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Saving the mountain bongo (*Tragelaphus eurycerus isaaci*): Assessment of the genetic status of captive bongos as a source for genetic reinforcement of wild populations

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Abstract
Fewer than 140 individuals of the rare and critically endangered mountain bongo (*Tragelaphus eurycerus isaaci*) remain in the wild. This population has eroded genetic diversity, with only two haplotypes detected with mitochondrial DNA markers. The genetic diversity of mountain bongos from the European Endangered Species Programme (EEP) was assessed for this study. Genetic diversity of 10 captive individuals was measured by sequencing a portion of the mitochondrial DNA control region; the resulting sequences were compared to published data from this subspecies and used to establish levels of haplotype-sharing between wild and captive populations. Our data show that captive mountain bongo populations harbour a rare haplotype that is found in less than 5% of individuals in some wild populations and is absent in others. The findings suggest that captive individuals harbour valuable genetic diversity, making them potentially valuable candidates for a reintroduction programme to help reinforce the gene pools of wild populations. We further propose a two-way approach that also involves introducing wild individuals into captive populations, with the goal of maintaining the genetic health of both *in situ* and *ex situ* populations.

Introduction

A central goal of species conservation programmes is to develop strategies that will maintain or increase the genetic diversity of endangered species. Significant genetic diversity is a key feature of most healthy wild populations as it allows them to successfully respond to changing environmental conditions and adapt more successfully to local habitats (Willi et al. 2006; Shaffer 1990; Frankel et al. 1981; Westemeier 1998; Gilpin 1986). Severe decline in population size, owing to habitat fragmentation or other anthropogenic factors, can lead to a reduction in genetic diversity, potentially posing a risk to population viability through inbreeding and the fixation of deleterious genes (Shaffer 1990; Johnson 2006; Bijlsma et al. 1998; da Silva et al. 2006; Leberg 1990, 1993).

The bongo, *Tragelaphus eurycerus* Ogilby, 1837, is a large antelope with a wide historical distribution in the tropical forests of west, central and east Africa (Elkan and Smith 2013). Traditionally, this species has been divided into two subspecies, which occur either side of the Albertine Rift: the western bongo *T. e. eurycerus* from west and central Africa and the mountain bongo, *T. e. isaaci* Thomas 1902, from the montane forests of the Gregory Rift in Kenya and, formerly, Mount Elgon, Uganda (Reillo 2002; East 1999). Currently, the mountain bongo is critically endangered (IUCN category C2a(i)), with an estimated population of only about 75 - 140 individuals, mostly from the Aberdare Mountains in western Kenya (Elkan and Smith 2013; Faria et al. 2011).

Analyses of the control region of mitochondrial DNA (mtDNA) can provide a useful measure of the population structure and history of a species (Moritz 1994; Moritz et al. 1987; Kidd and Friesen 1998). In a recent study of mtDNA extracted from faecal pellets, mountain bongos were confirmed from four areas: the Mau Forest, Eburu Forest, the Aberdare Mountains and Mount Kenya (Faria et al. 2011). The authors found that the genetic diversity of the sampled bongos was very low, with only two identified haplotypes for the fragment of mtDNA control region that was investigated. Moreover, almost 70% of samples had a single haplotype (B02), which has almost reached fixation at Eburu Forest and Mount Kenya. Such limited genetic diversity may be the result of an original founder effect when populations of bongos became isolated in montane forests, or more likely it is a result of genetic drift following a dramatic decline in bongo numbers particularly during the 20th century, owing to loss of habitat and hunting.

Table 1. Locality and collection details for *Tragelaphus eurycerus isaaci* samples used in this study (HWAP: Howletts wild animal park; PLWAP: Port Lymne Wild Animal Park).

Genetic ID	Birth Locality	Birth Date	Sex	Status	Local ID	International Studbook Number
NMS.Z.2012.47.3	Zoo Frankfurt	Apr/18/2002	Female	Dead	EDINBURGH/M06J20	1513
NMS.Z.2012.47.2	Zoo de Barcelona	May/09/1992	Female	Dead	EDINBURGH/M05D07	659
NMS.Z.2006.35	Blackpool Zoo Park	Apr/14/2005	Male	Dead	BLACKPOOL/M05037	1764
Saffi	Howletts	Oct/05/2008	Female	Alive	BEKESBRNE/H20858	2090
Rafael	Zoo Dvur Kralove, a.s.	Jun/06/2001	Male	Alive	BEKESBRNE/H20443	1439
NMS.Z.2012.47.4	Zoo Warszawa	Jan/15/2004	Female	Dead	EDINBURGH/M04K01	1658
Tenouk	Port Lympne	May/02/2005	Female	Alive	BEKESBRNE/H21109	1773
Kuambi	Howletts	April/22/2009	Male	Alive	BEKESBRNE/H20911	2154
Mbonzi	Howletts	Mar/16/2009	Female	Alive	BEKESBRNE/H20903	2148
Embu	Howletts	Aug/24/2006	Female	Alive	BEKESBRNE/H20643	1866

Fortunately, European and North American zoos hold a large captive population of mountain bongos, which was established from 38 founders in the 1970s (currently ~200 individuals in Europe and ~450 in North America), and which is actively managed through an international studbook (Bosley 2015). The size of the captive population provides the prospect of reinforcing current wild populations or reintroducing mountain bongos to their former range, if sufficient habitat survives. In 2004, 18 individuals from North America were flown to a captive breeding facility at Mount Kenya Game Ranch as a first phase of a reintroduction programme. However, until now there has been no assessment of the genetic diversity of captive mountain bongos, in order to compare their genetic diversity with that of wild populations. Given the relatively low genetic diversity found in wild populations, it may be possible to genetically augment them with captive stock.

In this study, we assess the genetic diversity of captive bongo populations in Europe by sequencing a portion of the mitochondrial control region and comparing the resulting sequences to published data from extant populations in the wild. Our goal is to assess the suitability of captive bongos as a source for genetic augmentation of wild populations through a reintroduction programme.

Table 2: A comparison of genetics summary statistics for the EAZA population in July 2016 and the study sample.

	EAZA population July 2016	Sample used in this study
Founders	29	19
Living animals	185	10
Living descendants	184	10
Percentage ancestry certain	99	100
Gene diversity	0.9155	0.8425
Mean inbreeding	0.0822	0.0606

Methodology

Sampling

DNA was extracted from tissue samples obtained from biopsies or from deceased animals. A total of 10 individuals was used for DNA extraction; six individuals from The Aspinall Foundation and four individuals born in other European collections (Table 1). Total genomic DNA was extracted using a Blood and Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions.

A portion of the left domain of the mitochondrial DNA control region (D-loop) was amplified using standard conditions with primers: MT4 (50-CCTCCCTAAGACTCAAGGAAG-30 Arnason et al. 1993) and B16168H (50-GGTTGCTGGTTTCACGCGGCATG-30 Simonsen et al. 1998), as reported in Faria et al. (2011). The selection of primers followed their successful use for the amplification of the mitochondrial control region in African bovids (Nersting and Arctander 2001; Simonsen et al. 1998; Birungi and Arctander 2000). PCRs were performed using a Qiagen Multiplex Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's instructions with minor modifications. Briefly, PCR cycling conditions consisted of an initial activation step at 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 60°C for 90 sec and 72°C for 90 sec and a final elongation step at 72°C for 10 min. PCR products were visualized using 1.5% agarose gel stained with GelRed Nucleic Acid Gel Stain (Biotiom) and ultraviolet light. Positive amplifications were purified using Exonuclease I and Alkaline Phosphatase (SAP) enzymes (USB Corp, USA) starting with a digestion period of 60 min at 37°C, followed by an inactivation period of 15 min at 80°C. Purified PCR products where then sequenced using BigDye TMTerminator Sequencing kit (Version 3.1, ABI) and analysed using a semi-automated genetic analyser (ABI 3130XL).

Data Analyses

Chromatograms from the resulting DNA sequences were checked for accuracy using BioEdit (version 7.2.5, Hall 1999). Sequence identity was confirmed using the online BLAST search tool (blast.ncbi.nlm.nih.gov/). Sequence alignment was performed using ClustalW (Thompson et al. 1994) and the reliability of the alignment was checked manually, confirming base calls against chromatograms. The resulting bongo sequences were aligned with published mountain bongo sequences to confirm species

Table 3. Summary statistics for the mtDNA control region sequence variability obtained for *Tragelaphus eurycerus isaaci* wild and captive populations.

Tragelaphus eurycerus isaaci	n	H	Hn	h	SD	π	SD	S
Captive population	10		1	0	NA	0	NA	0
NMS.Z.2012.47.3 (Consensus 52)	-	B01	-	-	-	-	-	-
NMS.Z.2012.47.2 (Consensus 57)	-	B01	-	-	-	-	-	-
NMS.Z.2006.35 (Consensus 87)	-	B01	-	-	-	-	-	-
Saffi	-	B01	-	-	-	-	-	-
Rafael	-	B01	-	-	-	-	-	-
NMS.Z.2012.47.4 (consensus 76)	-	B01	-	-	-	-	-	-
Tanouk	-	B01	-	-	-	-	-	-
Kiambi	-	B01	-	-	-	-	-	-
Mbonzi	-	B01	-	-	-	-	-	-
Embu	-	B01	-	-	-	-	-	-
Wild population	2		2	1	0.5	0.00239	0.0012	1
EU040245	-	B02	-	-	-	-	-	-
EU040246	-	B01	-	-	-	-	-	-

Sample size (n), Haplotype (H), Number of haplotypes (Hn), haplotype diversity (h), Nucleotide diversity (π), Standard deviation (SD), Segregating sites (S)

identity. The resulting clean dataset was used to estimate the observed number of haplotypes (NH), haplotype diversity (HD) (Nei 1987) and nucleotide diversity (Nei 1987), using the software DNAsp version 4.1 (Rozas et al. 2003).

Phylogenetic analyses were performed using the neighbour-joining (NJ) and maximum-likelihood (ML) algorithms, as implemented in the program MEGA, version 5. The Tamura-Nei (Tamura and Nei 1993) model of nucleotide substitution was identified as the model that best fitted the data set, using the Akaike Information Criterion (AIC), and this was used for both ML and NJ phylogenetic reconstructions. As phylogenetic relationships can sometimes be reticulate, we also used statistical parsimony to build a haplotype network, as implemented in TCS (Clement et al. 2000).

Results

A 428 bp long fragment of the mtDNA control region was determined for the 10 individuals analysed in this study. No insertions or deletions were detected between the mountain and western bongo haplotypes. Our working alignment included the 10 sequences of captive mountain bongos, wild mountain bongo haplotypes B01 and B02 (EU04246 and EU04245, respectively, from Faria et al. 2011) and one western Bongo sequence (JN632703, Hassanin et al. 2012), and yielded 22 polymorphic sites (excluding the outgroup *Bos taurus* sequence) with an average pairwise difference of 4.98% between the two subspecies. No nucleotide variation was found within the 10 sequences of the captive mountain bongos.

Representativeness of study sample

Given that there are currently 184 living mountain bongos in the EAZA region, our sample of 10 may not be representative of the whole population. An analysis of the studbook data revealed that the 10 animals in our sample are descendants of 19 of the 29 founders of the EEP population (Table 2). Moreover, the 10 founders not represented in this study represent only 3.5% of the current population. Hence, our study sample is highly representative of the current population of mountain bongos in the EAZA region and we do not expect any sampling biases in the proportions of haplotypes seen in our sample (Figure 1).

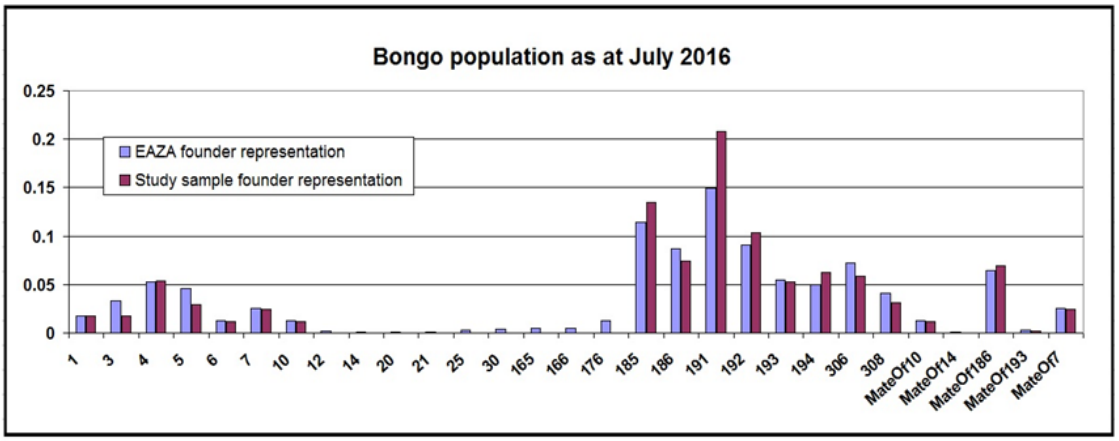

Figure 1: A comparison of the proportional representation of the 29 founders in current captive population in the EAZA region and the ten animals used in this study.

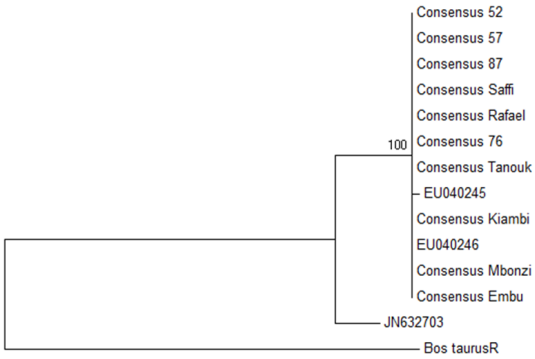

Figure 2. Maximum likelihood-neighbour joining tree estimated under the Tamura Nei model of nucleotide evolution, using the software Mega for all available mountain bongo sequences (Large clade), including one western bongo sequence obtained from GenBank. Bos taurus was used as outgroup. Bootstrap support was estimated using 10,000 replicates.

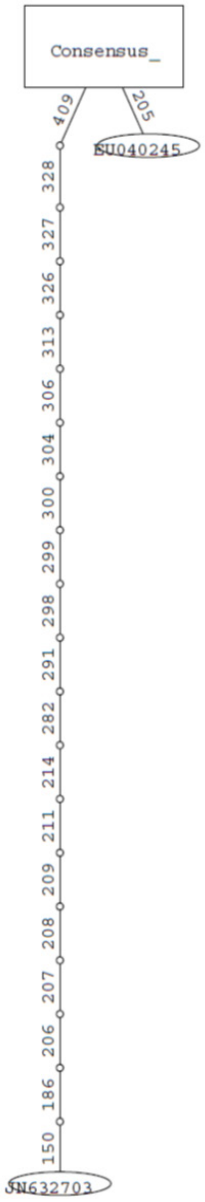

Figure 3. Statistical parsimony haplotype network calculated with TCS Software for the mountain and western bongo samples. The large rectangle represents haplotype B01, present in all captive mountain bongo samples (identical to EU040246). Haplotype B02 is represented by the published sequence EU040245. The small dots denote the mutational steps, and the number between dots refers to the position of the mutation.

Genetic diversity of mountain bongo populations

Only one mtDNA control region haplotype, BO1 was found in the EAZA captive population of mountain bongos (Table 3). Phylogenetic analysis (Figure 2) was performed on the total working alignment. The resulting NJ and ML phylogenies showed a clear and well-supported structure, with a clear separation between mountain (eastern) and lowland (central and western) bongo samples.

The haplotype network (Figure 3) also shows a clear pattern of separation between the two currently recognized subspecies of *Tragelaphus eurycerus*. The 10 individuals of captive mountain bongos clustered together with the published sequences for this subspecies (EU04246 and EU04245). Within the mountain bongo sequences, our recovery of a single base-pair substitution between the B01 and B02 mtDNA control region haplotypes is consistent with the findings of Faria et al. (2011). We found a significant separation between the lowland and mountain bongo subspecies, supported by 19 base pair substitutions of the analysed mtDNA fragment, which may be taken as evidence supporting the separate subspecies status of the western and eastern bongos. These results represent the first DNA-based evidence supporting the subspecies classification of this species.

Haplotype identity in captive bongos

The resulting alignment of mtDNA control region sequences in this study is shown in Figure 4, along with the sequences from published alleles B01 and B02. Our results provide convincing evidence for the existence of a single mtDNA control region haplotype within the sampled captive bongo individuals used in this study. This haplotype is identical to the wild mountain bongo haplotype B01, which Faria et al. (20011) found to be relatively frequent (30%) in the population in Aberdare National Park, but rare (> 5%) in Mt. Kenya National Park population and absent in the Eburu and Mau forest populations.

Discussion

A key advantage of using mtDNA for elucidating population histories resides in its power to detect signatures of demographic decline. Because of the smaller effective population size (Ne) of mtDNA, compared to that of nuclear DNA, mtDNA is the first to bear the evolutionary signature left by population decrease. The case of the mountain bongo is a typical example of a species with a once widespread distribution that is now confined to a few isolated localities, and is harbouring extremely low levels of genetic diversity. This is a likely consequence of drastic reductions in population size, owing to the combined effects of hunting, disease and habitat destruction (Kock et al. 1999; Estes et al. 2008).

Several examples have been reported of species showing reduced numbers of alleles due to population bottlenecks caused by human-mediated factors, such as habitat fragmentation and hunting. For example, the current whooping crane (*Grus americana*) populations harbour a single haplotype, but formerly hosted as many as six haplotypes, according to analyses of museum samples (Glenn et al. 1999). Haag et al. (2010) reported similar results for jaguars (*Panthera onca*) from recently fragmented regions of the Atlantic Forest in Brazil. Likewise, populations of the Cape mountain zebra (*Equus zebra zebra*) (Moodley and Harley 2006; Watson and Chadwick 2007), the Cape buffalo (*Syncerus caffer*) (Van Hooft et al. 2002), and the African black rhinoceros (*Diceros bicornis*) showed a significant reduction in genetic diversity in modern populations.

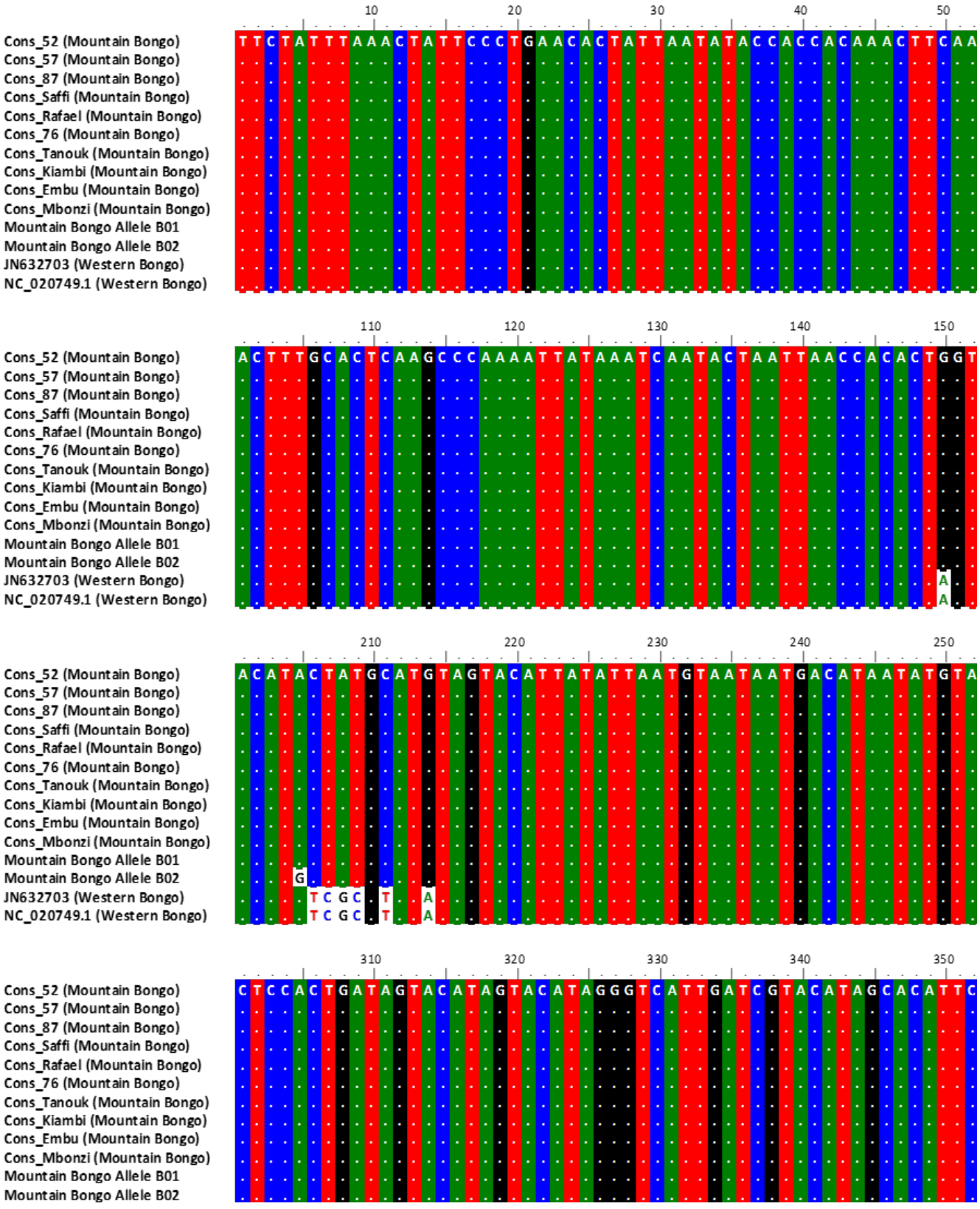

Figure 4. DNA alignment showing the two known mountain bongo haplotypes for the mtDNA control region (B01 and B02). Mountain bongo alleles B01 and B02 are those reported on Genbank, sequences EU04246 and EU04245, respectively.

The current status of surviving mountain bongo populations requires the urgent implementation of conservation actions to increase the numbers of this charismatic and critically endangered subspecies. Wild populations currently comprise a critically low number of individuals, and furthermore, remaining populations display low levels of genetic variability, represented by two mtDNA haplotypes at the most (Faria et al. 2011). These factors pose a potential high risk for the health and continued survival of the population (Brekke et al. 2010; Pomeroy 2013; Bilski et al. 2013; Johnson et al. 2011; Jamieson et al. 2011). Given that there are successful captive breeding programmes in Europe and in the USA for this species, the reintroduction of captive stock to augment wild populations is a viable conservation action that should be explored.

In recent years, several case studies have provided convincing evidence of successful reintroductions, which offer a solid hope of success in the case of the mountain bongo. Key examples include the black-footed ferret (*Mustela nigripes*) (Jachowski et al. 2011), the golden lion tamarin (*Leontopithecus rosalia*) (Kierulff et al. 2013), the Puerto Rican crested toad (*Peltophryne lemur*) (Beauclerc et al. 2010), African wild dogs (*Lycaon pictus*) (Gusset et al. 2010), the Australian trout cod (*Maccullochella macquariensis*) (Lyon et al. 2012) and the Arabian oryx (*Oryx leucoryx*) (Islam et al. 2011).

Current genetic status of wild and captive mountain bongo populations: the case for reintroduction.

The wild mountain bongo population contains only two surviving mtDNA control region haplotypes, of which one (B2) is more prevalent, being found at frequencies of 70–100% in sampled wild populations (Faria et al. 2011). The results of this study have shown that the captive population contains only the B01 haplotype, which is underrepresented in wild populations. The presence of this rarer haplotype in captive mountain bongos makes them an ideal source for genetic augmentation of wild populations, potentially reducing the detrimental consequences of inbreeding depression in the wild.

The samples used in this study represent individuals that span a wide range of lineages within the European breeding population (see Table 2). The apparent fixation of this haplotype in captivity suggests that this lack of genetic diversity could prove problematic for the long-term viability of the captive population. To preserve the genetic health of captive populations, an ideal approach would be a two-way exchange, whereby not only captive individuals are used to replenish wild stocks, but individuals from wild populations are also used to maintain and further enrich the genetic diversity of the captive population. Such metapopulation management is likely to become increasingly common in the management of endangered species in the wild and captivity.

One Plan Approach

A two-way management approach is now starting to be utilised for a number of critically endangered species, because there is simply not enough remaining genetic variability to manage captive and wild populations separately, but currently there are no published examples of species managed under such an approach. However, a new initiative, called The One Plan Approach to Conservation and proposed by the IUCN SSC Conservation Breeding Specialist Group (CBSG), "promotes integrated species conservation planning, which considers all populations of the species, inside and outside their natural range, under all conditions of management, engaging all responsible parties and all available resources from the very start of any species conservation planning initiative" (Byers et al. 2013). In essence, this novel approach seeks to holistically manage the conservation of endangered species. The key points of such an approach rely on the effective use of all and any available tools that might help towards the conservation of the endangered species. These include, for instance, "source populations for demographic or genetic supplementation, assurance populations against imminent threats such as disease or invasive species, research populations to develop monitoring or management techniques and headstart programmes that temporarily shelter juveniles from high mortality and promote population growth" (Byers et al. 2013).

We propose that the mountain bongo is an ideal candidate for such a two-way conservation and population management strategy that follows the One Plan Approach, and its implementation would ensure the sustained and optimal survival of both wild and captive mountain bongo populations, thereby securing the long-term survival of this subspecies. This approach would have a positive impact on captive mountain bongo populations by augmenting their genetic variability, and making them even more important as a back-up to the wild populations, which may be under additional threat owing to hunting, disease and habitat loss.

Future genetic work

Whilst mitochondrial markers are useful tools for assessing basic population parameters, they do not have the resolving power required for individual identification. Nuclear markers such as microsatellites or SNPs (Single Nucleotide Polymorphisms), need to be developed for utilisation on mountain bongos, in order to provide the resolution required to identify individuals, and assess relatedness and paternity. Importantly, these nuclear markers would allow for the effective monitoring of wild populations using non-invasive genetic sampling techniques.

Currently, it is difficult to accurately estimate numbers of wild mountain bongos because they are cryptic and elusive, so their presence is usually assessed from indirect signs, such as faeces or tracks or camera traps (Putman 1984; Bowkett et al. 2009). Although faecal counts are commonly used to detect mountain bongo presence, faecal samples often yield poor quality DNA, so that sample viability can become a significant problem when using microsatellites. Therefore, we suggest that any future development of markers should focus on generating population-specific SNPs, which will provide dual benefits over microsatellites by both providing increased resolving power and an increased likelihood of success on degraded DNA samples due to shorter fragment length amplification. Accurate estimation of the remaining numbers in wild mountain bongo populations should be a key conservation priority and the construction of a suitable SNP panel should be a fundamental component of this work

Conclusion

The captive mountain bongo population contains valuable genetic diversity, as shown by the presence of the B01 haplotype, which is rare in wild populations. Therefore, the use of captive individuals for a genetic augmentation programme of wild populations will likely help increase levels of wild bongo genetic diversity, increasing the long-term genetic viability of the remaining populations. Furthermore, the implementation of a One Plan Approach for the mountain bongo will help ensure that both wild and captive populations maintain healthier levels of genetic diversity.

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