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RESEARCH ARTICLE

Defining Management Units for European Captive Aardvarks

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The Aardvark (*Orycteropus afer*) is a very unique, but relatively widespread African mammal. Although some morphological variation has been observed between forest and savannah populations and among different African regions, they are all considered as a single species. However, no modern taxonomic revision is available. All captive aardvarks in Europe are believed to stem from wild born animals from Namibia, but recently several new wild-caught aardvarks from Tanzania have been integrated into the captive population. This raises the question, whether these specimens should be interbred with the existing captive population or whether there is a risk of outbreeding depression. We studied the genetic structure of the captive populations by sequencing two mitochondrial genes (cytochrome *b* and 16S rRNA) to assess the degree of genetic differentiation between the two source regions. Our data suggest that the aardvarks kept in European zoos belong to the same phylogenetic (mitochondrial) lineage as the differentiation in the two studied mitochondrial markers was extremely low. A more comprehensive analysis of a larger sample with well documented origin (covering the complete geographic range) and with more sensitive genetic markers is needed to infer any final conclusions concerning the aardvark's taxonomy and identification of suitable aardvark management units. Zoo Biol. 33:433–439, 2014.

Keywords: *Orycteropus*; genetic structure; taxonomy; mitochondrial

INTRODUCTION

The Aardvark, *Orycteropus afer* (Pallas 1766), is a very unique taxon among Mammalia, Placentalia or even Afrotheria [Seiffert, 2007; Tabuce et al., 2008; Kuntner et al., 2010; Taylor, 2011]. In spite of many morphological and behavioral specializations [e.g., tubulidentate teeth, large number of endoturbinals, specialization to myrmecophagy combined with powerful adaptations for digging—reviewed in Shoshani et al., 1988; Lehmann, 2013; Taylor, 2013], it is generally characterized as an ungulate-like member within the clade Afrotheria. Formerly, it was even considered a proto-ungulate close to the common ancestral stem of all modern ungulates and showing morphological similarities with "Condylarthra" [Kingdon, 1997; Shoshani and McKenna, 1998]. The distribution of aardvarks (*sensu* Tubulidentata) was once widespread across the entire Old World, but the only extant species is restricted to the sub-Saharan African continent [Lehmann, 2006, 2007, 2009]. Aardvarks prefer habitats ranging from woodlands to open savannahs with

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abundant ant and termite nests, but they have also been recorded from primary evergreen forests in Cameroon, Gabon, and the DR Congo [Pagès, 1970; Grubb et al., 1998; Lindsey et al., 2008; Taylor, 2011, 2013]. Considering this distribution and the marked differences observed between forest and savannah populations in several African large mammals [Grubb et al., 1998], it is of great interest to know how strong the genetic differentiation among aardvark populations is. Unfortunately, the taxonomic knowledge on aardvarks is very limited, partly because of the scarcity of museum material including information on the specific geographical origin of collected animals [Setzer, 1956; Meester, 1971; Lehmann, 2009]. This fact complicates attempts to revise the aardvark taxonomy using modern statistical packages and species concepts [cf. Groves, 2001; Groves and Grubb, 2011]. In total, 18 extant aardvark taxa have been described since 1766, which are currently considered as subspecies of *O. afer*. Their diagnosis was predominantly based on hair density, coloration of specific body regions, length of toes, tail, ear or nose, and skull/mandible/teeth shape or size [Matschie, 1898; Lönnberg, 1906; Grote, 1921; Hatt, 1934]. However, their validity is rather doubtful [Shoshani et al., 1988] and accurate geographic ranges remain unknown. No subspecies has yet been synonymized, which is mainly caused by the lack of any modern taxonomic revision [Meester, 1971; Shoshani et al., 1988]. Several authors consider the subspecies diversity of the Aardvark to be overestimated, like for instance in Tanzania where the presence of five subspecies seems improbable for an animal of this size [Meester, 1971; Lehmann, 2007; Taylor, 2011, 2013].

Since the late 19th century, European and American zoological institutions have kept and attempted to breed aardvarks in captivity, but breeding programs became fruitful only since the 1960s and 1970s after new founders from the wild were integrated to the captive population. The first successful breeding strategies (starting with the Frankfurt Zoological Garden, Germany in 1962) and the organized exchange of specific individuals among the breeding institutions helped minimizing inbreeding [Parys et al., 2012].

In September 2013, 35 males, 41 females and 3 specimens of unknown sex, 79 in total, were kept in 33 European, American and Asian zoological institutions [International Species Information System, http://www.isis.org/]. All captive aardvarks in Europe are believed to stem originally from wild born animals from Namibia [Schoo, 2008, 2009], but recently, several new wild-caught aardvarks from Tanzania have been integrated into the captive population [Parys et al., 2012]. This raises the question, whether these specimens should be interbred with the existing captive population or whether there is a risk of outbreeding depression.

In order to avoid negative effects of outbreeding, it is important to identify appropriate management units [MU] that should be based upon a large number of purebred individuals [Frankham et al., 2002; Allendorf and Luikart, 2007; Witzenberger and Hochkirch, 2011]. Every additional unrelated founder specimen is of great interest for *ex situ* conservation management in order to maintain the genetic diversity [Frankham et al., 2002]. Taking all these points into account, the integration of the recently arrived Tanzanian aardvarks could positively influence the genetic diversity of the European captive population, but only if they belong to the same MU. If this is not the case, an admixture of both populations in zoos might lead to genetic dilution and/or outbreeding depression [Parys et al., 2012].

Considering our limited knowledge about the taxonomy of aardvarks and the difficulty to work with wild individuals, a molecular analysis of aardvarks kept in European zoos seems to be the most effective tool to start answering these questions. In the present study we sequenced two mitochondrial genes (cytochrome *b* and 16S rRNA) from 28 zoo individuals to obtain a first assessment of the degree of genetic variability within the captive population and to analyze the degree of genetic differentiation between the two source regions. Mitochondrial DNA is often used in DNA barcoding and is useful to delimit conservation units as long as differentiation is strong [Hochkirch and Görzig, 2009]. It is thus ideal for a rapid assessment of evolutionary differentiation. However, it may fail to detect recent differentiation processes and might be influenced by introgression and incomplete lineage sorting [Husemann et al., 2013]. Therefore, this analysis will provide useful initial information for the current breeding program, although other markers would be necessary to obtain knowledge on potential regional adaptations, which can also be important to identify suitable MUs.

MATERIALS AND METHODS

To examine the genetic structure of European captive population of aardvarks, hair, tissue, and blood samples were collected from 21 alive and seven dead individuals out of the 45 aardvarks kept in Europe [ISIS, September 2013]. Among these, four were wild born individuals from Tanzania, whereas the remaining ones were captive-born individuals, which are believed to stem from Namibian wild born founders (for details see Table 1). Samples were usually taken during regular veterinary procedures. At the Prague Zoo, buccal swabs, blood and hair samples were obtained without general anesthesia in the morning, while the aardvark was still asleep. For blood samples, Mesocain® gel was first applied locally on the skin and let sit for 5 min. Blood was then drawn from the medial *vena saphena* using hypodermic needles (20G—0.9 mm × 25 mm). Buccal swabs from each side of the buccal mucosa were collected using special germfree brushes. Bristle hairs were easily sampled with germfree wide tweezers or forceps, and taken predominantly from the pelvic region of the animal at the Prague Zoo. Our sample collection procedures were well-tolerated as they did not induce any increased stress to the animals (e.g., the sleep was not

TABLE 1. List of voucher specimens included in the present study, with their origin (Tanzania or Namibia), detected haplotype for cyt *b* and 16S, studbook number (European studbook; ESB), name or local number, sex and current location (as of December 31, 2012) [Schoo, 2013]

Voucher number [origin]	Cyt *b* haplotype	16S haplotype	ESB	Name (local number)	Sex	Location
1_Tanzania	Haplotype 6[f]	Haplotype 1[a]	181	In nominate (loc. 209080)	M	Wroclaw Zoo
2_Tanzania	Haplotype 7[g]	Haplotype 1	187	In nominate (loc. 209081)	M	Wroclaw Zoo
3_Tanzania	Haplotype 8[h]	Haplotype 2[b]	194	Ermine	F	Frankfurt n./M. Zoo
4_Tanzania	Haplotype 4	Haplotype 1	195	Ernst	M	Frankfurt n./M. Zoo
5_Namibia	Haplotype 4[d]	Haplotype 1	72	Tata	M	Prague Zoo[†]
6_Namibia	Haplotype 1[a]	Haplotype 1	180	Draco	M	Prague Zoo
7_Namibia	Haplotype 1	Haplotype 1	44	Myška	F	Kessingland Zoo
8_Namibia	Haplotype 1	Haplotype 1	74	Dudley	M	Prague Zoo[†]
9_Namibia	Haplotype 1	Haplotype 1	156	Danny	M	Bioparc Valencia Rainforest
10_Namibia	Haplotype 1	Haplotype 1	159	Quote	F	Prague Zoo
11_Namibia	Haplotype 2[b]	Haplotype 1	161	Snuggy	F	Faunia Zoo Madrid
12_Namibia	Haplotype 1	Haplotype 1	177	Bovenkantje	F	Burgers' Zoo Arnhem
13_Namibia	Haplotype 1	Haplotype 1	103	Oryc	F	Burgers' Zoo Arnhem
14_Namibia	Haplotype 2	Haplotype 1	98	Snuffy	F	Burgers' Zoo Arnhem
15_Namibia	Haplotype 1	Haplotype 3[c]	145	Irmo	M	Burgers' Zoo Arnhem
16_Namibia	Haplotype 2	Haplotype 1	179	Henkjan	M	Bioparc Valencia Rainforest
17_Namibia	Haplotype 1	Haplotype 1	190	In nominate (loc. 615782)	F	Burgers' Zoo Arnhem[†]
18_Namibia	Haplotype 1	Haplotype 1	170	In nominate (loc. 613151)	F	Burgers' Zoo Arnhem[†]
19_Namibia	Haplotype 2	Haplotype 1	189	In nominate (loc. 615648)	F	Burgers' Zoo Arnhem[†]
20_Namibia	Haplotype 2	Haplotype 1	162	In nominate (loc. 612518)	F	Burgers' Zoo Arnhem[†]
21_Namibia	Haplotype 2	Haplotype 1	172	Misha	F	Zoological Society of London
22_Namibia	Haplotype 3[c]	Haplotype 1	147	Oq	F	Colchester Zoo
23_Namibia	Haplotype 3	Haplotype 1	151	Plucky	M	Berlin Zoo
24_Namibia	Haplotype 3	Haplotype 1	144	Curly	F	Antwerp Zoo
25_Namibia	Haplotype 1	Haplotype 1	39	Okahandja	F	Burgers' Zoo Arnhem[†]
26_Namibia	Haplotype 1	Haplotype 1	140	Puq	F	Colchester Zoo
27_Namibia	Haplotype 1	Haplotype 1	41	Pieta	F	Prague Zoo
28_Namibia	Haplotype 5[e]	Haplotype 2	127	Quiggly	M	Kessingland Zoo

GenBank accession number (NCBI; The National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) for cytochrome *b* haplotypes: KF984292 (a), KF984293 (b), KF984294 (c), KF984295 (d), KF984296 (e), KF984297 (f), KF984298 (g), KF984299 (h), and 16S rRNA haplotypes: KF984300 (a), KF984301 (b), KF984302 (c); (†) deceased animals.

interrupted). Tissue, blood, and buccal swabs samples were directly stored in 96% ethanol, whereas hair samples were put in plastic or paper envelopes at room temperature until transport to the laboratory, where they were stored at −20°C. DNA was extracted using JET QUICK Tissue DNA Spin Kit (Genomed, Löhne, Germany).

We sequenced the mitochondrial genes cytochrome *b* (cyt *b*) and 16S rRNA (16S). Cytochrome *b* is a standard marker, which proved to be very useful to determine genetic differentiation [Johns and Avise, 1998]. The level of genetic distance can therefore be compared with others mammal species [Bradley and Baker, 2001; Baker and Bradley, 2006]. The 16S rRNA gene has been used in several phylogenetic studies as it is easy to amplify [Lloyd, 2003]. It has proved suitable for identification of animal species [Mitani et al., 2009], even for mammal fecal samples [Yan et al., 2011], exhibiting a low degree of variation at the population level, but a high resolution at the species and subspecies level [Nijman and Aliabadian, 2010; Nicolas et al., 2012].

We used the universal primers L14724, L14841, and H15915 [Kocher et al., 1989; Irwin et al., 1991] for cyt *b* and 16sAL and 16sBH for 16S [Palumbi, 1996]. The polymerase chain reaction (PCR) [Sambrook et al., 1989] were done in 25 μl using 12.5 μl of Combi PPP Master Mix (Top-Bio, http://www.top-bio.cz) with $MgCl_2$ and Taq Purple DNA polymerase (50 U/ml), 1.5 μl of each primer (10 pmol/μl) and 1.5 μl of DNA extract. The amplification of cyt *b* consisted of an initial denaturation step at 95°C for 3 min, denaturation at 95°C for 30 sec, annealing at 50°C for 1 min, extension at 72°C for 1.5 min and final extension at 72°C for 10 min. This cycle was repeated 30–37 times. The PCR program for 16S consisted of initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 54°C for 1.5 min, extension at 65°C for 1.5 min and final extension at 72°C for 10 min. This was repeated 33 times. PCR products were purified using JET QUICK PCR Purification Spin Kit (Genomed) and sequences were obtained using Sanger sequencing [Sanger et al., 1977] on an ABI PRISM 3130xl automatic sequencer (Applied Biosystems) at the Laboratory of genomics, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice.

One sequence of *O. afer*, accession number Y18475, Namibian origin—Burgers' Zoo, Arnhem [Arnason et al.,

1999] and two other afrotherian mammals with a complete mitochondrial sequence—*Echinops telfairi* (accession number NC_002631.2) and *Chrysochloris asiatica* (accession number NC_004920.1) were downloaded from GenBank as outgroups. The obtained sequences were aligned using the automatic multiple alignment program for amino acid or nucleotide sequences MAFFT version 6 [Katoh et al., 2009]. They were subsequently adjusted manually using the Bioedit [Hall, 1999]. Prior to analyses, cyt *b* sequences were translated into amino acids in MEGA5 [Tamura et al., 2011] using the vertebrate mitochondrial translation code. This procedure did not detect any stop codons or gaps suggesting that all protein coding sequences were functional and no pseudogenes were amplified.

We employed a Bayesian inference method (BI) for separate and simultaneous phylogenetic analyses of both genes. The best-fitting substitution model of DNA sequence evolution was selected by jModelTest 0.1.1 [Posada, 2008] under the Akaike Information Criterion (AIC). The general time reversible model including gamma distribution parameters (GTR + G) was determined for all datasets (cyt *b*, 16S, and combined matrix). Bayesian phylogenetic analysis was conducted with a Metropolis-coupled Markov chain Monte Carlo algorithm [Altekar et al., 2004] as implemented in MrBayes version 3.1.2 [Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003]. The nucleotide data were run for 5,000,000 generations with two runs and four chains for each run. A sampling frequency of every 100th generation produced 50,000 sampled trees. The first 20% (10,000 trees) were discarded as a burn-in.

Haplotype networks were constructed in TCS version 1.21 [Clement et al., 2000] that implements statistical parsimony. Average genetic distance values of cyt *b* were calculated on the basis of Kimura two-parameter model of substitution by MEGA5 [Tamura et al., 2011]. The Kimura two-parameter model was selected for comparisons with other published distances for mammals [Bradley and Baker, 2001; Baker and Bradley, 2006].

RESULTS

The obtained cyt *b* sequences ranged from 950 to 1,130 bp (base pairs) from the total number of 1,144 bp. The final alignment consisted of 31 individuals (one tenrec, one golden mole, and 29 aardvarks) and 940 well-supported base pairs. A total of 14 positions were variable and only five of them were parsimony informative (outgroup excluded). Most 16S sequences comprised 550 bp and the final alignment consisted of the same 31 individuals and 491 well-supported pair bases. There were only three variable positions and one of them was parsimony informative (outgroup excluded).

Bayesian analyses (BI) for both genes and the concatenated data set exhibited extensive polytomous relationships among all investigated aardvarks (results not shown). We obtained nine haplotypes of cyt *b* and three of 16S (see Table 1), the haplotype networks of cyt *b* and 16S (Figs. 1 and 2) exhibited only limited overall structure.

Pairwise genetic distances among aardvark individuals varied from 0% to 0.7% in cyt *b* and from 0% to 0.2% in 16S. The genetic distance between sorted individuals according to the Tanzanian and Namibian origin was 0.2% for cyt *b* and 0.1% for 16S.

DISCUSSION

This study represents the first attempt to analyze the genetic structure of this unique and relatively widespread African mammal, albeit for a relatively small sample as is to be expected for this very secretive animal, rarely studied or kept in captivity. Our data suggest that all aardvarks kept in European zoos belong to the same phylogenetic lineage as the differentiation of the two studied mitochondrial markers was extremely low. Although the resolution of our data set was by far too low to confirm the Namibian origin of the captive population, there is also no evidence for the opposite.

Our phylogenetic analyses do not support the hypothesis that Namibian and Tanzanian aardvarks belong to different phylogenetic lineages. They do not even cluster according to their respective regional origin. In spite of a geographic distance of approximately 1,200 km, our results suggest that at least some Tanzanian aardvarks could have had rather recent contacts with Namibian ones. Remarkably, close phylogenetic relationships between East African and Southwest African populations have also been shown for other widespread African mammals [Grubb et al., 1999], such as antelopes [Arctander et al., 1999; Nersting and Arctander, 2001; Pitra et al., 2002; Moodley and Bruford, 2007; Lorenzen et al., 2010] and giraffes [Brown et al., 2007; Hassanin et al., 2007; Groves and Grubb, 2011]. Repeated faunal exchanges between both regions are generally assumed given the repeated glacial and interglacial changes with marked influences on climate and vegetation in Africa during the whole Quaternary [Van Zinderen Bakker, 1967; Hamilton and Taylor, 1991; Coetzee, 1993]. Glacial periods seem to have been particularly suitable for the development of faunal corridors involving savannah-dwelling mammals [Van Zinderen Bakker, 1962, 1967; Cerling, 1992; Morley and Kingdon, 2013].

Overall, our results indicate no (or very limited) differentiation in the European populations of captive aardvarks. All detected genetic distances of cyt *b* were significantly lower than 2%, which is considered by Bradley and Baker [2001], for the same gene and under the same model, to be a criterion marking the boundary between intraspecific and subspecies level. Therefore, all obtained results indicate that the aardvarks in the captive population belong to the same phylogenetic lineage and might be considered a single Management Unit (MU). However, one needs to consider that we only studied two mitochondrial genes. Therefore, it cannot be excluded that the Tanzanian population has adaptations to a different environment, which

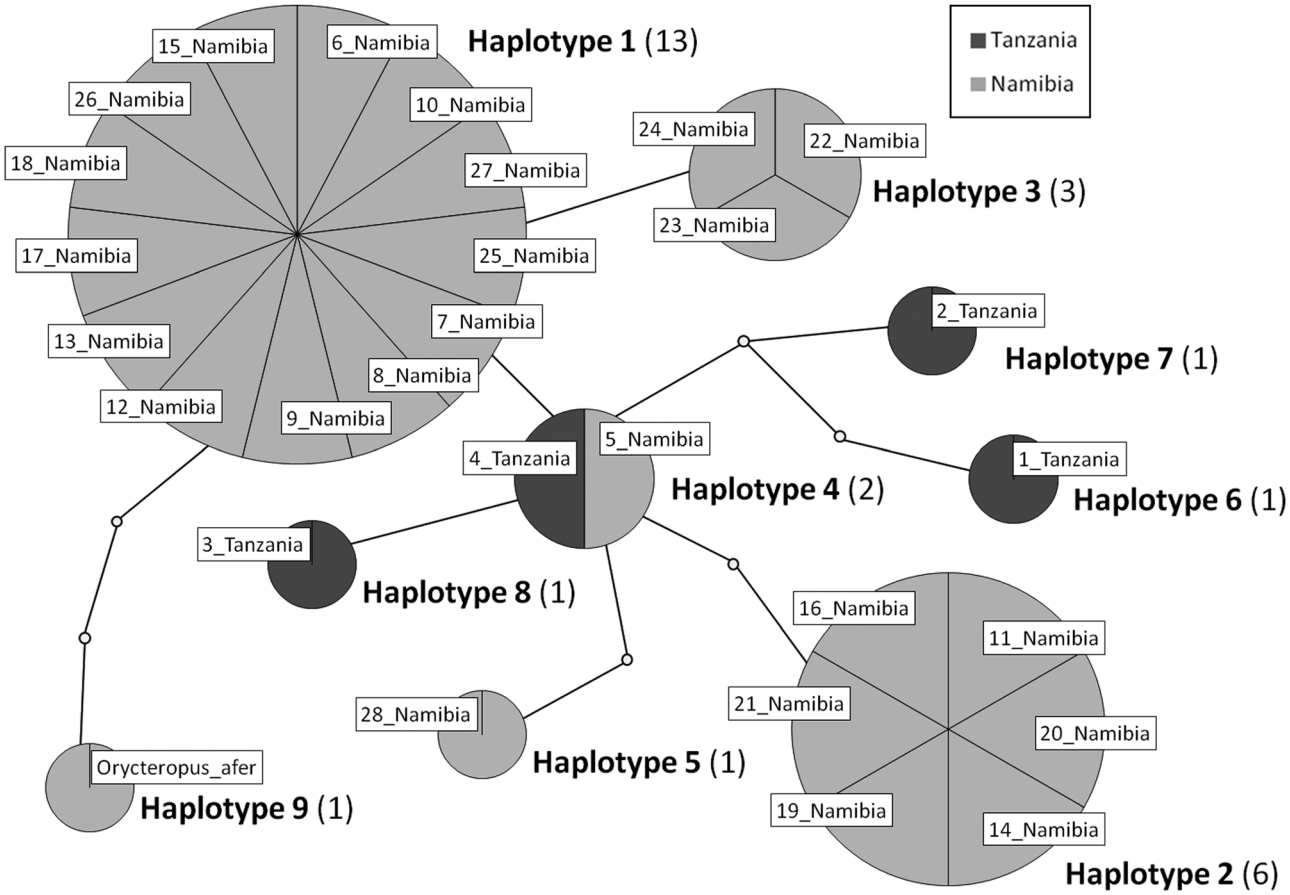

Fig. 1. Haplotype network (TCS) of cyt *b* with frequences for each haplotype. Each line connecting circle indicates one base pair substitution. Small circles indicate hypothetical internodes.

are not reflected by a neutral marker. Therefore, we recommend not mixing Namibian and Tanzanian individuals until more extensive studies have been conducted. So far, the zoological institutions housing the new Tanzanian aardvarks have been precautious in this regard, and favored Tanzanian–Tanzanian husbandry with already some offspring, for example, at the Frankfurt Zoological Garden, Germany [Parys et al., 2012].

PERSPECTIVE

Cytochrome *b* is a useful marker for inferring phylogenies [Amori et al., 2009]. It is also well comparable with many other mammalian species for a basic estimation of level of observed genetic divergences [Bradley and Baker, 2001; Baker and Bradley, 2006]. In spite of some criticisms towards cytochrome *b* (for a review see Amori et al. [2009]), it usually provides a good resolution, even higher than in COI, which is the preferred gene for DNA barcoding [Tobe et al., 2010]. Nevertheless, some more sensitive mitochondrial genes, for example, the mitochondrial control region [Arctander et al., 1999; Moodley and Bruford, 2007; Amori et al., 2009], might provide more insight into the phylogenetic differentiation of aardvarks. Furthermore, it would be useful to study also non-neutral genes, to test if genes under selection (e.g., MHC) differ among populations and show an adaptation to the different environments. A full genomic analysis could be helpful to identify potential genes under selection and to fully understand the degree of differentiation of aardvark populations. For a more detailed insight into the neutral genetic population structure, microsatellites are usually the recommended marker system [Witzenberger and Hochkirch, 2011].

As mentioned above, our study is influenced by the relatively small sample size, limited to aardvarks kept in European zoological institutions. Moreover, the precise geographical origin for these aardvarks is almost always unknown, with the exception of a few individuals (e.g., individual ESB 39 originates from Okahandja, central Namibia). In many cases, individuals may stem from local traders or "hunter's" acclimatization stations, which concentrate animals from more or less distant areas. This is why it remains difficult to identify MUs purely from individuals stemming from zoos. Tanzania for instance may be inhabited by as many as five aardvark subspecies [Lehmann, 2007]. A more comprehensive analysis based on a larger sample

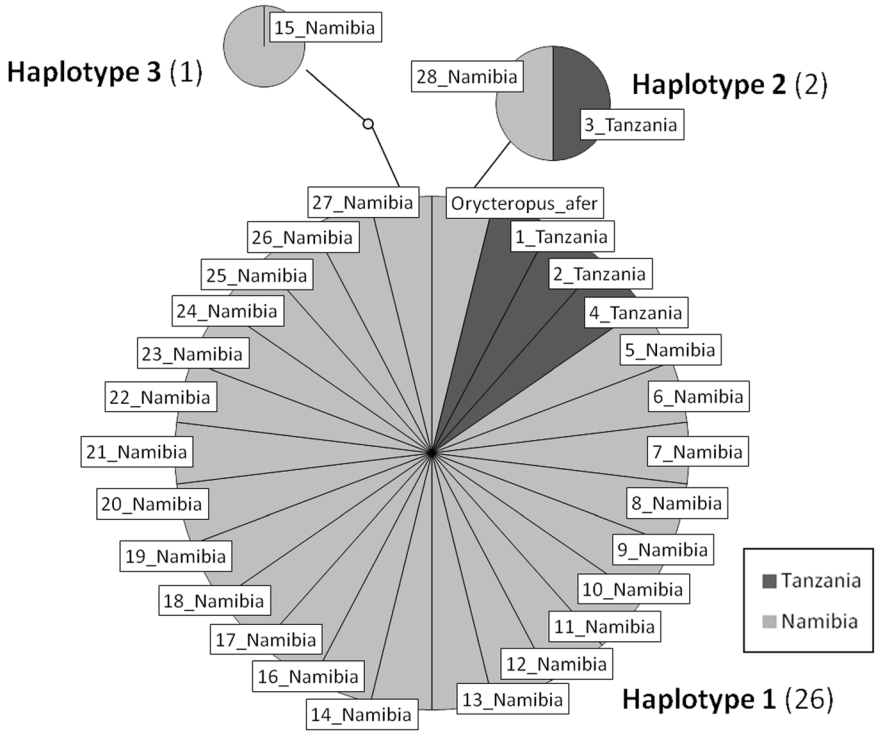

Fig. 2. Haplotype network (TCS) of 16S with frequences for each haplotype. Each line connecting circle indicates one base pair substitution. Small circles indicate hypothetical internodes.

consisting of aardvarks with well documented origin, representative of further sub-Saharan environments (i.e. forest and savannah) [Grubb, 1978; Taylor, 2011] and including ideally also the type specimens of all proposed aardvark subspecies would be necessary to confidently identify suitable aardvark MUs. Such study would also help deciphering aardvark's taxonomy [cf. Shoshani et al., 1988] and understanding its morphological evolution, as well as distribution pattern. These parameters are crucial to determine the conservation status of the recognized taxa/MUs and for establishing (if required) conservation actions.

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