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Research article

Genetic assessments for antelope reintroduction planning in four European breeding programmes

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Abstract

The potential reintroduction value of zoo animals is often cited as a reason for maintaining captive populations. To validate this argument, it is important for conservation breeding programmes to consider the evolutionary history and population genetic diversity of their founders, so that managers can understand the possible consequences of breeding decisions in captivity and to evaluate the options for releasing individuals back to the wild. For the European captive populations of roan antelope (*Hippotragus equinus*), greater kudu (*Tragelaphus strepsiceros* or *Strepsiceros spp.*), common eland (*Tragelaphus oryx*) and waterbuck (*Kobus ellipsiprymnus* or *Kobus spp.*), there is a need to understand more about their genetic status and to evaluate their likely geographic origin within their natural distribution. We employed DNA nucleotide sequencing of the mitochondrial (mtDNA) control region to identify the maternal lineage of captive animals and inform decision making concerning future possible translocations in each species. Sequence data from 60 individual antelope were compared against existing reference data from wild populations. Sequence analysis of roan, greater kudu and common eland allowed inference of the broad geographic origin and subspecies of each animal's maternal lineage. For waterbuck, clear discrimination of ellipsen and defassa subspecies was not possible due to a zone of hybridisation preventing unambiguous assignment of captive waterbuck to subspecies. Our findings highlight the application of molecular genetic research to a persistent challenge in zoo population management; namely, the need to understand captive genetic variation relative to that found in the wild.

Introduction

The importance of genetic management within zoos is well established and has become integral to the formulation of breeding recommendations (Ballou and Lacy 1995). The overarching aim of many zoo breeding programmes is to maintain population genetic diversity over the long term (~100 years) to minimise risks associated with inbreeding, reduce loss of evolutionary adaptive potential and ensure that populations are in optimal genetic condition to support future reintroduction and reinforcement activities (Frankham et al. 2010). In addition to minimising loss of diversity and co-ancestry, good management practice should also consider broad levels of population differentiation within species so that, wherever appropriate, groups, such as evolutionary significant units (ESUs), management units (MUs) or subspecies, are maintained as cohesive independent breeding populations. This approach reduces the risk of outbreeding depression within adaptively differentiated species (Frankham et al. 2011) and allows captive populations to contribute to translocation programmes that seek to adhere to IUCN translocation guidelines concerning the movement of animals in the wild (IUCN/SSC 2013). For widely-distributed taxa that may have experienced population differentiation over evolutionary timescales, the geographic and genetic origins of captive animals should be considered when deciding whether they

are suitable for incorporation into release projects. It is therefore necessary for species management programmes to consider the evolutionary history of population founders and to understand the possible consequences of subsequent mixing throughout the species' pedigree history in captivity.

The application of molecular genetic (DNA) analysis to studies of species diversity in the wild provides a strong reference base against which to investigate and interpret zoo population genetic diversity. By assessing wild and captive animals with a common set of DNA markers, observed ex-situ genetic variation can be interpreted in the context of historic or contemporary in-situ population data. A number of recent studies have employed such an in situ–ex situ comparative approach to evaluate relative levels of population genetic diversity (e.g. in golden eagles; Sato et al. 2017), to understand zoo population genetic structure (e.g. in okapi; Stanton et al. 2015; in dama gazelle; Senn et al. 2014) and to examine subspecies admixture within captive individuals (e.g. chimpanzees; Hvilsom et al. 2013). Here we extend this application of conservation genetic management to the assessment of captive-bred individuals belonging to four antelope species within EAZA (European Association of Zoos and Aquaria) conservation breeding programmes.

Within the European captive populations of roan antelope (*Hippotragus equinus*), greater kudu (*Tragelaphus strepsiceros* or *Strepsiceros spp.*), common eland (*Tragelaphus* or *Taurotragus oryx*) and waterbuck (*Kobus ellipsiprymnus* or *Kobus spp.*), there is a need to understand more about their genetic status and to evaluate their likely geographic origin based on species-wide phylogeographic variation. Each species has been the subject of serial taxonomic revisions and a number of phylogenetic studies of natural populations that provide the basis for assigning captive antelope to wild origin, with varying levels of precision. Here we employ DNA nucleotide sequencing of the mitochondrial DNA (mtDNA) control region to identify the maternal lineage of captive animals and inform decision making concerning future possible translocations. The use of mtDNA sequence data will enable the implementation of a precautionary exclusion approach, restricting translocation in cases where the mtDNA lineage of an individual does not correspond to observed genetic variation in the geographic region of introduction. The study is part of a developing programme to conduct genetic analysis of individual antelope to inform conservation managers about their wild origin.

Target species – taxonomy, phylogeography and management questions

The roan antelope has previously been considered to consist of six subspecies (Ansell 1971), which may be grouped into three broad geographic regions: western, central, and eastern and southern (Kingdon and Hoffman 2013). Studies of mitochondrial and nuclear DNA diversity recognised the western subspecies, *H. e. koba*, as clearly distinct from the others (Alpers et al. 2004). The taxonomy of Groves and Grubb (2011), adopted by Wilson and Mittermeier (2011), does not recognise any subspecies, but acknowledges the genetic distinctiveness of the western koba population. The central issue in our study was to assess the evidence for whether captive roan antelope individuals originate from the western, central or eastern/southern populations.

The greater kudu has been described as comprising four subspecies of *Tragelaphus strepsiceros* based on stripe patterns (Ansell 1971), while Kingdon (1997) recognised three subspecies. Groves and Grubb (2011), adopted by Wilson and Mittermeier (2011), recognised four full species, placing them in the *Strepsiceros* genus, as follows: Cape kudu *S. strepsiceros* (coastal south-eastern South Africa, plus isolated populations in central South Africa); Zambezi kudu *S. zambesiensis* (distribution from northern Tanzania/southern Kenya south to eastern South Africa and west to Namibia); northern kudu *S. chora* (northern Tanzania/ southern Kenya north to Eritrea and east Sudan); and western kudu *S. cottoni* (north Central African Republic, south-eastern Chad, western Sudan). Previous genetic studies based on mtDNA data (Nersting and Arctander 2001, illustrated in Lorenzen et al. 2012), have included a large portion of the range of Zambezi kudu, across which broad phylogeographic structuring is observed from southwest to northeast. However, this study apparently did not include Cape kudu or western kudu, and only one northern kudu. The aim of our study was to examine whether captive antelope displayed mtDNA haplotypes consistent with either the northern kudu or the Zambezi kudu.

The common eland is recognised by Wilson and Mittermeier (2011) as comprising two subspecies, in the genus *Taurotragus*, based on pelage: *Taurotragus oryx oryx* (southern Africa including Namibia, Botswana, Swaziland and South Africa); and T*aurotragus oryx livingstonii* (eastern Africa across to central west, including Sudan, Ethiopia, Somalia, Kenya, Tanzania, Uganda, Rwanda, Democratic Republic of the Congo, Angola). Kingdon and Hoffman

Table 1. Mitochondrial DNA control region sequencing results for 60 antelope samples in this study. Data for greater kudu, common eland and roan antelope enabled specific translocation management questions to be addressed, however introgression between wild waterbuck subspecies confounded assignment of individual to either defassa or ellipsen forms. Collection codes: H = Howletts, UK; PL = Port Lympne, UK; DK = Dvur Kralove, CZ; S = Sigean, FR.

Species	n	Collection (samples)	Sample type	Haplotypes observed	Translocation Issue	Results
Greater Kudu	6	H(5); PL(1)	FTA card	3	Zambezi kudu* or not (Z/NZ)	Z=100%
Eland	10	PL	FTA card	3	Southern (S) or Eastern (E)	S=50%; E=50%
Roan	20	PL(10); DK(10)	FTA (10); Hair (10)	2	Southern/eastern vs western	S/E (E) = 100%
Ellipsen waterbuck	12	DK	Hair	4	Ellipsen vs defassa	-
Defassa waterbuck	12	PL(4); DK(3); S(5)	FTA (9); Hair (3)	4	Ellipsen vs defassa	-

(2013) maintain eland in the *Tragelaphus* genus, and recognise a third subspecies *T. pattersonianus*, as the northern subspecies, from Tanzania northwards. Genetic studies by Lorenzen et al. (2010) found a significant regional divide between mtDNA lineages from southern and eastern Africa. In our study we addressed the issue of whether or not the captive population contained representatives of either lineage.

Waterbuck, are usually considered to comprise two subspecies (Kingdon and Hoffman 2013): ellipsen (*Kobus ellipsiprymnus ellipsiprymnus*) and defassa (*K. e. defassa*), based on differences in phenotype and geographical distribution (Ansell 1971; East 1998). Groves and Grubb (2011), adopted by Wilson and Mittermeier (2011), consider the two taxa to represent two monotypic species, *Kobus ellipsiprymnus* and *K. defassa*. Ellipsen waterbuck are found in eastern and southern Africa and defassa are distributed across western and central Africa, with a contact zone and intermediate forms occurring in Kenya (Lorenzen et al. 2006). One previous genetic study focusing on population genetic structure, primarily in East Africa, revealed a continuous cline in nuclear DNA diversity from ellipsen to defassa subspecies, which did not form reciprocally monophyletic clades under mitochondrial DNA control region analysis (Lorenzen et al. 2006). Some geographic structure was evident, however, with haplotypes typically clustering by subspecies across an east–west transition. In the current study, samples of captive ellipsen and defassa waterbuck were analysed to determine the relationship of their maternal lineage to those of wild waterbuck across the sampled distribution of the two subspecies.

Materials and Methods

Sampling

Samples for DNA analysis were obtained from four captive collections in Europe between 2011 and 2017 as bloods stored on Whatman FTA cards or plucked hairs stored in envelopes. Samples were collected from 20 roan antelope, six greater kudu, 10 common eland and 24 waterbuck (Table 1). Where possible, animals were selected to represent separate founder matrilines to maximise the proportion of the captive population represented in the study. According to the ZIMS database, currently animal numbers are approximately as follows: greater kudu, c230 animals (from c25 founders); roan, c140 animals (from only 5 reported founders); common eland, c550 animals; defassa waterbuck, c140 animals; ellipsen waterbuck, c170 animals (founder numbers for last three species unknown).

Molecular genetic analysis

DNA extraction was performed using the Qiagen DNeasy Blood & Tissue Kit (Qiagen 69504). Hair samples were prepared by cutting the shafts of 20 hairs per individual and retaining ~1cm of hair shaft with the follicle for digestion using 20µl of proteinase K and 20µl 1M DTT in 250µl of ATL buffer, vortexed and incubated at 56°C for one hour. PCR amplification of the control region was performed in greater kudu, common eland and waterbuck using the forward MT4 (Arnason et al. 1993) and reverse BT16168H (Simonsen et al. 1998) primer pair, amplifying a region of approximately 500bp in length. For roan antelope, DNA samples were initially amplified using the forward N777 (L15910) and reverse DLH1 (H164998) primer pair (~550bp) (Matthee and Robinson 1999); however, taxon-specific primers were subsequently designed and employed, targeting a longer, fully overlapping 568bp region to improve PCR amplification in lower quality samples (RoanF: 5'-AGCCTCCCTAAGACTCAAGGA-3'; RoanR: 5'-AGCGACCCCCACAAGTAATG-3'). PCR reactions were prepared in a total volume of 10µl using 1ul DNA, 7µl 2X Maxima Hot Start PCR master mix (ThermoFisher Scientific) and 1µl of 10nM of each primer. Thermocycling conditions for forward N777 (L15910) and reverse DLH1 (H164998) were: 95°C for 5 min; 40 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 1 min; 72°C for 10 min and for forward MT4 and reverse BT16168H were 95°C for 5 min; 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min; 72°C for 10 min. PCR product was purified using 1µl mix of Exonuclease I (ThermoFisher Scientific EN0581) and FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific EF0651) with 1:1 ratio and sequenced in both directions using a BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific 4337455) on an AB3130xl genetic analyser.

Sequence analysis

Raw sequence data were edited using Geneious v10.0.2 (Biomatters Inc.), forward and reverse sequences were combined and trimmed to a standard length for all samples (roan, 485bp; kudu, 431bp; eland, 442bp; waterbuck, 516bp). For each species, sample sequence data was analysed together with available reference sequence data for the species obtained from the NCBI nucleotide sequence database, including those sequence data used in the previous phylogeographic studies. Sequence results from the current study were compared to wild geolocated reference samples through phylogenetic reconstruction performed in Geneious, using default parameters for alignment and construction of Neighbour-Joining trees. The position of zoo animal sequence haplotypes within the resulting trees enabled inference of phylogeographic origin. For waterbuck, due to a lack of support for any particular tree topology, an additional TCS haplotype network analysis was performed to examine the relationships between sequences observed in wild and captive animals of the two subspecies (ellipsen and defassa).

Results

DNA nucleotide sequences were successfully produced for six greater kudu, 10 common eland, 20 roan and 24 waterbuck (12 defassa and 12 ellipsen) (35 FTA card samples and 25 hair samples) (Table 1).

Roan antelope

Roan samples displayed two different haplotypes, designated here as AFR1 and AFR2 (GenBank Acc. No. MG839214 and MG839215), both clustering within the southern/eastern clade and distinct from the western clade haplotypes (Figure 1).

Greater kudu

The results for greater kudu showed three different haplotypes within the six samples, one of which was identical to an existing reference sequence (AF301691), with the other two designated AFK1 and AFK2 (GenBank Acc. No. MG839216 and MG839217). All three haplotypes clustered within the Zambezi kudu clade, two within the south-western reference samples from Namibia (Figure 2) and the third associated with both Namibian (south-western) and Zambian (south-eastern intermediate) sequences.

Common eland

Three haplotypes were observed in the common eland, designated here as AFE1, AFE2 and AFE3 (GenBank Acc. No. MG839218, MG839219, MG839220). Haplotypes AFE1 and AFE2 clustered with the eastern clade, while AFE3 clustered with the southern clade (Figure 3).

Waterbuck

Ellipsen and defassa waterbuck each displayed four different haplotypes: KEE1-4 (GenBank Acc. No. MG839225-28); KED1-4 (GenBank Acc. No. MG839221-24), which were distributed widely

Figure 1. Roan antelope. Neighbour-joining tree based on mitochondrial control region DNA sequences showing relationships of individuals analysed in this study (AFR1 & AFR2) and in previous publications (Alpers et al. 2004)

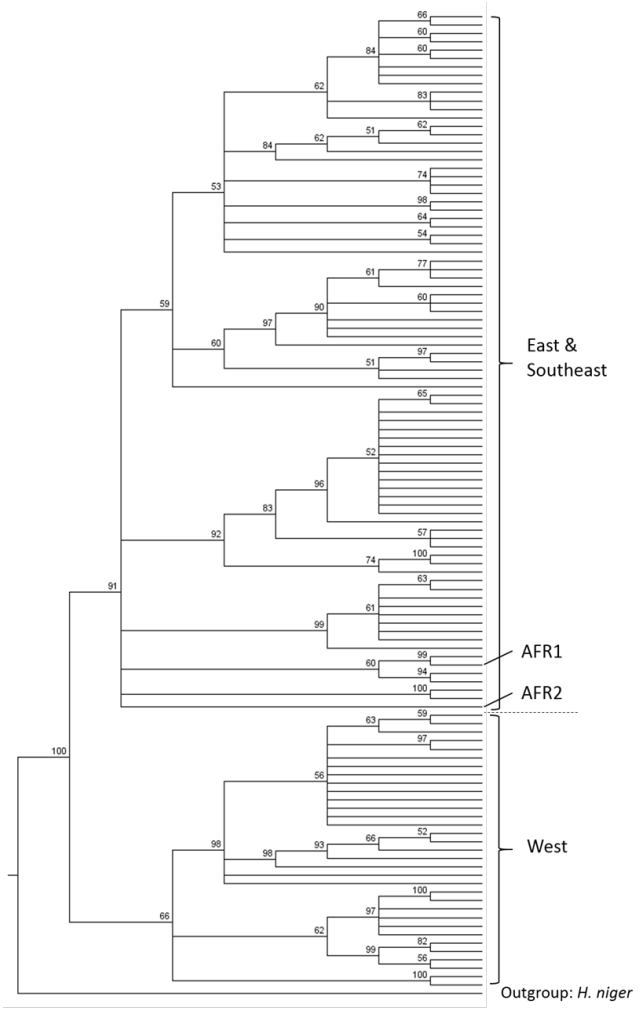
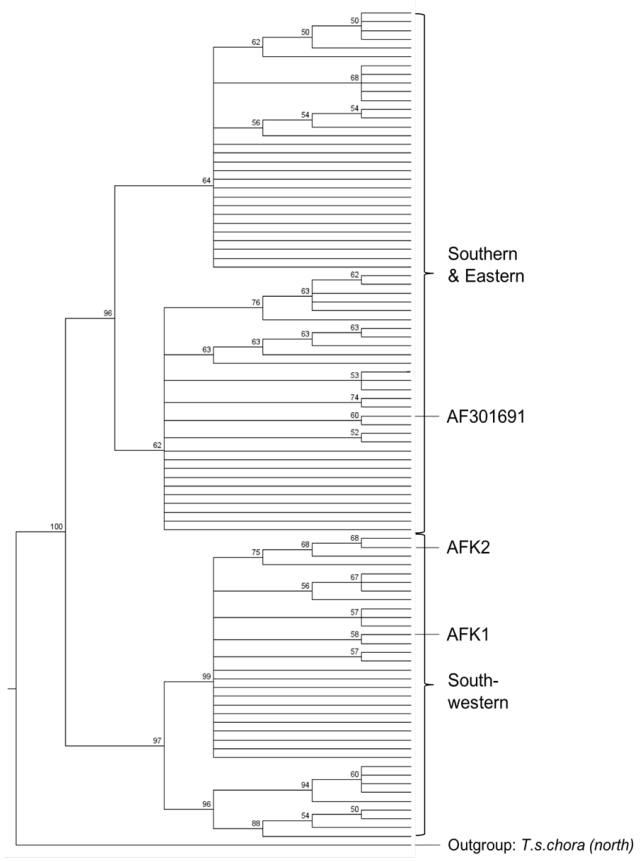


Figure 2. Greater kudu. Neighbour-joining tree based on mitochondrial control region DNA sequences showing relationships of individuals analysed in this study and in previous publications (Nersting and Arctander 2001). One sample matched to a previously observed haplotype (Acc. No. AF301691). Haplotypes AFK1 and AFK2 were not previously observed. All sequences clustered within the Zambezi kudu rather than the northern *T. s. chora* subspecies



across both the tree reconstruction (not shown) and haplotype network (Figure 4). The lack of haplotype clustering between the two subspecies limits definitive verification of subspecies status in captive samples based on maternally inherited DNA.

Discussion

The sequence results enabled individual roan, greater kudu and common eland to be assigned to particular mitochondrial clades based on existing reference data for each species. For roan antelope and common eland, the position of the samples in the neighbour-joining trees enabled their mitochondrial genetic lineage to be unambiguously determined and the specific project aims addressed. For kudu, the lack of widespread reference data for the northern subspecies, *T. s. chora*, does limit the interpretation of the data slightly, however it is reasonable to conclude that the three haplotypes observed in the samples originate from the Zambezi kudu (Groves and Grubb 2011) distributed in eastern and southern Africa. For waterbuck, the results are more ambiguous, due to the overlapping distributions of *K. e. defassa* and *K. e. ellipsiprymnus* mitochondrial haplotypes. As the mtDNA reference data were largely collected across a zone of known introgression, this finding is perhaps not surprising, however it does restrict the interpretation of sequence data observed in the samples.

The number of haplotypes observed among species varied considerably (2 haplotypes from 20 roan antelope, but three haplotypes from six greater kudu and eight haplotypes from 24 waterbuck). This indicates relative differences in zoo population genetic diversity among the four species, which in turn suggests differences in natural effective population size, or reflects differences in the number and representation of founder individuals for the respective zoo populations.

The use of mitochondrial DNA sequence data in this study was appropriate given the available reference data, however it is important to note that mitochondrial DNA can only provide information concerning the maternal ancestry of the antelope

Figure 3. Common eland. Neighbour-joining tree based on mitochondrial control region DNA sequences showing relationships of individuals analysed in this study and in previous publications (Lorenzen et al. 2010). Samples in this study were assigned to three novel haplotypes, AFE1, AFE2 and AFE3, which clustered with the Eastern (AFE1&2) and Southern (AFE3) clades

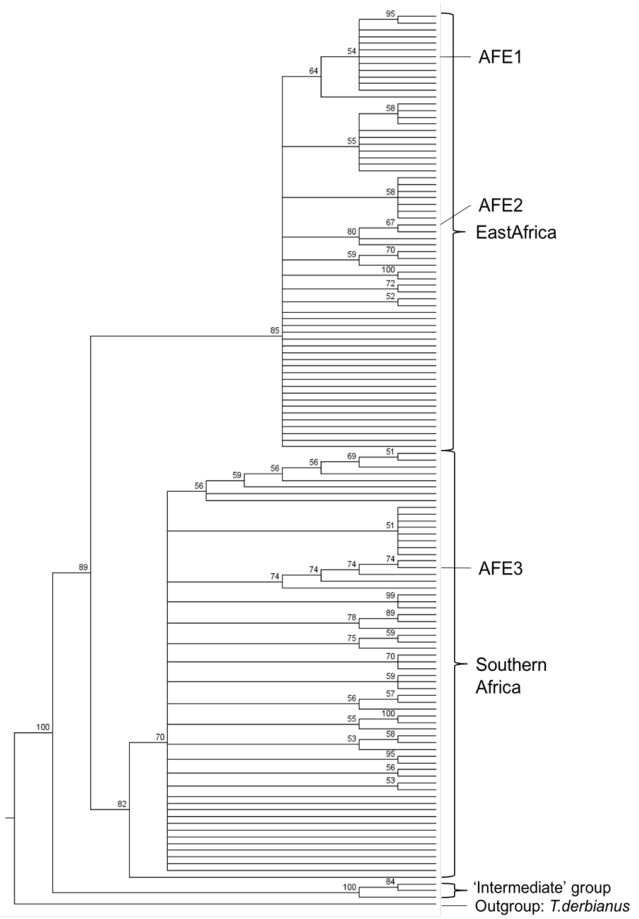
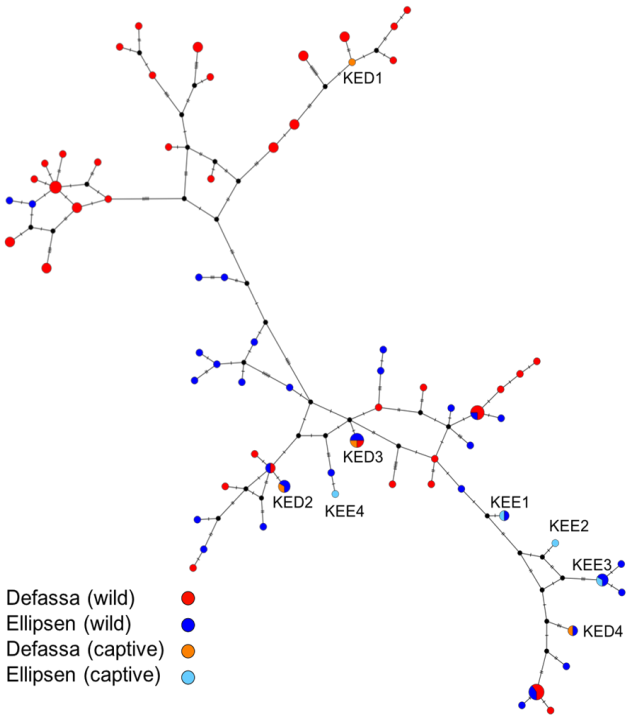


Figure 4. Waterbuck. TCS network diagram of mitochondrial control region DNA sequences showing relationships of individuals analysed in this study (KED 1-4; KEE 1-4) and in previous publications (Lorenzen et al. 2006). Red = defassa (wild); dark blue = ellipsen (wild); orange = defassa (captive); light blue = ellipsen (captive)



sampled and genetic introgression from male animals originating from different lineages cannot be ruled out. Applied under a precautionary principle, the approach used here can therefore provide strong evidence for where not to translocate individual animals to (i.e. where their mtDNA haplotype belongs to a different phylogeographic group), but results must be treated more cautiously in cases where the mtDNA haplotype is apparently consistent with a particular locality. In such instances, where the possibility of historic introgression between different forms exists in captivity or in the wild, further evaluation of nuclear DNA would be required to definitely assign an individual to its population of origin.

This paper highlights the application of relatively simple molecular genetic research to a clearly recognised but persistent challenge in zoo population management; namely, the need to understand the range of genetic variation present within zoo breeding programmes and how this relates to the distribution of genetic variation in the wild. The potential reintroduction value of zoo animals is often cited as a reason for maintaining captive populations and while this may not be the only justification given, it is an area that requires ground-truthing to validate such claims and to inform long term conservation breeding strategies. Assumptions regarding the genetic status of zoo populations have been questioned in recent years (Ito et al. 2016; Senn et al. 2014) and should continue to be the subject of ongoing testing and verification. Not until we really understand the diversity and origins of what we have in zoo breeding programmes can we start to incorporate captive animals into truly effective, integrated species conservation plans.

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