Received: 27 March 2017 | Accepted: 6 August 2017

DOI: 10.1111/zsc.12257

ORIGINAL ARTICLE

WILEY Zoologica Scripta

Deep divergence among mitochondrial lineages in African jackals

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Funding information
Alexander von Humboldt-Foundation; The Rufford Foundation

Recently, molecular analyses revealed that African and Eurasian golden jackals are distinct species. This finding suggests re-investigation of the phylogenetic relationships and taxonomy of other African members of the Canidae. Here, we provide a study on the phylogenetic relationship between populations of African jackals *Lupulella mesomelas* and *L. adusta* inferred from 962 bp of the mitochondrial cytochrome b (cytb) gene. As expected from its disjunct distribution, with one population in eastern Africa and the other one in southern Africa, we found two mitochondrial lineages within *L. mesomelas,* which diverged about 2.5 million years ago (Ma). In contrast, in *L. adusta* with its more continuous distribution in sub-Saharan Africa, we found only a shallower genetic diversification, with the exception of the West African population, which diverged around 1.4 Ma from the Central and East African populations. Both divergence ages are older than, for example the 1.1–0.9 million years between the grey wolf *Canis lupus* and the African golden wolf *C. lupaster*. One taxonomic implication of our findings might be that the two *L. mesomelas* populations warrant species status. However, genome-wide data with adequate geographical sampling are needed to substantiate our results.

KEYWORDS
Africa, Canidae, *Canis*, cytochrome b, *Lupulella*, mitochondrial DNA, phylogeny

> *While endeavouring to throw some light into the hopeless confusion the nomenclature of the jackals of Africa is now in, I do not expect the present communication to clear up all the disputable points.*
>
> *De Winton (1899 p. 533).*

1 | INTRODUCTION

The application of molecular methods in evolutionary biology and population genetics has provided striking insights into the evolutionary history of many taxonomic groups including canids (e.g., Bardeleben, Moore, & Wayne, 2005; vonHoldt et al., 2011, 2016; Lindblad-Toh et al., 2005; Wayne et al., 1997). In combination with the unified species concept of de Queiroz (2007), this approach has also led to the discovery of cryptic species even within well-known genera such as *Canis* (Koepfli et al., 2015; Rueness et al., 2011). For instance, molecular analyses have shown that the African subspecies of the golden jackal *C. aureus anthus* is more closely related to the northern grey wolf (*C. lupus*) than to the Eurasian golden jackal (*C. aureus*), and hence should be recognized as a distinct species within the genus *Canis* (Koepfli

et al., 2015; Viranta, Atickem, Werdelin, & Stenseth, 2017). It also became apparent that the genus *Canis* is a paraphyletic group, with the genera *Lycaon* (African wild dog) and *Cuon* (dhole) being more closely related to the wolf clade sensu lato (*C. lupus* including *C. lupaster* African golden wolf, *C. latrans* Coyote, *C. simensis* Ethiopian wolf, *C. aureus* golden jackal and *C. familiaris* dog) than the African black-backed (*C. mesomelas* Schreber, 1775) and side-striped (*C. adustus* Sundevall, 1847) jackals (Lindblad-Toh et al., 2005; Wayne & O'Brien, 1987). Because of this paraphyly, and as the two African jackal species fall significantly outside of the *Canis* clade, Viranta et al. (2017) re-established the genus *Lupulella* Hilzheimer, 1906, for these species.

The question of the nomenclature and definition of "*lupaster"* is far from being resolved as well as the question of whether *Canis* should encompass *Lycaon*, *Cuon* and *Lupulella* (Gippoliti & Groves, 2012; Gonzalez, 2012; Koepfli et al., 2015; Viranta et al., 2017). In this study, we adopted the provisional taxonomy as proposed by Geraads (2011), Dinets (2015) and Viranta et al. (2017), that is *Lupulella* as a genus for the African jackals and *C. lupaster* for the African golden wolf (for a discussion of the taxonomic implications, see Viranta et al., 2017).

The phylogenetic relationships among and within the two remaining African jackal species are not well resolved, partly because they have been used "only" as additional in-groups in phylogenetic analyses of canids, without a proper geographic sampling of the two species (Bardeleben et al., 2005; Koepfli et al., 2015; Lindblad-Toh et al., 2005; Wayne et al., 1997; Zrzavý & Řičánková, 2004) or, if the focus of a study, their sampling was geographically confined to Kenya and northern Tanzania (Wayne et al., 1990). Previous analyses based on nuclear markers suggested a common ancestry for *L. adusta* and *L. mesomelas* (Bardeleben et al., 2005; Lindblad-Toh et al., 2005), whereas in analyses of mitochondrial markers, both jackal species did not form a monophyletic clade (Bardeleben et al., 2005; Koepfli et al., 2015; Viranta et al., 2017; Wayne et al., 1997; Zrzavý & Řičánková, 2004). When comparing the genetic variation within the two species, we expect contrasting phylogeographical signals due to the different distribution patterns of *L. mesomelas* (disjunct) and *L. adusta* (continuous).

Lupulella adusta is found in large parts of sub-Saharan Africa (Figure 1) from Senegal in the West to Eritrea in the East and South through East Africa to northern South Africa and Angola (Hoffmann, 2014a; Loveridge & Macdonald, 2013). While most authors consider a white stripe from elbow to hip and black side stripes as the most important features in distinguishing the species as its name indicates (Walton & Joly, 2003), this is not always conspicuous. Given the large distribution range of *L. adusta*, geographic variation is expected and several subspecies have been proposed

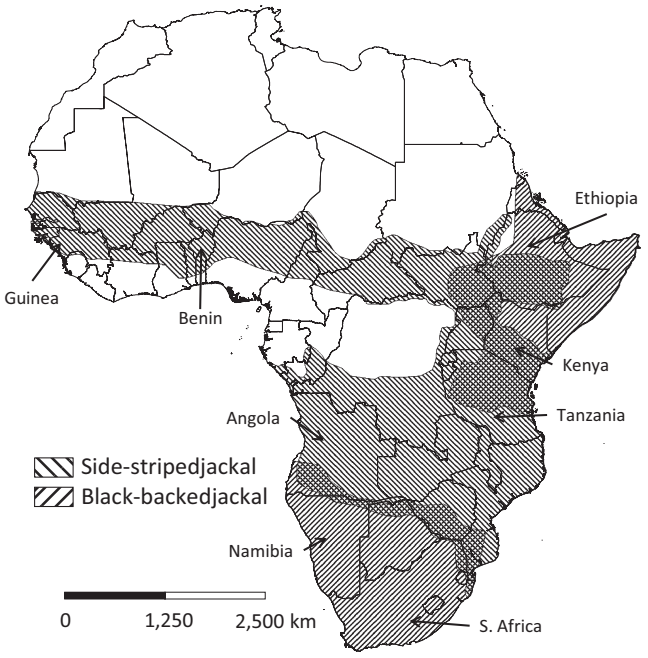

FIGURE 1 Distribution map of African jackals (IUCN species distribution maps, Hoffmann, 2014a, 2014b). Arrows indicate countries from where samples or cytb sequences were available for the study

(Allen, 1939; Coetzee, 1977; Kingdon, 1997; Wozencraft, 2005). However, the validity of these taxa is questionable (Loveridge & Macdonald, 2013; Sillero-Zubiri, 2009).

Lupulella mesomelas is found in southern Africa as well as in north-eastern Africa and the Horn of Africa (Figure 1) (Loveridge & Nel, 2013). Its two areas of occurrence are separated by a distribution gap of 1,000 km from central Tanzania to central Zimbabwe (Ansell, 1960; Loveridge & Macdonald, 2002; Loveridge & Nel, 2013). Five subspecies have been described (Coetzee, 1977), but given the disjunct distribution of *L. mesomelas*, these were later reduced to only two, the Cape jackal *L. m. mesomelas* in southern Africa and the East African jackal *L. m. schmidti* in eastern Africa (Kingdon, 1997; Walton & Joly, 2003).

So far no comprehensive study has been carried out to determine the genetic variation and phylogenetic relationships between species and populations of African jackals. But as de Winton nicely phrased it in 1899 (p. 533), we do not expect to clear up all the disputable points about the phylogeny and taxonomy of African Canini. We aim to contribute the first data to close the gap in our knowledge about the evolutionary history of canids by reconstructing the phylogenetic relationships among mitochondrial lineages of African jackals. We therefore generated sequence data of a 962-bp-long fragment of the mitochondrial cytochrome b (cytb) gene from modern and historic *Lupulella* specimens and compared them with orthologous data from related species.

2 | MATERIALS AND METHODS

2.1 | Sample collection

We collected samples from 15 *L. mesomelas* individuals (three modern tissue samples from South Africa and 12 museum samples from Kenya, Tanzania and Namibia) and 13 *L. adusta* samples (nine faecal samples from Ethiopia and four museum samples from Angola, Tanzania and Namibia) (Table S1). The museum samples consisted of small pieces of skin (ca. 5 × 5 mm) provided by the Museum für Naturkunde Berlin (MfN), Germany. The faecal samples were collected during field studies in Ethiopia, dried and stored on silica beads. The three tissue samples (dry ear tips) from South Africa were obtained from black-backed jackals that were legally killed by professional hunters employed by farmers on their private land as a means to protect their small livestock. No animal was disturbed, harmed or killed for the sake of this study.

2.2 | Laboratory methods

DNA from museum samples was extracted following methods described in Rohland, Siedel, and Hofreiter (2004), while DNA extraction from modern tissue and faecal samples was carried out with Dynabeads MyOneTM SILANE according to Atickem et al. (2013). The ca. 1,020-bp-long fragment of the cytb gene was amplified either in a single PCR reaction (modern tissue and faecal samples; primers Canis-Cyt-1_F and Canis-Cyt-6_R) or via six overlapping PCR products (museum samples) (Table 1). PCR reactions with a total volume of 25 µl and 10–50 ng genomic DNA were performed with the AmpliTaq Gold 360 Master Mix (Applied Biosystems) in a Labcycler (Sensoquest). PCR conditions consisted of a predenaturation step at 95°C for 10 min, followed by 40–50 cycles, each with denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s (museum samples) or 60 s (modern tissue samples). At the end, a final extension step at 72°C for 7 min was added. PCR performance was checked on 1% agarose gels, and PCR products were excised from the gel and purified with the QIAquick Gel Extraction Kit (Qiagen). Sequencing was conducted on an ABI 3130xL sequencer (Applied Biosystems) using the BigDye Terminator 3.1 Cycle Sequencing kit (Applied Biosystems) and the amplification primers. Reactions with a total volume of 10 µl contained 1 µl of BigDye, 1.5 µl of sequencing buffer, 3.3 pmol of primer and 10–25 ng of PCR product. Cycling was performed in a Labcycler with 25 cycles of denaturation at 96°C for 30 s, annealing at 50°C for 15 s and extension at 60°C for 4 min. Before sequencing, reactions were purified with the standard ethanol/EDTA/sodium acetate precipitation method (Applied Biosystems). Obtained sequence electropherograms were checked in 4Peaks 1.8 (nucleobytes.com/4peaks/), and sequences were assembled in SeaView 4.5.4 (Gouy, Guindon, & Gascuel, 2010). SeaView was also used to verify correct translation of nucleotide sequences into amino acid sequences. Sequences are available in GenBank (accession numbers MF196934-MF196961).

TABLE 1 Primers applied in this study

Primer ID	Sequence 5′–3′	PCR product length
Canis-Cyt-1_F	CGTTGTAYTTCAACTATAAG	205 bp
Canis-Cyt-1_R	CTGTRTCYGATGTATAGTG	
Canis-Cyt-2_F	GGAGTATGCYTRATTCTAC	220 bp
Canis-Cyt-2_R	CTATGAATACATAGGATCC	
Canis-Cyt-3_F	TACATGTRGGACGAGGC	235 bp
Canis-Cyt-3_R	GTTGCTTTGTCYACTGAG	
Canis-Cyt-4_F	TAGTAGARTGRATCTGAGG	193 bp
Canis-Cyt-4_R	TAGTARGGGTGRAATGGAA	
Canis-Cyt-5_F	TCAGGAATYACATCAGACT	216 bp
Canis-Cyt-5_R	GATRGCRTAGGCGAATAG	
Canis-Cyt-6_F	GAYAAYTACACCCCTGCA	223 bp
Canis-Cyt-6_R	AAARTCAGAAYARGCATTGG	

To avoid cross-sample contamination, particularly between museum samples as well as among museum and modern samples, we conducted all working steps (DNA extraction, PCR setup, gel electrophoresis, gel extraction, etc.) in separate laboratories and under Captair Bio PCR (Erlab) or Safe 2020 biological safety cabinets (Thermo Fisher Scientific). Analyses of modern and historic samples were strictly separated. Historic samples were handled in our ancient DNA laboratory, and modern samples were investigated in our standard molecular genetic laboratories. Further, gloves were regularly changed, and we used only dual-filter tips and ran various negative controls during all DNA extraction and PCR amplification steps of modern and historic samples. For museum samples, workbenches were decontaminated with UV light and bleach before and after each sample. For some samples, PCR amplification and sequencing were repeated. To minimize the risk of amplifying nuclear mitochondrial-like sequences (numts), we designed Canini-specific primers on the basis of Canini sequences deposited in GenBank and which we used as additional data for our phylogenetic tree reconstructions (Table S1).

2.3 | Phylogenetic analyses

To reconstruct phylogenetic trees, we expanded our data set with additional cytb sequences from GenBank (Table S1). The data set contained 73 sequences from Canini representatives as well as one *Vulpes vulpes* sequence, which was used for rooting purposes. After removing identical sequences, a

total of 53 sequences remained in the final data set. Forty-seven of them were full-length (962 bp), while the remaining six sequences, obtained from GenBank, were only 402 bp in length. The missing 560 bp in these sequences was substituted by Ns. The alignment was generated with Muscle 3.8.31 (Edgar, 2010) in SeaView (Gouy et al., 2010) and corrected by eye. We reconstructed phylogenetic trees with the maximum-likelihood (ML) and Bayesian algorithms using IQ-TREE 1.5.2 (Nguyen, Schmidt, von Haeseler, & Minh, 2015) and MrBayes 3.2.6 (Ronquist et al., 2012), respectively. For both reconstructions, we applied the optimal model of sequence evolution (TrN+I+G), which we determined with ModelFinder (Chernomor, von Haeseler, & Minh, 2016; Kalyaanamoorthy, Minh, Wong, von Haeseler, & Jermiin, 2017) in IQ-TREE under the Bayesian information criterion. The ML analysis was run with 10,000 ultrafast bootstrap (BS) replications (Minh, Nguyen, & von Haeseler, 2013). The Bayesian tree was reconstructed in four independent Markov Chain Monte Carlo runs. We ran all repetitions for 10 million generations with tree and parameter sampling occurring every 1,000 generations, and applied a burn-in of 25%. We checked convergence of all parameters and the adequacy of the burn-in by assessing the uncorrected potential scale reduction factor (Gelman & Rubin, 1992), calculated by MrBayes. Posterior probabilities (PP) for nodes and a phylogram with mean branch lengths were calculated from the posterior density of trees using MrBayes. Phylogenetic trees were visualized in FigTree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

We estimated divergence times using a Bayesian approach implemented in the BEAST 2.4.2 package (Bouckaert et al., 2014). As in Koepfli et al. (2015), we assumed a strict clock model of lineage variation and a Yule prior for branching rates. Likewise, for comparative reasons, we applied the same fossil-based calibration points and identical setting as Koepfli et al. (2015). Accordingly, we calibrated the split between Canini and Vulpini at 9–12 million years ago (Ma) and the most recent common ancestor (MRCA) of the genus *Canis* (sensu stricto; Figure 2) at 1–3 Ma. For both calibration points, we used a lognormal distribution with following settings: mean = 0.0, standard deviation = 0.7 and offset = 8.7 for the Canini-Vulpini split resulting in a 95% highest posterior density (HPD) of 9.0–11.9 Ma, and mean = 0.0, standard deviation = 0.5 and offset = 0.7 for the MRCA of *Canis* resulting in a 95% HPD of 1.1–3.0 Ma. We performed two independent analyses each with 25 million generations and tree and parameter sampling occurring every 1,000 generations. To assess the adequacy of the burn-in and convergence of all parameters, we inspected the trace of the parameters across generations using Tracer 1.6 (http://beast.bio.ed.ac.uk/Tracer). Sampling distributions of independent runs were combined with LogCombiner 2.4.2, and trees with mean node heights were summarized with TreeAnnotator 2.4.2 using a burn-in of 10%. Trees were visualized in FigTree.

3 | RESULTS

We successfully sequenced 962 bp of the mtDNA cytb gene from 15 *L. mesomelas* and 13 *L. adusta* individuals, resulting in eight and eleven unique haplotypes, respectively. All obtained sequences were correctly transcribed into amino acid sequences without any premature stop codons and were highly similar to those of conspecifics derived from GenBank, thus limiting the possibility that we amplified numts. In the alignment consisting of 53 unique haplotypes, we found 348 variable sites, of which 264 were parsimony-informative.

The topology of our time tree is similar to the cytb tree reported by Koepfli et al. (2015). The first divergence (≈6 Ma) within the Canini separates the Neotropical species, here the maned wolf (*Chrysocyon brachyurus*) and the bush dog (*Speothos venaticus*), from the Old World and Nearctic taxa (Figure 2, Fig. S1). Subsequently (≈5.1 Ma), a clade containing *Lycaon*, *Cuon* and *Lupulella adusta* diverged from a clade comprising *Canis* and *Lupulella mesomelas*. *L. adusta* diverged from a common clade of *Cuon* and *Lycaon* around 4.6 Ma, whereas the lineages of *L. mesomelas* and *Canis* split around 3.8 Ma. The major *Canis* clade consists of two wolf clades (grey wolves [*C. lupus* II] and wolves of China [*C. lupus* I]), probably two lineages of African golden wolves (*C. lupaster* North Africa, *C. lupaster* Kenya), Ethiopian wolves (*C. simensis*), Eurasian golden jackals (*C. aureus*) and coyotes (*C. latrans*). The most interesting findings of our phylogenetic reconstruction are, however, a confirmation of the mitochondrial paraphyly of the two African jackal taxa (*Lupulella*) and their deep intraspecific splits. The two geographic lineages of *L. mesomelas* split around 2.6 Ma, and the extreme West African *L. adusta* lineage diverged from its presumed conspecifics in Central and Eastern Africa around 1.4 Ma. The terminal clades in our tree are all strongly supported with the exception of the non-West African *L. adusta* clade and the North African *C. lupaster* clade. Most of the deeper splits, however, are only poorly supported, which most likely results from the relatively low genetic information available for this study (only 962 bp). One of the museum specimens (ZMB_Mam_52394) from the Caprivi Strip in Namibia (Fig. S2c) was classified as *L. adusta*, but its cytb sequence clustered clearly with those of *L. mesomelas* (haplotype 15 in Fig. S1).

4 | DISCUSSION

Although our geographic sampling was limited and we had to restrict our analysis to the genetic information available at

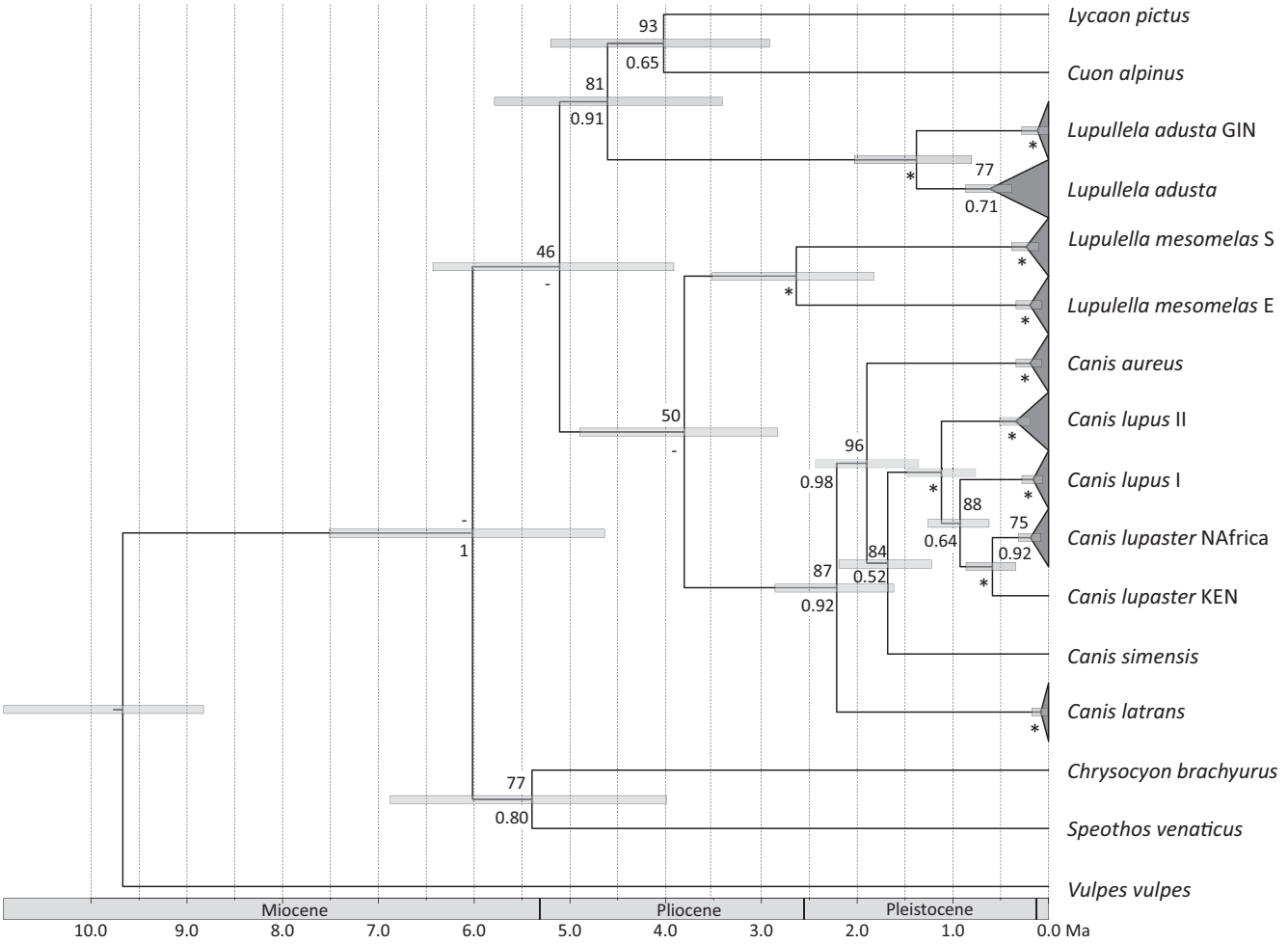

FIGURE 2 Ultrametric tree showing phylogenetic relationships and divergence times within Canini based on 962 bp of the cytb gene. Node bars indicate 95% HPDs of estimated divergence times. Numbers at nodes refer to ML BS and Bayesian PP support values; * denotes ML BS ≥ 95% and Bayesian PP ≥ 0.99. The uncollapsed tree is presented in Fig. S1

GenBank, we found some interesting results. The phylogenetic reconstruction revealed a deep divergence between the two geographically disjunct populations of the black-backed jackal. Although our analysis was based on just a short fragment of the mitochondrial genome, reciprocal monophyly of both clades is strongly supported. The divergence age of the two clades is most likely older than the divergence between coyotes and other members of *Canis* (Figure 2). Similarly, we found a relatively deep divergence between side-striped jackals from West Africa (Guinea) and those from Central/East Africa. The divergence age in this case is similar to that between different wolf clades (*C. lupus* I, *C. lupus* II and *C. lupaster*) (Figure 2).

Due to the use of only a small fragment of the mitochondrial genome, most of the deeper relationships among various Canini clades are not well supported. Therefore, our results do not contribute much to the resolution of their phylogenetic relationships. However, at least the phylogenetic position of the African jackals outside the *Canis* clade seems unambiguous. It remains open whether the two African jackal taxa are monophyletic as suggested by nuclear DNA studies (Koepfli et al., 2015; Lindblad-Toh et al., 2005; Perini, Russo, & Schrago, 2010) or, as in our reconstruction and in other mitochondrial DNA studies, paraphyletic (Koepfli et al., 2015; Viranta et al., 2017). Paraphyly would then justify the re-establishment of the genus name *Schaeffia* Hilzheimer, 1906; for the *L. adusta* clade (Viranta et al., 2017). Gene tree discordance is a common phenomenon and may be explained by differences in nucleotide composition, homoplasy, incomplete lineage sorting or hybridization (Ballard & Whitlock, 2004; Funk & Omland, 2003; Koepfli et al., 2015; Roos et al., 2011). However, to determine which process is responsible for the discordance between nuclear and mitochondrial phylogenies needs further investigations.

In two early papers, Wayne et al. (1990, 1997) found a large genetic divergence among *L. mesomelas* from Kenya and northern Tanzania. Among the detected four haplotypes, three were closely related, whereas one was significantly different (8%). This is in contrast to our results. Although our geographic sampling of *L. mesomelas* in East Africa covered

the same area (Kenya and northern Tanzania), we found only five closely related haplotypes. A possible explanation for this contrast might be that the divergent haplotype found by Wayne et al. (1990, 1997) is in fact a nuclear mitochondrial DNA segment (numt) (Thalmann, Hebler, Poinar, Pääbo, & Vigilant, 2004).

Lupulella mesomelas and *L. adusta* have overlapping ranges in both eastern and Southern Africa. So far, no evidence for hybridization between the two species or between *Lupulella* and other Canini has been reported, although hybridization seems to be common in other Canini (Galov et al., 2015; Gottelli et al., 1994; Randi et al., 2014; Stronen et al., 2012). At least two *Canis* taxa, red wolves (*Canis rufus*) and eastern wolves (*Canis lycaon*), are most likely results of genetic admixtures between grey wolves and coyotes (vonHoldt et al., 2016). We have at least one case in our study, where introgression between the two jackal species cannot be excluded, if there was no misidentification. The museum specimen ZMB_Mam_52394 is labelled as *Canis adustus,* and its fur pattern supports this classification (Fig. S2c). However, its haplotype is clearly *L. mesomelas*. The geographic provenance of the sample is the region of range overlap between the two jackal species within the Caprivi Strip, Namibia (S19.65° E17.33°), where hybridization might be possible. The fur pattern of the specimen suggests *L. adusta*, which can be the case if the hybridization event happened several generations before with subsequent backcrossing with *L. adusta* (e.g., mitochondrial capture, Zinner, Arnold, & Roos, 2009, 2011). Further molecular analysis might help to solve the question about hybridization.

A disjunct distribution pattern as of *L. mesomelas* is found in other savannah-dwelling species, including bat-eared fox *Otocyon megalotis*, aardwolf *Proteles cristatus* (Kingdon & Hoffmann 2013) and several ungulates (Lorenzen, Heller, & Siegismund, 2012). In particular with respect to the other carnivores, it would be interesting to test whether the divergence ages between eastern and southern populations are similar to that found for *L. mesomelas*, which would indicate a common climatic–ecological trigger for the distribution patterns.

4.1 | Possible taxonomic and conservation implications

Due to the short fragment of the cytb gene we used in our analysis, our results remain preliminary, in particular with respect to taxonomic revisions. However, if subsequent studies based on more genetic information confirm our results, the two populations of black-backed jackals that were previously considered as subspecies *L. m. schmidti* and *L. m. mesomelas* might be elevated to two distinct species. There are also morphological differences between the two populations. For instance, the skulls of *L. mesomelas* from East Africa have a shorter total length and are wider and less variable than the skulls of *L. mesomelas* from southern Africa (Valkenburgh & Wayne, 1994). Similarly, the two clades of *L. adusta* might constitute two separate taxa, whereby it would be interesting to investigate whether the earlier *Canis anthus* Cuvier, 1820, from Senegal matches genetically the West African *L. adusta* clade. Of course, for such an analysis, one would need to examine DNA from the type specimen, but unfortunately, this seems to be untraceable according to Viranta et al. (2017). The "traditional" taxonomy of *Canis* assumes that four species occur in Africa: *C. adustus*, *C. aureus*, *C. mesomelas* and *C. simensis* (e.g., Wozencraft, 1993, 2005), but historical papers on Libyan mammals (e.g., Hufnagl, 1972) already stressed the presence of two kinds of canids, a larger one (*lupaster*) and a smaller one (*variegatus* = *anthus* = *soudanicus,* etc.). A more complete geographical sampling probably will indeed reveal even more cryptic lineages within African canids.

A possible revision of the jackal taxonomy may also have implications on their conservation status that might need reassessment (Agapow et al., 2004; Gippoliti, Cotterill, Zinner, & Groves, 2017; Groves et al., 2017). To date, both species have been listed as Least Concern by the IUCN Red List of Threatened Species (Hoffmann, 2014a, 2014b), and in some countries, they are even regarded as vermin (e.g., South Africa, Bergman et al., 2013). However, if the taxa exhibit smaller distribution ranges or lower population densities following a revision, their respective conservation status might need to be changed. The first population to prioritize for a new assessment would probably be the West African *L. adusta*.

5 | CONCLUSION

Our study brings new evidence that the evolutionary history of African canids is more complicated than previously thought. Within *L. mesomelas,* we found that the two disjunct geographical populations comprise two old mitochondrial lineages, probably older than the divergence between grey wolves and Eurasian golden jackals. We also found that within *L. adusta,* no spatial genetic structure is obvious, besides the divergence between the West and the Central/East African lineages. Our study highlights the need for more taxonomic and molecular studies on African canids based on consistent geographic sampling. Genomic data are needed to solve some of the questions we have discussed above, for example hybridization among *Lupulella* species, the rank of the *Lupulella* clades and the possible relationships among *Canis anthus* and the West African *L. adusta* clade.

ACKNOWLEDGEMENTS

We are grateful to Christiane Schwarz for help in the laboratory and Håkan Pohlstrand for help during fieldwork in Ethiopia. We also thank two reviewers for their constructive

critique. Further, we thank the Alexander von Humboldt-Foundation (Georg Forster-Research Grant [HERMES]) and The Rufford Foundation for financial support of this project.

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Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Atickem A, Stenseth NC, Drouilly M, Bock S, Roos C, Zinner D. Deep divergence among mitochondrial lineages in African jackals. *Zool Scr*. 2018;47:1–8. https://doi.org/10.1111/zsc.12257