al. 2010; Cuarón 2005). It is thus crucial to understand the genetic structure of threatened species in the wild as well as in captive populations in order to enhance the success of any conservation action (Attard et al. 2016; Grueber et al. 2015).

The Northern Bald Ibis, *Geronticus eremita*, is listed as critically endangered on the IUCN red list of threatened species (BirdLife International 2015) and listed on Appendix S1 in Supplementary material of the convention on international trade in endangered species of wild fauna and flora (CITES). Until the 17th century, this migratory and colonial species had a disjunct distribution, with populations in Central Europe, North Africa and the Middle East (Bowden et al. 2008; Böhm and Pegoraro 2011; Schenker 1977). The main reasons for its extinction in Europe at the beginning of the seventeenth century were anthropogenic threats, mainly overhunting and habitat loss (Böhm and Pegoraro 2011; Bowden 2015; Unsöld and Fritz 2011). The last remaining wild and sedentary population is located in the Souss Massa Region (Souss Massa National Park and Tamri), Morocco, and comprised about 584 individuals in 2016 (Bowden et al. 2003; Böhm and Pegoraro 2011; Böhm and Bowden 2016; Grepom BirdLife Maroc 2014). Furthermore, a semi-wild population of about 217 individuals is managed in the Birecik breeding centre, in southern Turkey (Böhm and Bowden 2016). Another wild population was rediscovered in Syria in 2002, but is no longer breeding (Fritz and Unsöld 2015; Serra et al. 2004). The International Advisory Group for the Northern Bald Ibis assumes, that this population in now likely to be extinct (Böhm and Bowden 2016). The European zoo population, which is managed in the European Endangered Species Program (EEP) since 1988 counts about 1300 individuals (Böhm 2015). It descends from 100 to 150 Moroccan birds imported between the 1950s and 1978 (Böhm and Pegoraro 2011). While the exact origin of the founder individuals is unknown, it is assumed that the majority originates from the Atlas Mountains (Böhm and Pegoraro 2011). About 50–90 of those birds have bred, but exact information on breeding success is not available. The European zoo population has increased to ca. 1300 individuals within the last 50 years.

A first sedentary colony of the Northern Bald Ibis from zoo offspring (mainly Innsbruck, Stuttgart and Rosegg zoo), comprising 27 founder individuals, was established in 1997 at the Konrad-Lorenz research station in Grünau, Upper-Austria (Tintner and Kotrschal 2001), based on the experience from a prior experiment by the Innsbruck zoo (Thaler et al. 1981). Currently 63 birds belong to the Grünau colony. Later, similar projects in Scharnstein/Germany ('Waldrappteam') and Andalusia/Spain ('Proyecto Eremita') followed (Fritz 2004; Quevedo et al. 2004). These projects aim on re-establishing the Northern Bald Ibis in the wild. Based on the experience made in Grünau and Scharnstein and after a ten years feasibility study, a reintroduction project for the Northern Bald Ibis funded by the European Union was initiated 2014 in Austria with the objective of reintroducing the species in the European Alps (Germany, Austria and Italy). A team of Northern Bald Ibis experts (the so called 'Waldrappteam') is now establishing a self-migrating population, which breeds in Burghausen (Germany 48°9.495′N, 12°49.458′E) and Kuchl (Salzburg 47°38.040′N, 13°9.618′E) and overwinters in Laguna di Orbetello (Italy 42°29.023′N, 11°12.685′E) (Fritz et al. 2017; Böhm and Bowden 2016). This population is based on 116 founder individuals, mainly from Rosegg zoo in Carinthia and the colony of the Konrad-Lorenz research station in Grünau, Upper-Austria. In June 2016 it consisted of 99 individuals (Böhm and Bowden 2016). Intensive management (i.e. escorting juvenile birds by microlite aircraft) is conducted to support the birds in becoming a stable migrating and reproducing population. A second group of Northern Bald Ibis was reintroduced close to the Bay of Cádiz in Andalusia, Spain, within the 'Proyecto Eremita' and is managed as a sedentary colony (Muñoz and Ramírez 2017). The colony was founded by 23 hand- and parent-raised birds from Jerez zoo. In January 2016 a total of 78 individuals were part of this colony (Böhm and Bowden 2016).

Choosing the most suitable individuals from the ex situ pool is a major factor determining the success of reintroduction projects (Saura et al. 2008). We therefore conducted a study of the genetic diversity within the European zoo population (ZOO = all individuals belonging to European ex situ projects pooled) of the Northern Bald Ibis and the founder populations of the reintroduction projects in Burghausen (WRT = 'Waldrappteam' population) and the Bay of Cádiz (PE = 'Proyecto Eremita' population). It is still under debate whether the individuals of the wild population in North Africa (wild western population) and the potentially extinct population in the Middle East (wild eastern population) may represent two distinct genetic lineages (Pegoraro et al. 2001) qualifying as evolutionary significant units (ESUs).

This would have consequences for the management as they would need to be managed separately to avoid outbreeding depression (Edmands 2007). While the European zoo population originates from approximately 74 Moroccan founders (Böhm and Pegoraro 2011), it might be important to identify individuals originating from the eastern wild population early enough, and evaluate the potential consequences of (past or future) admixture between both wild populations. The existence of two distinct genetic lineages was hypothesized by Pegoraro et al. (2001), based upon a single common substitution in the cytochrome *b* (cyt *b*) gene when analysing 12 individuals. Analyses of more individuals and loci are therefore necessary to assess whether the two populations represent ESUs.

Our study represents the first systematic genetic analysis of the European zoo population of the Northern Bald Ibis using 15 polymorphic microsatellite loci and two mitochondrial genes. The aims were:

1. to test if more substitutions are found between the western wild (WB_W = museum samples and ZOO = European zoo population, which originated from Morocco) compared to the eastern wild population (WB_E = museum and blood samples), confirming the hypothesis that these represent two distinct ESUs,
2. to analyse the genetic structure within the European zoo population (ZOO) and evaluate the level of admixture between zoo colonies,
3. to test whether the two source populations for reintroductions (WRT and PE) cover a similar level of genetic diversity as the complete European zoo population (ZOO) and thus are likely to be diverse enough to ensure reintroduction success,
4. to explore if historical samples from the eastern wild population (WB_E = museum and blood samples) and the western wild population (WB_W = museum samples) have a similar genetic diversity as the samples in the European zoo population (ZOO).

Methods

Sampling and DNA extraction

The Northern Bald Ibis is kept in 70 European zoos, which are listed in the EEP. A total of 43 zoos and institutions followed our request to support us with genetic material for DNA analysis (Appendix S1). Furthermore, three museums provided samples from their Northern Bald Ibis collections (Appendix S1). In total, we received samples of 836 individuals, 747 of which were chosen for further analyses (596 blood samples, 82 buccal swabs, 18 feathers and 51 tissue samples). The remaining 89 samples were excluded because these individuals were direct siblings or the relevant zoo was already overrepresented in the sampling. Buccal swabs were taken with sterile cotton dry swabs (Copan Diagnostics Inc., Murrieta, CA, USA), blood was stored in pure ethanol (99.9%) or fixed on FTA™ MicroCards (Whatman, GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK). Feathers were stored in sterile reaction tubes (Eppendorf AG, Hamburg, Germany). Tissue samples were stored in absolute ethanol. To keep the sampling minimally invasive, blood samples were taken during routine veterinary medical screening in the zoos. Tissue samples originated from museum material (n = 23) or dead birds from zoos (n = 28).

For blood, buccal swabs, tissue and feathers, DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) or the QIAamp DNA Investigator Kit (Qiagen GmbH, Hilden, Germany) for museum samples following the manufacturer's protocol. Museum samples were isolated in a distinct laboratory unit, separated from the tissue samples to avoid contamination. Furthermore, the complete data set was tested for identical genotypes to cross-check for contamination. DNA quality was quantified using the Qubit Invitrogen Fluorometer (ThermoFisher Scientific, Darmstadt, Germany). DNA quality was on average 26.0 ng/µl for blood samples, 15.8 ng/µl for swaps, 35.0 ng/µl for tissue samples and 8.3 ng/µl in feathers.

Genotyping

The samples were genotyped at 15 microsatellite loci developed for the Northern Bald Ibis (Wirtz et al. 2016). Each forward primer was labelled with a fluorescent dye at the 5′-end (HEX or FAM). According to the expected fragment lengths, fluorescent dye and annealing temperature, microsatellite primers were pooled in five multiplex reactions (multiplex reaction I: A07, A09, D07; multiplex reaction II: A11, B05, B12; multiplex reaction III: A06, A12, B08; multiplex reaction IV: B01, B04, C02; multiplex reaction V: A02, B06, B11). PCRs were conducted with the Qiagen Type-it Microsatellite PCR Kit (Qiagen GmbH, Hilden, Germany) in a MultiGene Gradient Thermocycler (Labnet International, Inc., Woodbridge, USA). A 10.5 µl PCR reaction batch was used, consisting of 5.0 µl Type-it MasterMix, 1.0 µl Aqua bi.dest., 2.0 µl Q-solution, 1.0 µl primer mix (2.0 pmol/µl forward and reverse primer) and 1.5 µl genomic DNA. Detailed PCR conditions are described in Wirtz et al. (2016). Fragment length analysis of the diluted PCR products (1:20) was conducted on an ABI 3500 sequencer (Applied Biosystems, ThermoFisher Scientific, Darmstadt, Germany). For genotype scoring, GeneMapper 5.0 (ThermoFisher Scientific, Darmstadt, Germany) was used. For samples with low DNA quality, genotyping was repeated three times.

Sequencing

Pegoraro et al. (2001) did not find any difference between eastern and western populations in 16S rDNA and a single substitution in cytochrome *b* (cyt *b*). To test, if more region-specific substitutions can be detected, we sequenced the NADH hydrogenase subunit 5 (ND5), which is known to be a highly variable gene (Paton and Baker 2006). We sequenced this marker in 98 individuals (five samples of Turkish and Syrian origin and 93 from zoo populations). The fragment was amplified using the self-designed 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′. Furthermore, we sequenced cyt *b* in 16 individuals five samples of Turkish and Syrian origin and 11 from zoo populations with presumably Moroccan origin) to confirm the findings of Pegoraro et al. (2001). For amplification of the cyt *b* gene, we used the primers cytbL 5′-CCA TCC AAC ATC TCA GCA TGA TGA AA-3′ and cytbH 5′-CCC CTC AGA ATG ATA TTT GTC CTC A-3′ (Kocher et al. 1989; Bartlett and Davidson 1991).

PCR was conducted with the 5PRIME HotMasterMix (5PRIME, Hamburg). The reaction batch consisted of 20.0 µl 5PRIME HotMasterMix, 26.0 µl aqua bi dest. 1.0 µl primer (each forward and reverse) and 1.4 µl DNA. Amplification for both ND5 and cyt *b* was conducted as follows: initial denaturation at 94 °C for 2 min, 33 cycles of denaturation at 94 °C for 30 s, annealing at 56.9 °C for 30 s and elongation at 65 °C for 1 min. The final elongation was done for 10 min at 65 °C. PCR products were purified with the High Pure PCR Product Purification Kit (Roche) and sequenced with the BigDye Terminator V3.1 Cycle Sequencing Kit (ThermoFisher Scientific) following the manufacturer's protocol. For purification of the sequencing product, the BigDye®XTerminator™ Purification Kit (ThermoFisher Scientific) was used. DNA sequencing was run on an ABI 3500 sequencer (Applied Biosystems, ThermoFisher Scientific).

Data analysis

Genotyping

All individuals with data for more than two microsatellite loci missing (n = 105) were excluded from further analysis. To rule out potential scoring errors (e.g. stutter bands, large allele dropout) we tested our data set with Micro-Checker 2.2.3 (van Oosterhout et al. 2004). FreeNA (Chapuis and Estoup 2007) was used to test for null alleles. We tested for deviation from Hardy-Weinberg-Equilibrium (HWE) in GenAlEx 6.5 (Peakall and Smouse 2012).

To detect genetic structure in the microsatellite data set, we ran a STRUCTURE 2.3.4 analysis (Pritchard et al. 2000) with a burn-in period of 10^5 and 10^6 Markov chain Monte Carlo (MCMC) simulations with the number of possible clusters set from K = 1 to K = 25. Each K was tested with 10 iterations. We used the admixture model in combination with the correlated allele frequencies model. To determine the most likely number of genetic clusters, we calculated ΔK (Appendix S2) as proposed by Evanno et al. (2005) in STRUCTURE Harvester 0.6.93 (Earl and von Holdt 2012). As the ΔK method tends to result in underestimates of genetic structuring (Janes et al. 2017), we selected the most likely number of genetic clusters by exploring the Q-values within the clusters and discarded K values in which at least one cluster had Q-values below 0.9 in all individuals as proposed by Schulte et al. (2012). We used the software CLUMPP (Jakobsson and Rosenberg 2007) to obtain means out of multiple runs (10 iterations) for the selected number of K.

Additionally, we used BAPS v.6.0 (Corander and Marttinen 2006) for Bayesian inference of the genetic structure in our data set. We ran the 'clustering of individuals' algorithm for K = 2–25 with ten iterations followed by the 'admixture based on mixture clustering' algorithm for each K.

For subsequent analyses, two different data partitions were used. Data set 1: To test for the genetic constitution of the source populations for reintroductions, 'Waldrappteam' and 'Proyecto Eremita', compared to the European zoo population, we distinguished five groups: wild individuals (i.e. museum samples) from the eastern part of the range (WB_E) and the western part of the range (WB_W), the European zoo population (ZOO), the 'Waldrappteam' population (WRT) for the reintroduction in the Alps and the 'Proyecto Eremita' population (PE) for the reintroduction in Spain. Data set 2: Individuals were assigned to the genetic clusters as determined by the highest assignment probability (Q) of the STRUCTURE analysis (Table 1). This partition was chosen to test for the overall differentiation among breeding lines and genetic variation within them, as a priori assignment based upon geography is not possible within the European zoo population, because zoos exchange individuals permanently (see Witzenberger and Hochkirch 2014). Therefore, the origin of a zoo sample does not reflect a permanent place of origin, but merely the current location of the individual. Testing for genetic differentiation between STRUCTURE clusters thus helps to understand the maximum differentiation between breeding lines.

To compare genetic diversity between groups within data set 1 or clusters within data set 2, we calculated the mean number of alleles (N_a), number and frequency of private alleles, fixation index (F_{IS}), expected (H_e) and observed heterozygosity (H_o) in GenAlEx 6.5 (Peakall and Smouse 2012) as well as allelic richness (A_r) in FSTAT 2.9.3.2 (Goudet

Table 1 Number of individuals in each cluster detected by STRUCTURE (data set 2) and dominating institutions

	Number of individuals	Dominating institutions per cluster
Cluster 1	44	**Waldrappteam Burghausen, Germany/Austria (12 of 62 individuals)**
Cluster 2	30	Parco Natura Viva Bussolengo, Italy (16 of 16 individuals) Dublin Zoo, Ireland (8 of 23 individuals)
Cluster 3	36	Naturzoo Rheine, Germany (15 of 15 individuals)
Cluster 4	51	Konrad-Lorenz-Forschungsstelle Grünau, Austria (23 of 96 individuals)
Cluster 5	20	Wilhelma Zoologischer Garten Stuttgart, Germany (9 of 12 indviduals)
Cluster 6	54	**Waldrappteam Burghausen, Germany/Austria (13 of 62 individuals)** Tiergarten Schönbrunn Wien, Austria (12 of 29 individuals) Tierpark Rosegg, Austria (10 of 25 individuals)
Cluster 7	36	Blackpool Zoo, UK (5 of 5 individuals) Zoo Duisburg, Germany(5 of 5 individuals) Nordens Ark Zoo Hunnebostrand, Sweden (8 of 16 individuals)
Cluster 8	52	Bioparc—Zoo De Doué La Fontaine, France (20 of 29 individuals) Zoologischer Garten Berlin, Germany (8 of 14 individuals)
Cluster 9	42	Konrad-Lorenz-Forschungsstelle Grünau, Austria (30 of 96 individuals)
Cluster 10	43	Alpenzoo Innsbruck Tirol, Austria (12 of 26 individuals) Konrad-Lorenz-Forschungsstelle Grünau, Austria (18 of 96 individuals)
Cluster 11	41	**Waldrappteam Burghausen, Germany/Austria (13 of 62 individuals)** Tiergarten Schönbrunn Wien, Austria (13 of 29 individuals)
Cluster 12	31	WB_E (8 of 8 individuals)
Cluster 13	55	Cotsworld Wildlife Park and Garden, UK (9 of 10 individuals) RZSS Edinburgh Zoo, Scottland (8 of 15 individuals) **Zoobotánico Jerez, Spain (9 of 21 indivduals)**
Cluster 14	48	Natur- und Tierpark Goldau, Switzerland (17 of 19 individuals) Bioparc—Zoo De Doué La Fontaine, France (7 of 29 individuals)
Cluster 15	59	Zoo Zürich, Switzerland (23 of 26 individuals) Nordens Ark Zoo Hunnebostrand, Sweden (8 of 16 individuals)

Numbers in parentheses show the number of individuals from the respective zoo/colony belonging to the cluster; eastern wild birds are underlined, reintroduced birds are in bold

1995), for the populations considered. To explore the level of genetic differentiation between groups (data set 1) or clusters (data set 2), we performed AMOVAs in GenAlEx 6.5 with 9999 iterations and calculated F_{ST}. The effective population sizes (N_e) of the three larger groups in data set 1 (ZOO, WRT, PE) were determined using the linkage disequilibrium method implemented in the software NeEstimator V2.01 (Do et al. 2014), where alleles with a frequency lower than 0.05 were excluded and 95% confidence intervals (CIs) were calculated using the jackknife method (Waples and Do 2008).

To illustrate the genetic relationships among groups, we calculated a Principal Component Analysis (PCA) using the R package adegenet 2.0.2 (Jombart 2015) in R 3.3.2 (R Core Team 2016). Missing data (NA) was replaced by the mean frequency for the allele concerned.

Sequencing

DNA base calling was done in MEGA 6.0 (Tamura et al. 2013), sequence alignment with ClustalW. For pairwise and multiple alignment, the Gap Opening Penalty was set to 15, the Gap Extension Penalty to 6.66. For the DNA Weight Matrix IUB was chosen, while the Transition Weight was set to 0.5. The delay divergent cutoff was set to 30%. The species *Nipponia nippon* was used as outgroup (accession number AY137078.1 and YP_637007 in GenBank®). No phylogenetic inference was conducted due to the small number of substitutions.

Results

Evolutionary lineages DNA sequencing

The cyt *b* sequences were 229 base pairs, the ND5 sequence 732 base pairs long. Only one substitution was found in each gene, both of which were silent substitutions (GenBank® accession numbers of all haplotypes MF682059 to MF682062). The substitution in cyt *b* (position 185) was identical to the one documented by Pegoraro et al. (2001) and reflected the origin of the samples (i.e. differed between wild eastern and wild western individuals), whereas the substitution in ND5 (position 75) was not related to the origin of

the sample. All ZOO individuals as well as all WRT and PE individuals possessed the western cyt *b* haplotype.

Population structure microsatellites

All microsatellite loci amplified successfully in general, even though some loci could not be amplified for certain individuals. Evidence for null alleles (with frequencies > 0.2) were detected at locus B04 in two populations (WRT, PE) and locus B05 in one population (WB_E). Neither large allele dropout nor stutter bands were indicated. Within the five groups (data set 1) we found deviations from Hardy-Weinberg-Equilibrium at 2–13 loci with the highest number of deviations found in the ZOO population. None of the 15 loci deviated systematically from HWE in many populations (or clusters). Therefore all loci were kept in the subsequent analyses. The BAPS analysis assigned all individuals to 18 different clusters [Fig. 2 (K = 18)]. For the following analyses only the STRUCTURE results were considered (Fig. 1). In the STRUCTURE analysis, the highest value for ΔK was found at K = 2, with some further peaks at K = 4, K = 9, K = 15 and K = 23 (selected STRUCTURE plots are

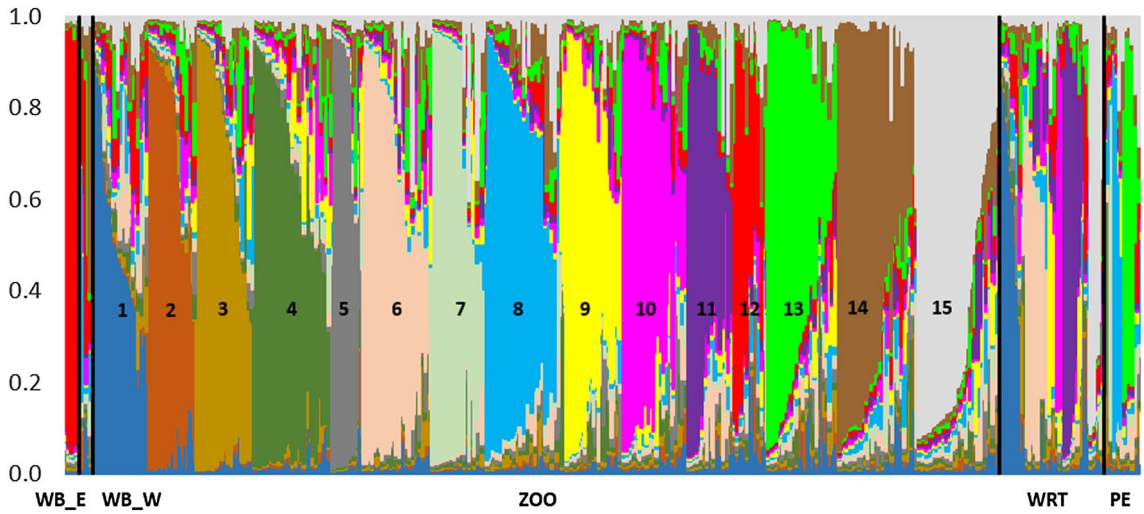

Fig. 1 Genetic clusters inferred by STRUCTURE (K = 15). Each individual is represented by a vertical bar, with the colour representing the estimated proportion of membership to the respective cluster (K) (n = 642). Black bars separate the five groups (WB_E, WB_W, ZOO, WRT, PE). The numbers (1–15) indicate the different clusters. The ZOO population is sorted by cluster affiliation. (Color figure online)

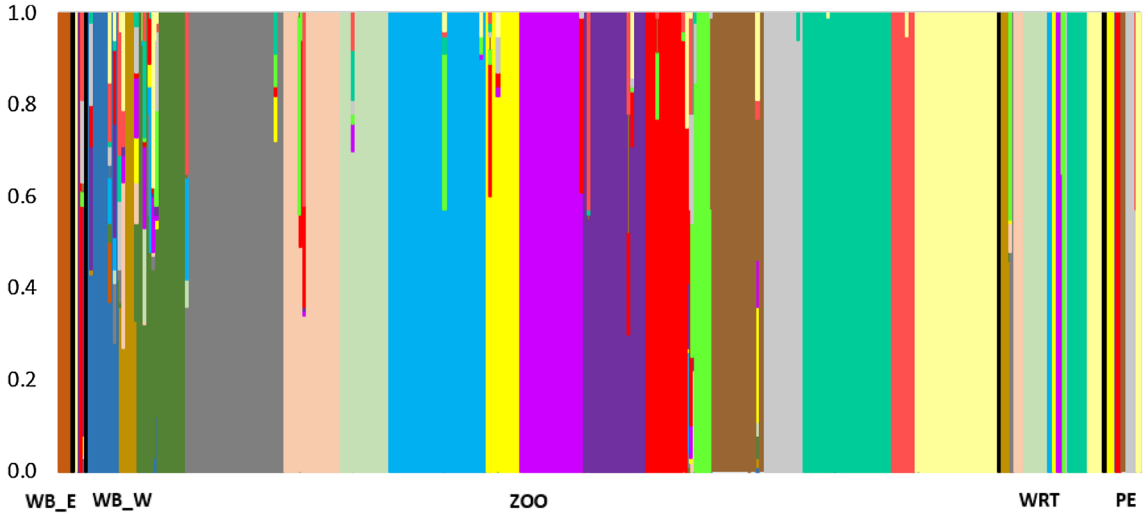

Fig. 2 Genetic clusters inferred by BAPS (K = 18). Each individual is represented by a vertical bar, with the colour representing the estimated proportion of membership to the respective cluster (K) (n = 642). Black bars separate the five groups (WB_E, WB_W, ZOO, WRT, PE). The ZOO population is sorted by cluster affiliation. (Color figure online)

shown in the supplement). Using the Q > 0.9 rule (Schulte et al. 2012), K = 15 was chosen as the most likely number of genetic clusters. The assignment of birds from the different facilities to the respective STRUCTURE cluster is shown in Table 1. Birds from Goldau zoo [Cluster 14, (Switzerland)], Zurich zoo [Cluster 15, (Switzerland)], Rheine zoo [Cluster 3, (Northwest Germany)], and Parco Natura Viva [Cluster 15, Northern Italy)] grouped together in one genetic cluster each. It was also noticeable that birds from three zoos of the United Kingdom (London, Cotsworld, and Edinburgh) grouped together, whereas birds from the nearby Blackpool zoo (Cluster 7) were assigned to birds from Duisburg zoo (reflecting former exchange between these zoos) and Nordens Ark Zoo (Sweden). The individuals from the WRT population (n = 62) were assigned to 11 different clusters with three clusters dominating (cluster 1: 19.3%, cluster 6: 21.0%, cluster 11: 21.0%). Birds from PE population were assigned to six genetic clusters with one cluster dominating (cluster 13: 42.8%). Wild birds from the western part of the range (WB_W) were assigned to five clusters.

The PCA showed a clear differentiation between the eastern wild birds (WB_E) and all the other birds (Fig. 3). The cumulative projected inertia was 46.16% for the first axis and 50.18% for the second axis. The first axis separated WB_E from the groups representing the captive birds (ZOO, WRT, PE) as well as the western wild birds (WB_W). Individuals from the 'Waldrappteam' and 'Proyecto Eremita' showed a complete overlap with the individuals from the European zoo colony.

Based on the AMOVA on data set 1, only 2% of the molecular variance was explained by subdivision into the five groups. The highest level of differentiation was found between WB_E and the four other groups (mean F_{ST} = 0.13 ± 0.01; Table 2). In data set 2, 13% of the molecular variance was explained by the genetic clusters, with the highest differentiation (F_{ST} = 0.18 ± 0.04) between cluster 3 (which consisted to 53.3% from individuals from Rheine zoo) as well as between cluster 5 (45% from Stuttgart zoo) and all other clusters. The mean F_{ST} values for data set 2 are illustrated in Appendix S3.

Table 2 Genetic differentiation (F_{ST} values) between the four groups (data set 1)

	WB_O	WB_W	ZOO	WRT	PE
WB_O	–				
WB_W	0.078	–			
ZOO	0.123	0.016	–		
WRT	0.129	0.044	0.009	–	
PE	0.110	0.000	0.018	0.041	–

All values were significant (P < 0.0001)

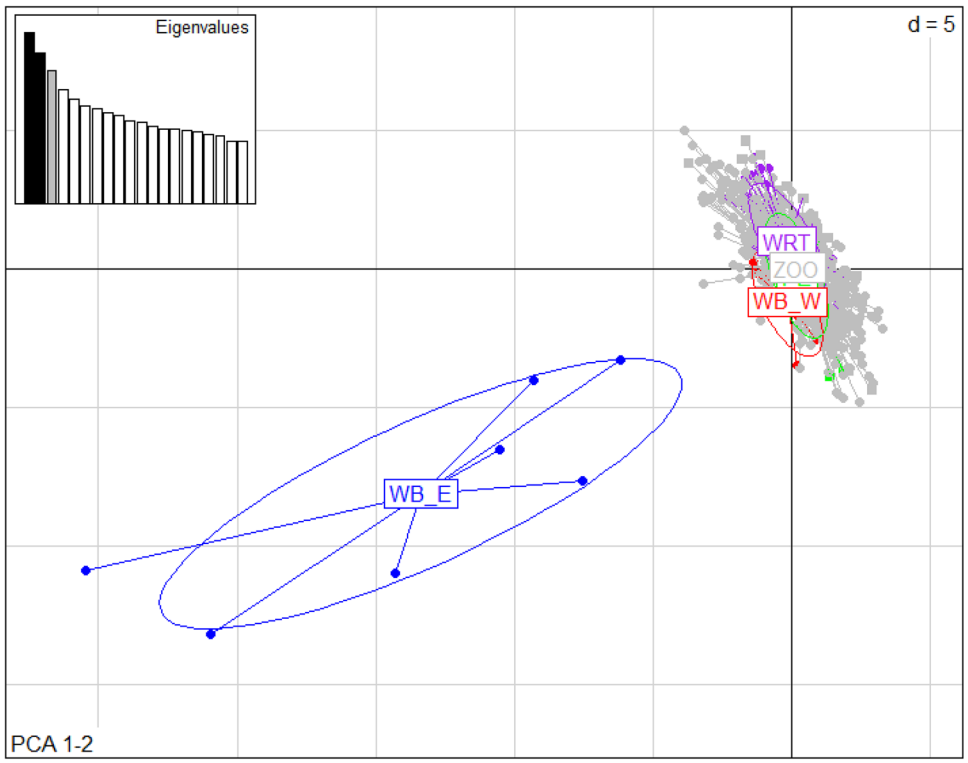

Fig. 3 Plot of the first two axes of the Principal Component Analyses including all multilocus genotypes for the five groups (WB_E: blue, WB_W: red, ZOO: grey, WRT: purple, PE: green). The distribution of Eigenvalues is shown by the graph in the upper left corner. The length of each line shows the distance of each individual from the group centroid

Genetic diversity: microsatellites

A total of 92 alleles was found in our data set. The mean number of alleles per locus varied from 4.00 (±0.24) to 5.40 (±0.36) among the five main groups (data set 1) and from 3.73 (±0.23) to 5.20 (±0.26) among the STRUCTURE based clusters (data set 2). Private alleles were detected in WB_E and WB_W as well as in the European zoo population (ZOO). No sign of inbreeding was detected. The effective population sizes for the groups of data set 1 were 48.3 (ZOO, CI 43.4–53.5), 23.9 (WRT, CI 18.6–31.2) and 16.6 (PE, CI 11.8–24.8).

When comparing genetic diversity in data set 1, allelic richness was highest in WB_E and lowest in WRT (Table 3). Allelic richness in ZOO and PE differed only marginally. In data set 2, the highest allelic richness was found in cluster 12, which was mainly composed of eastern wild birds (WB_E), while some clusters which comprised only zoo individuals (e.g. cluster 2) had a substantially lower genetic diversity. Among the alleles detected ($N_a = 92$), we found 90% in the captive individuals (ZOO), while the remaining 10% were found only in wild birds (WB). The 'Waldrapp-team' population (WRT) contained 73.9% of the alleles available in the European zoo colony, the 'Proyecto Eremita' population (PE) 71.7%.

Discussion

Two evolutionary significant units of the Northern Bald Ibis?

Our DNA sequence data do not confirm the hypothesis of two distinct ESUs in the Northern Bald Ibis. Although our results confirm the single substitution in the cyt *b* gene documented by Pegoraro et al. (2001), we did not detect any other region-specific substitution in ND5. The use of neutral genetic markers is quite common in defining ESUs (Allendorf et al. 2010; Ishtiaq et al. 2015; Wan et al. 2004). Based upon our DNA sequence data there is currently no basis for treating both populations as ESUs. However, it must be considered that neutral markers are not suitable to detect adaptations to local environmental conditions that may occur at a low frequency in the genomes (Selkoe and Toonen 2006). It remains unknown whether differences in migratory behaviour between birds from North Africa and the Middle East are genetically determined or caused by the different environmental conditions (Fritz and Unsöld 2015; Landmann 2015). Hence, mixing the populations should be avoided as long as no indication of inbreeding depression is observed in any of the populations. It would be advisable to study the eastern and western populations also by using

Table 3 Genetic diversity in the STRUCTURE based clusters (data set 2) and the five main groups (data set 1)

Cluster/group	N	N_a	A_r	H_o	uH_e	F_{IS}
CL_1	44	4.40 (±0.27)	3.93	0.59 (±0.04)	0.59 (±0.04)	0.01 (±0.04)
CL_2	30	3.73 (±0.23)	3.57	0.63 (±0.04)	0.59 (±0.03)	− 0.07 (±0.05)
CL_3	36	4.20 (±0.24)	3.87	0.57 (±0.04)	0.57 (±0.04)	0.00 (±0.04)
CL_4	51	4.20 (±0.24)	3.75	0.63 (±0.03)	0.60 (±0.02)	− 0.05 (±0.03)
CL_5	20	3.80 (±0.22)	3.70	0.60 (±0.05)	0.58 (±0.03)	− 0.06 (±0.06)
CL_6	54	4.27 (±0.23)	3.86	0.61 (±0.04)	0.59 (±0.03)	− 0.05 (±0.04)
CL_7	36	4.20 (±0.26)	3.94	0.58 (±0.04)	0.57 (±0.04)	− 0.04 (±0.04)
CL_8	52	4.47 (±0.29)	3.99	0.64 (±0.03)	0.65 (±0.02)	0.00 (±0.04)
CL_9	42	4.20 (±0.24)	3.80	0.58 (±0.04)	0.56 (±0.03)	− 0.04 (±0.03)
CL_10	43	4.33 (±0.21)	3.84	0.64 (±0.05)	0.59 (±0.04)	− 0.08 (±0.03)
CL_11	41	4.20 (±0.28)	3.73	0.58 (±0.04)	0.57 (±0.03)	− 0.02 (±0.04)
CL_12	31	5.20 (±0.26)	4.91	0.69 (±0.03)	0.70 (±0.03)	0.08 (±0.06)
CL_13	55	4.67 (±0.33)	4.09	0.62 (±0.03)	0.62 (±0.02)	0.00 (±0.03)
CL_14	49	4.10 (±0.28)	3.89	0.57 (±0.05)	0.57 (±0.04)	0.00 (±0.05)
CL_15	59	4.27 (±0.28)	3.73	0.61 (±0.04)	0.61 (±0.02)	0.00 (±0.03)
WB_E	8	4.27 (±0.23)	4.04	0.69 (±0.05)	0.72 (±0.02)	− 0.02 (±0.07)
WB_W	7	4.00 (±0.24)	3.87	0.58 (±0.06)	0.69 (±0.03)	0.08 (±0.08)
ZOO	551	5.40 (±0.36)	3.66	0.60 (±0.03)	0.68 (±0.02)	0.12 (±0.03)
WRT	66	4.47 (±0.26)	3.53	0.64 (±0.04)	0.66 (±0.02)	0.03 (±0.04)
PE	26	4.33 (±0.32)	3.80	0.68 (±0.22)	0.70 (±0.02)	− 0.04 (±0.05)

N sampling size, N_a number of alleles, A_r allelic richness, H_o observed heterozygosity, $_uH_e$ unbiased expected heterozygosity, F_{IS} fixation index

adaptive genetic markers to evaluate potential adaptations to the environment.

Contrary to the two sequenced genes, the PCA plot based on microsatellite data (Fig. 3) showed a strong differentiation of the wild bird population (WB_E) from the eastern part of the range compared to the European zoo population (ZOO), the wild western birds (WB_W) and the two source populations for reintroductions (WRT and PE). This result is not surprising and suggests that there is no recent gene flow between the eastern and western population (or the European zoo population). It also confirms that the complete captive population originated from Morocco. However, the genetic differentiation on the microsatellite level is probably not a sufficient argument to treat both populations as different management units (Palsbøll et al. 2007). Differentiation in microsatellite loci can arise quite rapidly as shown by our own data, i.e. the strong genetic structure within the ZOO population. To derive final conclusions regarding the status of the western and eastern birds, more detailed knowledge on the fitness effects of interbreeding and potential adaptations would be required. Furthermore it would be necessary to study morphological, behavioural or ecological differences (as indicated by the study of Böhm and Pegoraro 2011 who mention that western birds have shorter bills).

Genetic structure of the captive population

We found significant genetic structure of the European zoo population of the Northern Bald Ibis, reflecting known breeding lines in most cases. For example, birds from Blackpool zoo grouped with birds from Duisburg zoo (Cluster 7), which is in line with the exchange of individuals recorded in the EEP. Nearly all individuals from Goldau, Rheine and Zurich zoo grouped together in one genetic clusters with only slight signs of admixture (Fig. 1; Table 1), reflecting the limited breeding management in these facilities. A similar pattern was found in the zoo population of the European wildcat, which is completely unmanaged (Witzenberger and Hochkirch 2014). This suggests that exchange between zoos could be optimized to increase admixture and ensure the maintenance of genetic diversity of the Northern Bald Ibis ex situ population. This needs to include exchange across national borders to support admixture. It must be prevented that specific zoos constitute distinct genetic clusters due to limited exchange with other institutions. Our results can help to reach this goal and help the EEP to prevent reproduction of closely related individuals and minimize kinship within the captive colonies (Ballou and Lacy 1995; Ivy et al. 2009).

Despite the potential to optimize gene flow among the European zoo population, the levels of heterozygosity were generally high and no indications of inbreeding were detected (F_{IS} varied from −0.08 to 0.12). The higher F_{IS} value for the European zoo population (ZOO) could be explained by the deviations from HWE for this population due to the Wahlund effect. The results suggest that the captive population of the Northern Bald Ibis is generally vital and does not suffer strongly from loss of genetic diversity.

Genetic diversity of the source populations for reintroductions

To avoid negative consequences of low genetic diversity in reintroduced populations, it is recommended that the genetic relatedness of reintroduced individual should be kept as low as possible (Alcaide et al. 2010; Willoughby et al. 2015). A high genetic diversity is important to avoid founder effects, which in turn could promote inbreeding due to the small number of alleles available (Frankham et al. 2010). Furthermore, a high genetic diversity is necessary to allow a population to adapt to potentially changing environmental conditions in their new habitat (Frankham 2005; Keller and Waller 2002; Holderegger and Segelbacher 2016).

The genetic diversity of the source population for reintroductions in Austria (WRT) is slightly lower than in the general ZOO population. However, this source population maintains the majority of alleles detected in the ZOO population and only some rare alleles were missing. The 'Proyecto Eremita' source population (PE) shows marginally higher values ($A_{r,\ u}H_e$) than the European zoo population. The source populations for both reintroduction projects were dominated by a few genetic clusters (as determined by the STRUCTURE analysis; Fig. 1) and it is therefore advisable to add some individuals from underrepresented breeding lines to the reintroduced population. The effective population sizes are still low when considering the general recommendations on the number of individuals needed to establish viable populations (Frankham 1995). However, it is unlikely that these values can be strongly increased given the low effective population size of 8.8%, of the European zoo population. Integrating individuals from the wild might be the only way to increase N_e values, but this is currently no feasible option due to the high conservation status of the Northern Bald Ibis in the wild. Samples of extant wild Moroccan birds could improve our analyses, as the available western wild birds were probably part of the founders of the ZOO population. It would thus be important to clarify the genetic diversity in the last extant wild populations.

Comparison with the historic samples from wild populations

Despite our small sample size of eight individuals from the eastern wild population (WB_E), it is striking that this population had a higher genetic diversity than any of the ex situ groups or the western wild population, including a relatively high proportion of private alleles. While this might

be an effect of the genetic differentiation between the eastern and western population (as discussed above), it might also point to an initial founder effect caused by a low number of founders of the European zoo population or by the decline of the western population prior to captive breeding. Furthermore, the duration and the captive breeding itself could have triggered a loss of genetic diversity. While the eastern population in Syria is now possibly extinct, a semi-wild colony still exists in Birecik (Turkey). It is likely that this population still maintains some private alleles compared to the European zoo population, and inclusion of specimens from the eastern population into the gene pool of the ex situ population would certainly increase genetic diversity in the latter. However, this would first require a genetic study on the Birecik colony, and even more important, a study on potential genetic adaptations of the eastern and western populations to avoid any negative effects of outbreeding, such as a loss of alleles which may show adaptations to the Middle East habitats, but may be detrimental to the life in western environments (Edmands and Timmerman 2003; Frankham 2008; Lynch 1991). If based upon this study mixing both populations appears feasible in the future, we recommend an experimental approach in captivity first with strict monitoring of the genetic effects and fitness (in terms of survival and reproductive success) of the offspring. However, given the high restrictions of genetic sampling in Turkey and the high conservation status of the only wild Northern Bald Ibis population in Morocco, sampling these populations will require concerted action of the authorities involved. Closing this knowledge gap is one of the key factors identified by the AEWA Northern Bald Ibis International Working Group to support conservation measures (Bowden 2015).

Conclusion

Our study shows that the genetic diversity of the source populations for reintroductions largely reflect the genetic diversity found in the European zoo population. The European zoo population of the Northern Bald Ibis is genetically less diverse than the few historic samples from the Middle East, but generally vital and does not suffer strongly from this loss of genetic diversity. The strong genetic structure suggests that admixture within the European zoo population can be improved by targeted exchange of individuals from different breeding lines. Genetic diversity in the source populations for reintroductions can be further increased by specifically adding individuals from underrepresented breeding lines to these stocks. We can currently not confirm a significant differentiation of the eastern and western population of the Northern Bald Ibis, justifying their treatment as different ESUs. However, as a precaution, we still advise to manage them separately as long as no specific study on genetic adaptations is available and as long as no negative effects of inbreeding are apparent. Our study confirms that genetic studies provide a suitable basis for improving the management of captive populations and reintroductions.

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References

Alcaide M, Negro JJ, Serrano D, Antolín JL, Casado S, Pomarol M (2010) Captive breeding and reintroduction of the lesser kestrel Falco naumanni: a genetic analysis using microsatellites. Conserv Genet 11:331–338. https://doi.org/10.1007/s10592-009-9810-7

Allendorf FW, Hohenlohe PA, Luikart G (2010) Genomics and the future of conservation genetics. Nat Rev Genet 11:697–709

Armstrong DP, Seddon PJ (2008) Directions in reintroduction biology. Trends Ecol Evol 23:20–25

Attard CRM, Moller LM, Sasaki M, Hammer MP, Bice CM, Brauer CJ, Carvalho DC, Harris JO, Beheregaray LB (2016) A novel holistic framework for genetic-based captive-breeding and reintroduction programs. Conserv Biol 30:1060–1069. https://doi.org/10.1111/cobi.12699

Ballou JD, Lacy RC (1995) Identifying genetically important individuals for management of genetic diversity in pedigreed populations. In: Ballou JD, Gilpin M, Foose TJ (eds) Population management for survival & recovery. Analytical methods and strategies in small population conservation. Columbia Univ. Press, New York

Ballou JD, Lees C, Faust LJ, Long S, Lynch C, Lackey LB, Foose TJ (2010) Demographic and genetic management of captive populations. In: Kleinmann DJ, Lumpkin S, Allen M, Harris H, Thompson K (eds) Wild mammals in captivity: principles and techniques for zoo management. University of Chicago Press, Chicago, pp 219–252

Bartlett SE, Davidson WS (1991) Identification of *Thunnus tuna* species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochrome b genes. Can J Fish Aquat Sci 48:309–317

BirdLife International (2015) Species factsheet: *Geronticus eremita*, BirdLife. http://datazone.birdlife.org/species/factsheet/22697488. Accessed 06 April 2017

Böhm C (2015) Northern Bald Ibis *Geronticus eremita* EEP studbook. Alpenzoo, Innsbruck

Böhm C, Bowden CGR (2016) Northern Bald Ibis Conservation and Translocation Workshop. Report of 4th International Advisory Group for the Northern Bald Ibis (IAGNBI) Meeting Seekirchen, Austria; August 2016; p. 121

Böhm C, Pegoraro K (2011) Der Waldrapp. Die Neue Brehmbücherei 659. Westarp, Hohenwarsleben

Bowden CGR (2015) International Single Species Action Plan for the Conservation of the NorthernBald Ibis (*Geronticus eremita*). AEWA Technical Series No. 55. Bonn

Bowden CGR, Aghnaj A, Smith KW, Ribi M (2003) The status and recent breeding performance of the critically endangered Northern Bald Ibis *Geronticus eremita* population on the Atlantic coast of Morocco. Ibis 145:419–431

Bowden CGR, Smith KW, El Bekkay M, Oubrou W, Aghnaj A, Jimenez-Armesto M (2008) Contribution of research to conservation action for the Northern Bald Ibis *Geronticus eremita* in Morocco. Bird Conserv Int 18:S74–S90. https://doi.org/10.1017/S0959270908000403

Bowkett AE (2009) Recent captive-breeding proposals and the return of the ark concept to global species conservation. Conserv Biol 23:773–776

Chapuis MP, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. Mol Biol Evol 24:621–631

Conde DA, Flesness N, Colchero F, Jones OR, Scheuerlein A (2011) An emerging role of zoos to conserve biodiversity. Science 331:1390–1391

Conway WG (2011) Buying time for wild animals with zoos. Zoo Biol 30:1–8

Corander J, Marttinen P (2006) Bayesian identification of admixture events using multilocus molecular markers. Mol Ecol 15:2833–2843. https://doi.org/10.1111/j.1365-294X.2006.02994.x

Cuarón AD (2005) Further role of zoos in conservation: monitoring wildlife use and the dilemma of receiving donated and confiscated animals. Zoo Biol 24:115–124. https://doi.org/10.1002/zoo.20040

Do C, Waples RS, Peel D, Macbeth GM, Tillett BJ, Ovenden JR (2014) NeEstimator v2: re-implementation of software for the estimation of contemporary effective population size (Ne) from genetic data. Mol Ecol Resour 14:209–214. https://doi.org/10.1111/1755-0998.12157

Dolman PM, Collar NJ, Scotland KM, Burnside R (2015) Ark or park: the need to predict relative effectiveness of ex situ and in situ conservation before attempting captive breeding. J Appl Ecol 52:841–850. https://doi.org/10.1111/1365-2664.12449

Earl DA, von Holdt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Geneti Resour 4:359–361. https://doi.org/10.1007/s12686-011-9548-7

Edmands S (2007) Between a rock and a hard place: evaluating the relative risks of inbreeding and outbreeding for conservation and management. Mol Ecol 16:463–475

Edmands S, Timmerman CC (2003) Modeling factors affecting the severity of outbreeding depression: factores de Modelaje que Afectan la Severidad de la Depresión Exogámica. Conserv Biol 17:883–892. https://doi.org/10.1046/j.1523-1739.2003.02026.x

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Frankham R (1995) Effective population size/adult population size ratios in wildlife: a review. Genet Res 66:95–107

Frankham R (2005) Stress and adaptation in conservation genetics. J Evol Biol 18:750–755. https://doi.org/10.1111/j.1420-9101.2005.00885.x

Frankham R (2008) Genetic adaptation to captivity in species conservation programs. Mol Ecol 17:325–333

Frankham R, Ballou JD, Briscoe DA (2010) Introduction to conservation genetics, 2nd edn. Cambridge University Press Cambridge

Fritz J (2004) The Scharnstein Project: establishing a migratory tradition with handraised Waldrapp Ibises. WAZA Magazine 5: 16–19

Fritz J, Unsöld M (2015) Internationaler Artenschutz im Kontext der IUCN reintroduction guidelines: Argumente zur Wiederansiedlung des Waldrapps Geronticus eremita in Europa. Vogelwarte 53:169–179

Fritz J, Kramer R, Hoffmann W, Trobe D, Unsöld M (2017) Back into the wild: establishing a migratory Northern bald ibis *Geronticus eremita* population in Europe. Int Zoo Yearb 51:107–123. https://doi.org/10.1111/izy.12163

Goudet J (1995) FSTAT (Version 1.2): A computer program to calculate F-statistics. J Hered 866:485–486

Grepom BirdLife Maroc (2014) Rapport sur la reproduction de l'Ibis chauve dans la région de Souss-Massa. Saison 2014. http://grepom.org/programme-de-suivi-de-la-population-delibis-chauve-rapport-de-reproduction-saison-2014/. Accessed 06 April 2017

Grueber CE, Hogg CJ, Ivy JA, Belov K (2015) Impacts of early viability selection on management of inbreeding and genetic diversity in conservation. Mol Ecol 24:1645–1653. https://doi.org/10.1111/mec.13141

Hedrick PW, Garcia-Dorado A (2016) Understanding inbreeding depression, purging and genetic rescue. Trends Ecol Evol 31:940–952. https://doi.org/10.1016/j.tree.2016.09.005

Holderegger R, Segelbacher G (eds) (2016) Naturschutzgenetik. Ein Handbuch für die Praxis, Hauptverlag, Bern

Ishtiaq F, Prakash V, Green RE, Johnson J (2015) Management implications of genetic studies for ex situ populations of three critically endangered Asian Gyps vultures. Anim Conserv 18:259–270

Ivy JA, Miller A, Lacy RC, DeWoody JA (2009) Methods and prospects for using molecular data in captive breeding programs: an empirical example using parma wallabies (*Macropus parma*). J Hered 100:441–454

Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23:1801–1806. https://doi.org/10.1093/bioinformatics/btm233

Janes JK, Miller JM, Dupuis JR, Malenfant RM, Gorrell JC, Cullingham CI, Andrew RL (2017) The K = 2 conundrum. Mol Ecol 26:3594–3602. https://doi.org/10.1111/mec.14187

Jombart T (2015) An introduction to adegent 2.0.0, R-tutorial, Imperial College London

Keller LF, Waller DM (2002) Inbreeding effects in wild populations. Trends Ecol Evol 17:230–241

Kirkwood JK (2003) Welfare, husbandry and veterinary care of wild animals in captivity: changes in attitudes, progress in knowledge and techniques. Int Zoo Yearb 38:124–130

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Nat Acad Sci USA 86:6196–6200

Lacy RC (1994) Managing genetic diversity in captive populations of animals. In: Bowles ML, Whelan CJ (eds) Restoration of endangered species. conceptual issues, planning and implementation. Cambridge University Press, Cambridge, pp 63–89

Landmann A (2015) Bestandsschutz, Bestandsstützung, Wiederansiedlung oder Auswilderung-Wie kann oder soll der Waldrapp Geronticus eremita geschützt werden? Vogelwarte 53:169–180

Lynch M (1991) The genetic interpretation of inbreeding depression and outbreeding depression. Evolution 45:622–629

Muñoz AR, Ramírez J (2017) Reintroduced northern bald ibises from Spain reach Moroco. Oryx 51:204–205. https://doi.org/10.1017/S0030605317000138

Palsbøll PJ, Berube M, Allendorf FW (2007) Identification of management units using population genetic data. Trends Ecol Evol 22:11–16

Paton TA, Baker AJ (2006) Sequences from 14 mitochondrial genes provide a well-supported phylogeny of the Charadriiform birds congruent with the nuclear RAG-1 tree. Mol Phyl Evol 39:657–667

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28:2537–2539. https://doi.org/10.1093/bioinformatics/bts460

Pegoraro K, Föger M, Parson W (2001) First evidence of mtDNA sequence differences between Northern Bald Ibises (*Geronticus eremita*) of Moroccan and Turkish origin. Journal für Ornithologie 142:425–428

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Quevedo MA, Sánchez I, Aguilar JM, Cuandrado M 2004: Projecto Eremita: a study of different releasing techniques. WAZA Magazine 5:20–23

R Core Team (2016) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/

Saura M, Perez-Figueroa A, Fernandez J, Toro MA, Caballero A (2008) Preserving population allele frequencies in ex situ conservation programs. Conserv Biol 22:1277–1287. https://doi.org/10.1111/j.1523-1739.2008.00992.x

Schenker A (1977) Das ehemalige Verbreitungsgebiet des Waldrapps Geronticus eremita in Europa. Ornithologische Beobachtungen 74:13–30

Schulte U, Veith M, Hochkirch A (2012) Rapid genetic assimilation of native wall lizard populations (*Podarcis muralis*) through extensive hybridization with introduced lineages. Mol Ecol 21:4313–4326

Seddon PJ, Armstrong DP, Maloney RF (2007) Developing the science of reintroduction biology. Conserv Biol 21:303–312. https://doi.org/10.1111/j.1523-1739.2006.00627.x

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecol Lett 9:615–629

Serra G, Abdallah M, Assaed A, Abdallah A, Al Qaim G, Fayad T, Williamson D (2004) Discovery of a relict breeding colony of northern bald ibis *Geronticus eremita* in Syria. Oryx 38:106–108

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. https://doi.org/10.1093/molbev/mst197

Thaler E, Ettel E, Job S (1981) Zur Sozialstruktur des WaldrappsGeronticus eremita—Beobachtungen an der Brutkolonie des Alpenzoos Innsbruck. Journal für Ornithologie 122:109–128

Tintner A, Kotrschal K (2001) Waldrappen im Freiflug-Das Grünauer Waldrapp-Projekt. Zoologischer Garten 71:118–127

Tracy LN, Wallis GP, Efford MG, Jamieson IG (2011) Preserving genetic diversity in threatened species reintroductions: how many individuals should be released? Anim Cons 14:439–446

Unsöld M, Fritz J (2011) Der Waldrapp: Ein Vogel zwischen Ausrottung und Wiederkehr. Wildbiologie 2:1–16

van Dyke F (2008) Conservation biology: foundations, concepts, applications. Springer Science & Business Media, Berlin

van Oosterhout C, Hutchinson WF, Wills DM, Shipley P (2004) MICROCHECKER:MICROCHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538

Wan Q, Wu H, Fujihara T, Fang S (2004) Which genetic marker for which conservation genetics issue? Electrophoresis 25:2165–2176

Waples RS, Do C (2008) LDNE: a program for estimating effective population size from data on linkage disequilibrium. Mol Ecol Resour 8:753–756

Willoughby JR, Sundaram M, Wijayawardena BK, Kimble SJ, Ji Y, Fernandez NB, Antonides JD, Lamb MC, Marra NJ, DeWoody JA (2015) The reduction of genetic diversity in threatened vertebrates and new recommendations regarding IUCN conservation rankings. Biol Cons 191:495–503. https://doi.org/10.1016/j.biocon.2015.07.025

Wirtz S, Böhm C, Fritz J, Hankeln T, Hochkirch A (2016) Isolation of microsatellite loci by next-generation sequencing of the critically endangered Northern Bald Ibis, *Geronticus eremita*. J Hered 107:363–366. https://doi.org/10.1093/jhered/esw013

Witzenberger KA, Hochkirch A (2011) Ex situ conservation genetics: a review of molecular studies on the genetic consequences of captive breeding programmes for endangered animal species. Biodivers Conserv 20:1843–1861. https://doi.org/10.1007/s10531-011-0074-4

Witzenberger KA, Hochkirch A (2014) The genetic integrity of the ex situ population of the European Wildcat (*Felis silvestris silvestris*) is seriously threatened by introgression from domestic cats (*Felis silvestris catus*). PLoS ONE 9:e106083. https://doi.org/10.1371/journal.pone.0106083