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ORIGINAL ARTICLE

Genetic diversity, evolutionary history and implications for conservation of the lion (*Panthera leo*) in West and Central Africa

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ABSTRACT

Aim In recent decades there has been a marked decline in the numbers of African lions (*Panthera leo*), especially in West Africa where the species is regionally endangered. Based on the climatological history of western Africa, we hypothesize that West and Central African lions have a unique evolutionary history, which is reflected by their genetic makeup.

Location Sub-Saharan Africa and India, with special focus on West and Central Africa.

Method In this study 126 samples, throughout the lion's complete geographic range, were subjected to phylogenetic analyses. DNA sequences of a mitochondrial region, containing cytochrome *b*, tRNAPro, tRNAThr and the left part of the control region, were analysed.

Results Bayesian, maximum likelihood and maximum parsimony analyses consistently showed a distinction between lions from West and Central Africa and lions from southern and East Africa. West and Central African lions are more closely related to Asiatic lions than to the southern and East African lions. This can be explained by a Pleistocene extinction and subsequent recolonization of West Africa from refugia in the Middle East. This is further supported by the fact that the West and Central African clade shows relatively little genetic diversity and is therefore thought to be an evolutionarily young clade.

Main conclusions The taxonomic division between an African and an Asian subspecies does not fully reflect the overall genetic diversity within lions. In order to conserve genetic diversity within the species, genetically distinct lineages should be prioritized. Understanding the geographic pattern of genetic diversity is key to developing conservation strategies, both for *in situ* management and for breeding of captive stocks.

Keywords

Central Africa, evolutionary history, genetic diversity, lion, *Panthera leo*, phylogenetics, phylogeography, West Africa.

INTRODUCTION

Currently, two subspecies of the lion are recognized by the International Union for the Conservation of Nature (IUCN): the African lion, *Panthera leo leo* (Linnaeus, 1758), and the Asiatic lion, *Panthera leo persica* (Meyer, 1826) (Bauer *et al.*, 2008). This distinction has been confirmed in recent genetic studies (Driscoll *et al.*, 2002; Burger *et al.*, 2004; Dubach *et al.*, 2005; Barnett *et al.*, 2006b; Antunes *et al.*, 2008). However, the genetic diversity within the species is greater than this taxonomic classification implies; several studies based on genetic data have reported distinct phylogenetic groups within the African

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subspecies (Table 1), partially overturning earlier categorizations based on morphological traits and geographic distribution. A recent publication on lion phylogeny based on craniometric data concluded that two major evolutionary clusters can be distinguished: sub-Saharan Africa and North Africa/Asia (Mazák, 2010), also deviating from the former Africa–Asia separation.

When setting up management strategies to preserve genetic variation in a species, one has to determine which (meta)populations efforts need to be focused on. When the existing taxonomy does not sufficiently reflect the genetic diversity, a smaller scale should be used, such as evolutionarily significant units (ESUs) or management units (MUs) (Moritz, 1994). The phylogenetic approach emphasizes protection of (meta)populations with a unique evolutionary history. Insight into the geographic pattern of genetic variation is not only crucial for management of wild populations, but also for breeding of captive stocks.

The lion is classified as 'Vulnerable' on the Red List of Threatened Species (Bauer *et al.*, 2008), meaning that it faces 'a high risk of extinction in the wild'. Ninety per cent of the estimated continental population is located in southern and East Africa (Chardonnet, 2002; Bauer & Van Der Merwe, 2004), with many large and stable lion populations. However, in West and Central Africa lion populations are generally small and isolated (Chardonnet, 2002; Bauer & Van Der Merwe, 2004). There is an increasing number of lions in peripherally isolated populations or in wildlife parks with little to no gene flow. Lions may face genetic erosion and inbreeding in these regions (Björklund, 2003). Several studies show that inbreeding depression is much more pervasive in wild populations than previously realized (Lacy, 1997; Hedrick & Kalinowski, 2000; Keller & Waller, 2002; Tallmon *et al.*, 2004), and it has been observed that there is a strong correlation between genetic variation and reproductive parameters in lions (O'Brien, 1994). The number of mature individuals in West Africa has been estimated by two separate surveys as 850 (Bauer & Van Der Merwe, 2004) and 1163 (Chardonnet, 2002), and the lion was therefore classified as 'Regionally Endangered' according to the IUCN criteria (Bauer & Nowell, 2004).

It is known that West and Central Africa have a different climatic history than southern and East Africa, as West Africa and the northern part of Central Africa were characterized by hyperarid conditions during the Holocene glacial periods (Sarnthein, 1978; Klein & Martin, 1984; Dupont *et al.*, 2000; Gasse, 2000). This may have had a significant impact on local wildlife populations, related to climatic niches and food availability, possibly resulting in the development of distinct genetic lineages in this region. A dichotomy among genetic

Table 1 Overview of the genetic studies reporting phylogenetic units within *Panthera leo*.

Authors (year)	Findings	Units distinguished	Method
Dubach *et al.* (2005)	6 maternal haplotypes	South–west Africa East of the Rift Valley West of the Rift Valley Sabi Sands (RSA) (Asiatic lions not included)	Cytochrome *b* and NADH subunit 5 + 6 genetic markers
Barnett *et al.* (2006b)	11 maternal haplotypes	India North Africa West Africa Central Africa Eastern–southern Africa Southern Africa	Control region (HVR1) genetic marker
Antunes *et al.* (2008)	Nuclear data:		
	11 microsatellite groups 5 ADA haplotypes 3 TF haplotypes	India East Africa Southern Africa	22 microsatellites, ADA, TF (autosomal), SRY (Y-chromosome), 12S, 16S (mitochondrial) genetic markers + assessment of prevalence and genetic variation of the lion-specific FIV
	SRY + mitochondrial data:		
	1 paternal haplotype	India	
	12 maternal haplotypes	North/Central Africa Southern/East Africa Southern Africa East Africa	
	Six FIV subtypes:	Southern/East Africa Southern Africa East Africa	

NADH, nicotinamide adenine dinucleotide reduced form; RSA, Republic of South Africa; HVR1, hypervariable region 1; ADA, adenosine deaminase; TF, transferrin; SRY, sex-determining region Y; FIV, feline immunodeficiency virus.

haplotypes between West and Central Africa and southern and East Africa has been observed in seven African bovids (Arctander *et al.*, 1999; Nersting & Arctander, 2001; Pitra *et al.*, 2002; Van Hooft *et al.*, 2002), African elephant (*Loxodonta africana*) (Eggert *et al.*, 2002), cheetah (*Acinonyx jubatus*) (Freeman *et al.*, 2001), black rhinoceros (*Diceros bicornis*) (Brown & Houlden, 2000), roan antelope (*Hippotragus equinus*) (Alpers *et al.*, 2004) and giraffe (*Giraffa camelopardalis*) (Brown *et al.*, 2007). A similar genetic pattern is expected in lions, which would illustrate the need for stronger conservation efforts for the small and isolated West and Central African lion populations.

In this study we illustrate the phylogenetic relationships between lion populations from their entire geographic range, based on a sequence analysis of a large mitochondrial region. We know of only two previous studies that have included samples from West and Central Africa in phylogenetic analyses (Barnett *et al.*, 2006a,b). With information on the genetic makeup of lions from their West and Central African range, we may be able to conclude whether these form one or more distinct groups, with possible implications for a revised phylotaxonomy. This could have consequences not only for *in situ* wildlife management, but also for the management of zoo populations and for captive breeding programmes.

MATERIALS AND METHODS

Origin of the samples

For this study, scat, hair, blood or tissue samples were obtained from wild-ranging lions and from captive animals in zoos. In total, 53 individuals from 15 countries were sampled (see Table S1 in Appendix S1 in Supporting Information), and 73 sequences from GenBank (see Table S2 in Appendix S1) were added at a later stage for phylogenetic analysis. Six samples, which are indicated with question marks in the table and figures, had a doubtful origin: Angola (no. 9), Democratic Republic of the Congo (DRC) (no. 10) and Somalia (nos 7 and 19). In an earlier study the Moroccan lions from Rabat Zoo, which were originally thought to be descendants of the extinct Barbary subspecies, were identified to contain a haplotype from Central Africa (Barnett *et al.*, 2006a). The origin of all other lions or, in the case of captive lions, the origin of their ancestors, is known.

DNA isolation and sequence analysis

For this study, sequences of a mitochondrial region, containing the cytochrome *b* gene, tRNAPro, tRNAThr and the left domain of the control region, were analysed. The latter part contains the hypervariable region 1 (HVR1), which is the most variable part of the mitochondrial genome in the genus *Panthera* (Jae-Heup *et al.*, 2001).

DNA was extracted from tissue, blood, hair and scat samples. The targeted region was amplified using the primers shown in Table S3 in Appendix S1. Details of extraction methods, polymerase chain reaction (PCR) amplification and sequencing are given in Appendix S2.

Sequences were aligned visually and deposited in the GenBank database under accession numbers GU131164–GU131185, AY781195–AY781210 and DQ018993–DQ018996. Coding regions did not contain any stop codons or nonsense mutations, nor did they contain deletions or insertions that would lead to a frame shift. No known nuclear pseudogene insertions of cytoplasmic mitochondrial DNA sequences (NUMTs) were amplified.

Phylogenetic analysis

To increase the sample size for the phylogenetic analysis, 28 cytochrome *b* sequences (sample group 4) from five countries (Dubach *et al.*, 2005) and 45 control region sequences (sample group 5) from 19 countries (Barnett *et al.*, 2006a) were obtained from GenBank (see Table S2 in Appendix S1). To gain more insight in the recent evolutionary history of the lion, control region sequences from extinct lion populations were also included. Figure 1 shows the locations of origin of the samples processed in our laboratory and the sequences obtained from GenBank that were combined for phylogenetic analyses.

Sequences were divided into three sets for the analysis to obtain sequences of the same length (Table 2): cytochrome *b* + control region (A), cytochrome *b* (B) and control region (C). Samples of which only partial sequences could be obtained were either included in a subset of analyses or were completely excluded and were only used for direct sequence comparison (see Table S4 in Appendix S1).

Sequences from the Moroccan samples (no. 18) contain an insertion of 80 bp (also visible as a longer PCR product on the gel). This insertion proved to be a duplication of 1382–1462 bp and was treated as one mutational event in every analysis. A second region, present in all samples (including the GenBank samples), was excluded from the analysis based on unknown homology. This region contains a repeat of cytosines of variable length at 1382–1393 bp.

For Bayesian and maximum likelihood analyses three outgroup species were added: two sequences of tiger (*Panthera tigris*: EF551003 and DQ151550), leopard (*Panthera pardus*: NC_010641) and snow leopard (*Panthera uncia*: EF551004). In addition, one sequence of extinct European cave lion (*Panthera leo spelaea*: DQ899900) and one sequence of extinct American cave lion (*Panthera leo atrox*: DQ899912) were added for the analysis of the control region.

Bayesian analysis

MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001) was used for the Bayesian analysis of each of the sets of sequences. The appropriate models for molecular evolution were determined using MrModeltest2 (v.2.3) (Nylander, 2004). Stationary nucleotide frequencies of the HKY85 rate matrix were set to a flat Dirichlet distribution for the substitution rate priors and the state frequency priors. The Markov chain Monte Carlo search was continued for 1,000,000 generations, sampling

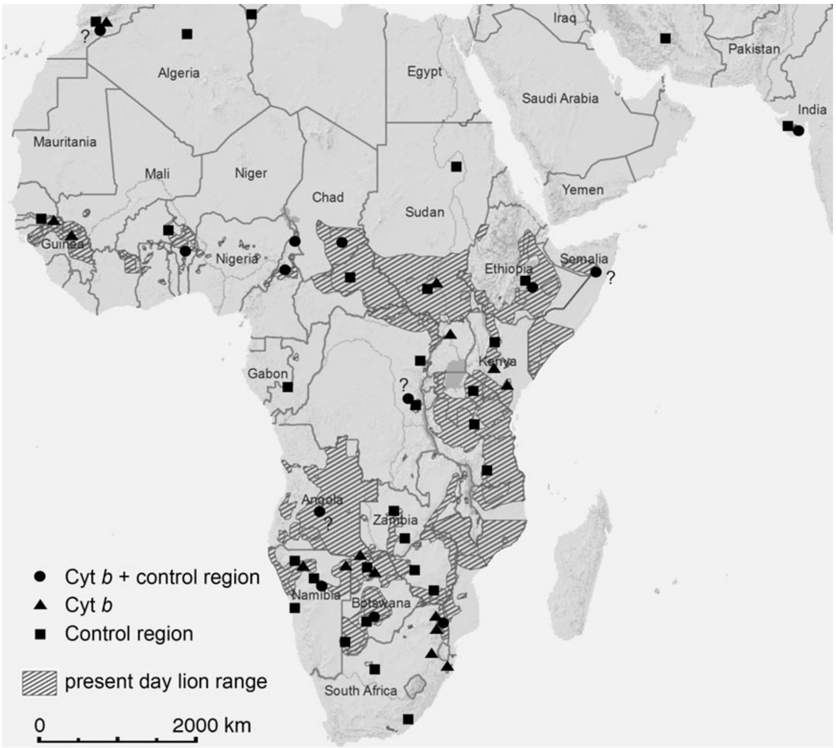


Figure 1 Map showing the origin of the lion (*Panthera leo*) samples that were used for the phylogenetic analyses. Dots indicate the samples from which cytochrome *b* (cyt *b*), tRNAThr, tRNAPro and the left domain of the control region sequences are known, triangles are cytochrome *b* sequences, and the squares show sample locations from which only a part of the control region was sequenced. For several samples only the country of origin was known, and there was no information available on the exact locality. In these cases the geographical centre of the known lion range within the country is indicated (lion range data are from Bauer *et al.*, 2008).

every 100 generations, and the first 2500 trees were discarded as burn-in.

Maximum likelihood analysis

Clusters of samples with an identical haplotype for the marker(s) studied were pooled and analysed as a single sample to reduce the time needed for analysis. Maximum likelihood (ML) analyses were performed using PAUP* 4.0 (Swofford, 2000). Heuristic ML searches [single random addition sequence, tree bisection–reconnection (TBR) without steepest descent] were performed for 100 bootstrap replicates. In each bootstrap replicate, all parameter settings were estimated by PAUP*, except for the base frequencies for which empirical data were used.

Maximum parsimony analysis

For each set of sequences, a haplotype network was generated, using TCS v.1.21: phylogenetic network estimation using statistical parsimony (http://darwin.uvigo.es/software/tcs.html).

Isolation by distance

The samples from Angola (no. 9), DRC (no. 10), Somalia (nos 7 and 19) and Morocco (nos 18 and 20) were excluded from the isolation-by-distance analysis because of their doubtful origin (see above). Two matrices were generated for each of the sets of sequences: one with the genetic distances between the samples, expressed in the number of variable sites in the sequences, the other with the geographical (Euclidean) distance. For some samples only the country of origin was known. In these cases the coordinates of the geographic centre of the lion range within the country was chosen. The Isolation by Distance Web Service (IBDWS) v.3.15 was used for performing a Mantel test for matrix correlation between genetic and geographic distance (http://ibdws.sdsu.edu/~ibdws/).

Table 2 Overview of the sets into which the lion (*Panthera leo*) sequences were subdivided for the phylogenetic analyses.

Sets for analyses	Genetic region	Position (bp)	Samples
A	Cytochrome *b*, tRNAPro, tRNAThr, control region	1–1764	1–2a, 2c–15, 17–19
B	Cytochrome *b*	1–1140	1–14, 16–23, 25–32
C	Control region	1355–1570	1–2a, 2c–15, 17–19, 34–73

RESULTS

Molecular phylogenetic relationships

Bayesian analysis

The HKY85 model was chosen as the model for DNA evolution by MrModeltest, supported by hierarchical likelihood ratio tests and the Akaike information criterion for each of the sets of sequences. Rate variation across sites was modelled allowing invariable sites in all sets. Phylogenetic trees with posterior probability (PP) values derived from Bayesian analysis are shown in Fig. 2 (cytochrome *b* + control region, and cytochrome *b* alone).

Maximum likelihood analysis

Phylogenetic trees derived from ML analyses are shown in Fig. 3 (cytochrome *b* + control region, and cytochrome *b* alone). Samples that share the same haplotype are joined on one branch, in clusters that are identical to the clusters found in the maximum parsimony analyses (see below).

Tree topology

Both Bayesian and ML analyses of cytochrome *b* + control region sequences (Figs 2a & 3a) support four basal clades: (1) the two Botswanan samples (PP > 0.95; bootstrap value >70%), (2) a southern clade with lions from Namibia (PP > 0.95; bootstrap value > 70%) and the Republic of South Africa (RSA) (PP > 0.95; bootstrap value > 70%), (3) Ethiopian and Somalian samples (bootstrap value > 70%), and (4) a geographically widespread clade, grouping lions from West and Central Africa, also including Angola and India (PP > 0.95; bootstrap value > 70%). In the ML analysis, the first three branches form a polytomy within the sister group of the widespread West and Central Africa clade, while in the Bayesian tree all branches have an equally basal position. Within the West and Central Africa group, the India clade is well supported in both analyses (PP > 0.95; bootstrap value > 70%). The branch leading to the rest of the group has significant branch support in the Bayesian analysis (PP > 0.95), and the position of the two Benin samples in this clade remains unresolved in both analyses. Among the West and Central Africa lions, the Bayesian analysis gives significant branch support (PP > 0.95) for: (1) a subclade with the two DRC samples, (2) a subclade containing 10 Cameroon samples, and (3) a subclade with all Chad samples together with one from Cameroon.

The tree based on Bayesian analysis of cytochrome *b* sequences (Fig. 2b) shows a basal split into two clades: (1) a clade from southern African countries, and (2) a clade with samples from West, Central and East Africa, plus Angola and India, and Botswana (no. 2) and RSA (nos 29–31) samples. There is significant branch support (PP > 0.95) within the southern African clade for two out of three subclades: (1) a subclade containing two Namibian lions (no. 6), and (2) a subclade consisting of samples from RSA (no. 8) and RSA (no. 32) (PP > 0.95). The ML analysis tree (Fig. 3b) shows a basal polytomy with: (1) samples from West, Central and East Africa, plus Angola and India, and Botswana (no. 2) and RSA (nos 29–31); (2) Namibia (no. 6); (3) a well-supported branch containing RSA samples (nos 8 and 32) (bootstrap value > 70%); (4) one containing sequences from Botswana and Namibia; and (5) Namibia (28b,d). The clade that contains the sequences from West, Central and East Africa shows significant branch support in both analyses (PP > 0.95; bootstrap value > 70%) for two subclades: (1) the Ethiopian (PP > 0.95) and Somalian samples, and (2) a subclade containing all samples from West and Central Africa, including Angola and India. The third branch, leading to the samples from Botswana (no. 2), Kenya and RSA (nos 29–31) is significantly supported by the Bayesian analysis (PP > 0.95). Within the West and Central African subclade, the branch leading to the clade with the Indian lions is significantly supported in the Bayesian tree (PP > 0.95) and the Benin samples have an unresolved position in both analyses.

The trees of the control region sequences (not shown) are not well resolved. The well-supported clades contain the two extinct cave lion subspecies *P. leo spelaea* and *P. leo atrox* (PP > 0.95; bootstrap value > 70%), and in the case of the Bayesian analysis there is significant support (PP > 0.95) for a branch with RSA samples (nos 8a,c).

Maximum parsimony analysis

A haplotype network was generated for each of the sets of sequences (Fig. 4). The patterns resulting from analysis of cytochrome *b* + the control region (Fig. 4a) and from analysis of cytochrome *b* alone (Fig. 4b) are strongly consistent. In both cases there is a clear distinction between West and Central African lions and southern and East African lions, indicated by numerous mutations between the two groups. In general, variation amongst the West African lions is relatively small, with many individuals sharing the same haplotype, and little distance between the different haplotypes. Indian samples branch off close to the West and Central African group. As was the case in previous phylogenetic analyses, Angolan lions share their haplotype with (or cluster close to) lions from West and Central African countries. Lions from southern and East African regions show more variation, illustrated by numerous mutations between the different haplotypes.

The haplotype network derived from the control region (Fig. 4c) shows a more complex structure. A short loop is formed by the extinct lion populations from North Africa and the Middle East.

Information derived from partial sequences

Because of partial sequences, Guinea (no. 16), Kenya (no. 24) and Uganda (no. 33) were excluded from these analyses. Comparing these partial sequences with the rest of the samples,

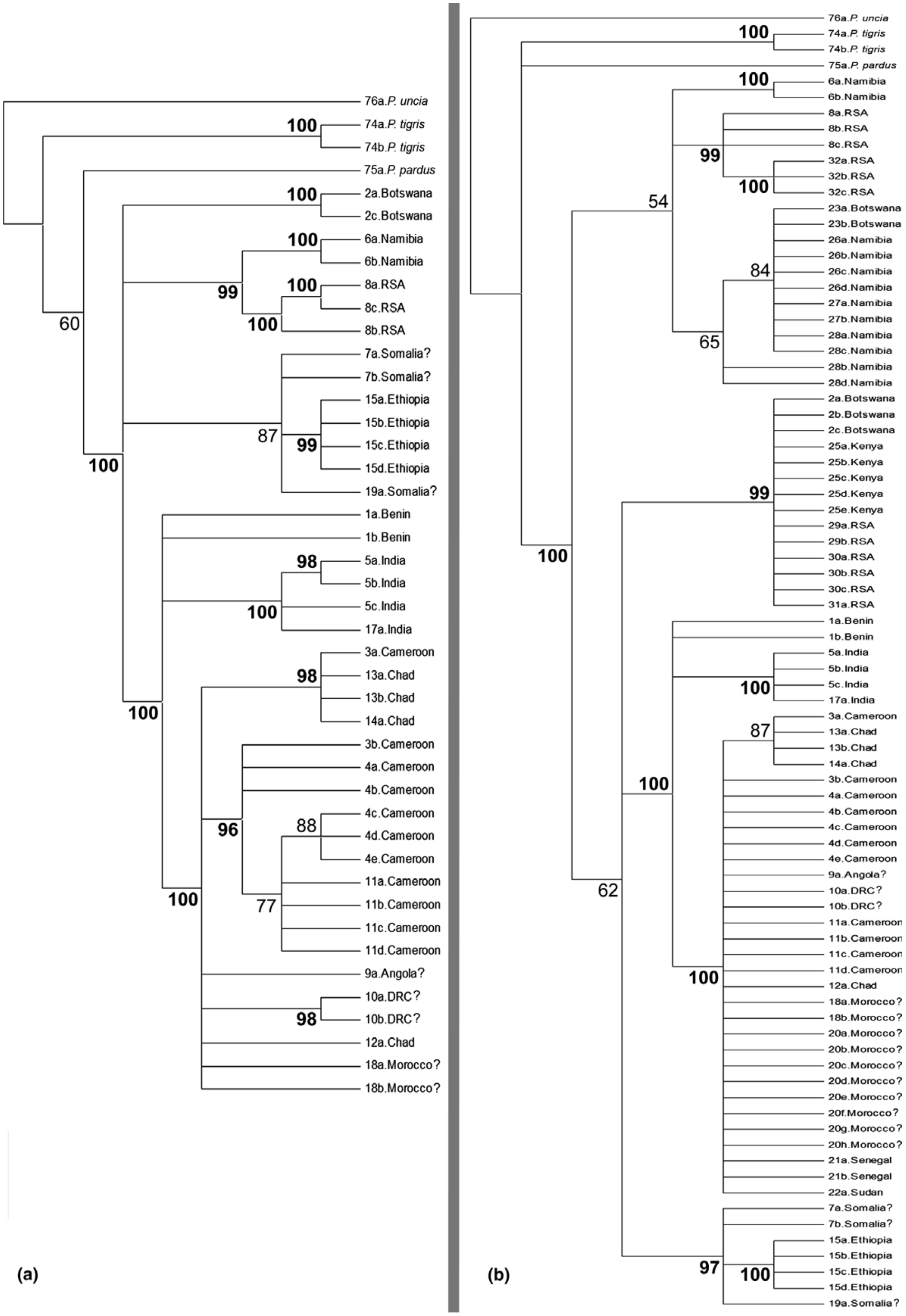


Figure 2 Phylogenetic trees resulting from Bayesian analysis of two sets of lion (*Panthera leo*) sequences: (a) cytochrome *b* + the control region, (b) cytochrome *b* alone. The numbers represent the percentages for Bayesian posterior probability (PP). DRC, Democratic Republic of the Congo; RSA, Republic of South Africa.

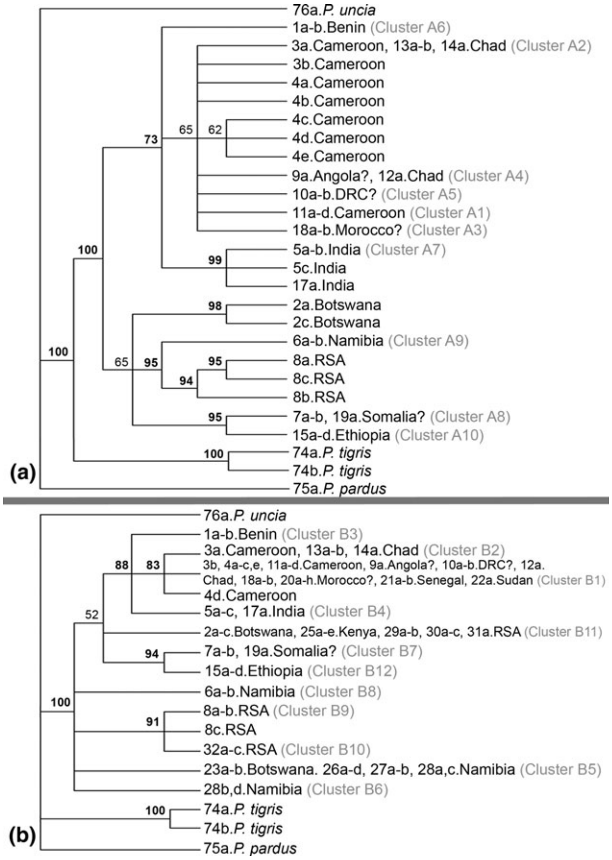


Figure 3 Phylogenetic trees resulting from maximum likelihood (ML) analysis of two sets of lion (*Panthera leo*) sequences: (a) cytochrome *b* + the control region, (b) cytochrome *b* alone. The numbers indicate the percentage for bootstrap support. Identical sequences were pooled. These clusters correspond to the clusters distinguished in the maximum parsimony analysis (Fig. 4). DRC, Democratic Republic of the Congo; RSA, Republic of South Africa.

it is very likely that the samples from Kenya and Uganda would cluster with the samples from Somalia and Ethiopia. The Guinean sample shows two point mutations that are not present in any of the other sequences and one point mutation they only share with the Benin samples. Based on the rest of the sequence, Guinea is likely to be positioned close to samples from Benin and Cameroon. In all three cases the partial sequences seem to be related to sequences of close or neighbouring countries.

Isolation by distance

A Mantel test and a linear regression analysis were performed for matrix correlation between genetic and geographic distances for each set of sequences (see Appendix S3). The R^2 value is the highest for the analysis of cytochrome *b* + the control region, 0.349. For cytochrome *b* alone and the control region (not shown), the R^2 values were 0.311 and 0.150, respectively. All these values are highly significant (*F*-test, $P < 0.0001$).

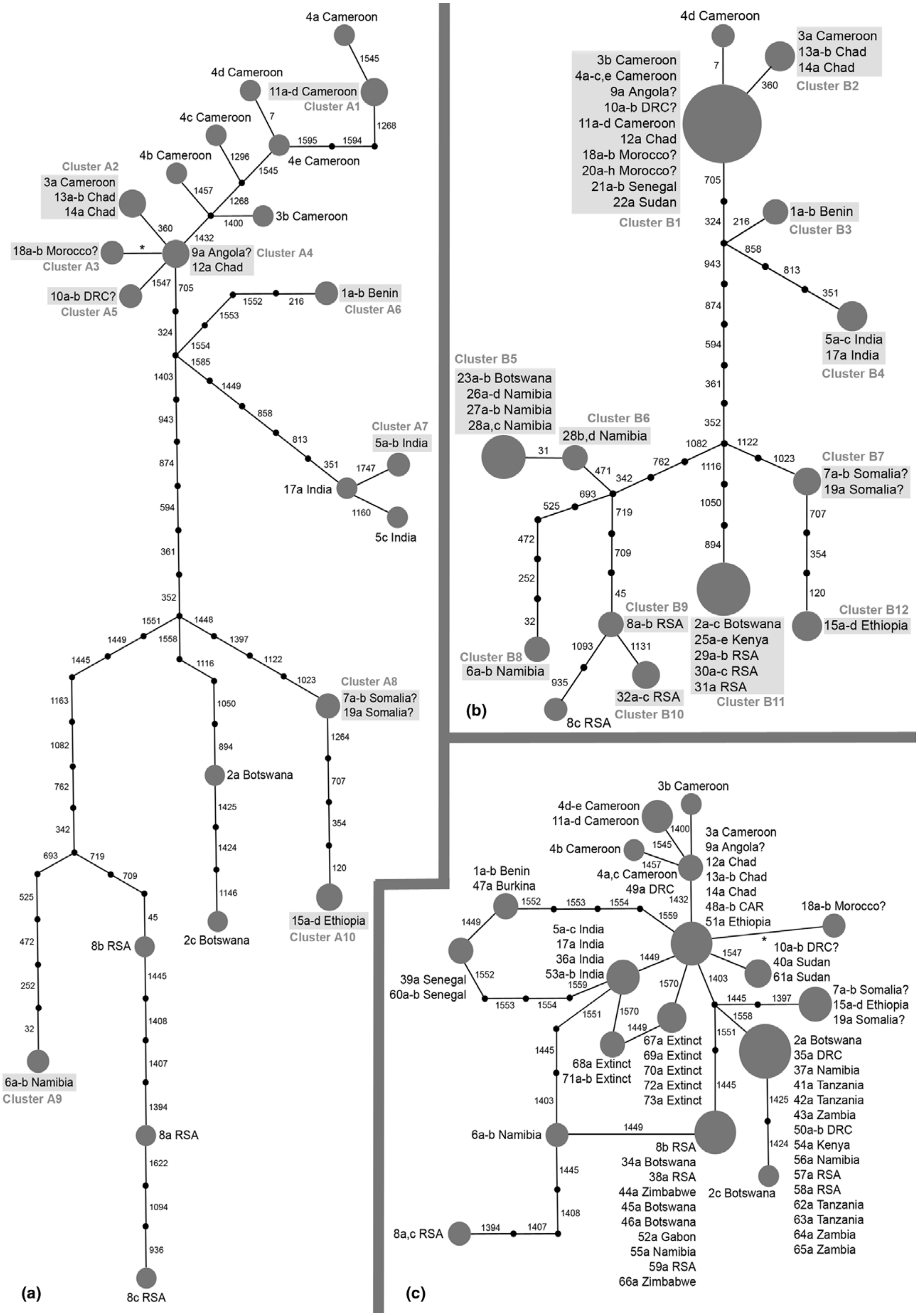
DISCUSSION

In this study, the divergence of mitochondrial sequences of the cytochrome *b* gene, tRNAThr, tRNAPro and the left domain of the control region was assessed in a large number of individual lions from different populations. The analyses consistently show similar patterns when using diverse algorithms. In general, samples from neighbouring countries cluster together and there is a distinction between West and Central Africa, and southern and East Africa.

This can partially be explained by the unique climatological history of western Africa, leading to a dichotomy as has been witnessed in other African mammals (Arctander *et al.*, 1999; Brown & Houlden, 2000; Freeman *et al.*, 2001; Nersting & Arctander, 2001; Eggert *et al.*, 2002; Pitra *et al.*, 2002; Van Hooft *et al.*, 2002; Alpers *et al.*, 2004; Brown *et al.*, 2007). The low genetic diversity in and between the West and Central African lion populations indicates that they have a shorter evolutionary history than the more diverse southern and East African lions. We hypothesize that this is caused by regional extinction followed by recolonization. During the Late Pleistocene, 40–18 thousand years ago (ka), large parts of West and Central Africa were characterized by hyperarid conditions (Sarnthein, 1978; Dupont *et al.*, 2000; Gasse, 2000). The resulting lack of prey might have led to regional extinction of lions. This hypothesis is supported by several studies on large mammals, based on genetic research (Arctander *et al.*, 1999; Van Hooft *et al.*, 2002; Alpers *et al.*, 2004) and fossil data (Klein & Martin, 1984). This bottleneck in lion populations coincides with the well-known cheetah bottleneck (Menotti-Raymond & O'Brien, 1993; Driscoll *et al.*, 2002) and Late Pleistocene megafaunal extinctions that occurred over much of the globe (Cardillo & Lister, 2002; Barnosky *et al.*, 2004; Lyons *et al.*, 2004). More humid conditions 15–11 ka (Gasse, 2000) probably made recolonization of West and Central Africa possible.

Because of the strong relationship between West and Central African lions and Asiatic lions, it is likely that recolonization took place from refugia in close geographic proximity to India, which may have been located in the Middle East. Historical records suggest that there was a continuous Eurasian–North African lion population, which was distributed from Morocco through the Middle East to India (Blanford, 1876; Vogt & Specht, 1889; Flower & Lydekker, 1891). The extinction of the

Figure 4 Haplotype networks for each of the analysed sets of lion (*Panthera leo*) sequences: (a) cytochrome *b* + the control region, (b) cytochrome *b* alone, (c) the control region alone. The numbers indicate the location of each mutation. In (a) and (c) one of these mutations is indicated by *, representing the 80-bp insert found in two of the Moroccan samples. Clusters correspond to the pooled sequences used for maximum likelihood analysis (Fig. 3). DRC, Democratic Republic of the Congo; CAR, Central African Republic; RSA, Republic of South Africa.


4a Cameroon
1545
4d Cameroon
11a-d Cameroon
Cluster A1
1268
4c Cameroon
7
1595
1594
4e Cameroon
4b Cameroon
1296
1545
Cluster A2
3a Cameroon
13a-b Chad
14a Chad
1457
1268
1400
3b Cameroon
360
1432
18a-b Morocco?
Cluster A3
*
9a Angola?
12a Chad
Cluster A4
10a-b DRC?
Cluster A5
1547
705
1552
216
1a-b Benin
Cluster A6
1553
324
1554
1585
1403
1449
943
858
813
Cluster A7
5a-b India
351
1747
874
17a India
1160
5c India
594
361
352
1551
1448
1449
1558
1397
1445
1116
1122
1023
Cluster A8
7a-b Somalia?
19a Somalia?
1163
1050
1264
1082
894
2a Botswana
707
762
1425
354
342
1424
693
719
709
120
525
1146
45
2c Botswana
15a-d Ethiopia
Cluster A10
472
8b RSA
1445
252
1408
32
1407
6a-b Namibia
Cluster A9
1394
8a RSA
1622
1094
936
(a)
8c RSA
4d Cameroon
3a Cameroon
13a-b Chad
14a Chad
Cluster B2
7
360
3b Cameroon
4a-c,e Cameroon
9a Angola?
10a-b DRC?
11a-d Cameroon
12a Chad
18a-b Morocco?
20a-h Morocco?
21a-b Senegal
22a Sudan
Cluster B1
705
324
216
1a-b Benin
Cluster B3
858
943
813
351
874
5a-c India
17a India
Cluster B4
594
Cluster B5
23a-b Botswana
26a-d Namibia
27a-b Namibia
28a,c Namibia
361
Cluster B6
28b,d Namibia
352
1082
1122
31
762
1116
1023
Cluster B7
7a-b Somalia?
19a Somalia?
471
342
693
525
719
1050
707
472
709
894
354
252
45
120
Cluster B9
8a-b RSA
Cluster B12
15a-d Ethiopia
32
2a-c Botswana
25a-e Kenya
29a-b RSA
30a-c RSA
31a RSA
Cluster B11
Cluster B8
6a-b Namibia
1093
1131
935
32a-c RSA
Cluster B10
(b)
8c RSA
3b Cameroon
4d-e Cameroon
11a-d Cameroon
1400
3a Cameroon
9a Angola?
12a Chad
13a-b Chad
14a Chad
48a-b CAR
51a Ethiopia
1545
4b Cameroon
1457
4a,c Cameroon
49a DRC
1a-b Benin
47a Burkina
1552
1553
1554
1432
1559
18a-b Morocco?
1449
5a-c India
17a India
36a India
53a-b India
1449
*
1547
10a-b DRC?
40a Sudan
61a Sudan
39a Senegal
60a-b Senegal
1552
1559
1570
1403
1445
1397
7a-b Somalia?
15a-d Ethiopia
19a Somalia?
1553
1554
1551
1570
1558
1449
1551
67a Extinct
69a Extinct
70a Extinct
72a Extinct
73a Extinct
2a Botswana
35a DRC
37a Namibia
41a Tanzania
42a Tanzania
43a Zambia
50a-b DRC
54a Kenya
56a Namibia
57a RSA
58a RSA
62a Tanzania
63a Tanzania
64a Zambia
65a Zambia
1445
68a Extinct
71a-b Extinct
1445
1425
1403
1449
1424
6a-b Namibia
8b RSA
34a Botswana
38a RSA
44a Zimbabwe
45a Botswana
46a Botswana
52a Gabon
55a Namibia
59a RSA
66a Zimbabwe
1445
2c Botswana
1408
8a,c RSA
1394
1407
(c)

lion in Europe, Middle East and North Africa has effectively severed gene flow from Asian lions to Africa (Mazák, 1970).

A complementary argument for the observed pattern in lion genetic diversity is the location of current natural barriers such as the African rain forest and the Rift Valley (Pitra *et al.*, 2002; Burger *et al.*, 2004; Dubach *et al.*, 2005; Barnett *et al.*, 2006b), as already proposed by Barnett *et al.* (2006b), and the connective Sahel savanna belt, which sustains numerous lion populations (Bauer & Van Der Merwe, 2004).

The Ethiopian samples show dispersion in the analyses: no. 15 clusters with samples from East Africa in every cytochrome *b* and cytochrome *b* + control region analysis, and, to a lesser extent, also in the control region analyses; however, no. 51 shows a closer genetic relationship to samples from West and Central Africa. It is possible that no. 15 comes from a population east of the Rift Valley, while no. 51 was sampled west of the Rift Valley, and is therefore connected to the Sahelian belt. Samples from DRC and Botswana also show some dispersion, reflecting genetic diversity within these countries. Botswana no. 2 groups with Kenya and RSA, while Botswana no. 23 (Moremi GR) shows close genetic relationship with the Namibian samples. The same dichotomy has been described by Antunes *et al.* (2008).

Our results confirm that the lions that are thought to be of Moroccan origin share their haplotype mainly with Central African countries, which was already discussed by Barnett *et al.*, 2006b;. Angola (no. 9) was positioned in the West and Central Africa group in every analysis. The Angolan sample shows little genetic relationship to samples from neighbouring countries such as Namibia, Botswana, Zambia, Tanzania and Uganda. Earlier published articles that include pedigrees (Steinmetz *et al.*, 2006) show that there is no certainty about the purity of the maternal line of the Angolan lions that are currently held in European zoos. This also explains why a similar pattern was found with the Angolan sample analysed by Antunes *et al.* (2008).

The isolation-by-distance analysis resulted in a highly significant correlation between genetic and geographic distance. A better model would be developed if possible migration routes as opposed to linear distances were used. Unfortunately, these routes are difficult to assess and probably changed extensively during the last millennia. We think that the inclusion of the Indian samples does not lead to an abnormally high correlation, as these samples show relatively few genetic differences when compared with West and Central Africa, despite the distance. The data points derived from the Indian samples do not form a separate group in the isolation-by-distance analysis, even in the analyses that do not include intermediate extinct lion populations from North Africa and the Middle East. It is also debatable if a linear model gives the best fit for the observed correlation, since it is expected that the variable sites in a genetic region can become saturated.

In this study lions from West and Central African countries are well represented, while samples from these regions were rare in other studies (Dubach *et al.*, 2005; Barnett *et al.*, 2006a,b; Antunes *et al.*, 2008; Mazák, 2010). West African countries were included in two previous studies, but only part of the control region was analysed, and samples connecting West to Central Africa were absent (Barnett *et al.*, 2006a,b). In general, a pattern was found of two major clades, one being located west of the Rift Valley, and one confined to eastern and southern Africa (Barnett *et al.*, 2006b). In the same study it was concluded that sub-Saharan lions are basal amongst modern lions, being in line with the high genetic diversity we observe in southern and East Africa.

The data from Barnett *et al.* (2006b) seem to indicate that West African lions are more closely related to lions from southern and East Africa than they are to Central African lions. India falls between West and Central Africa, while one would expect West and Central Africa to be directly related. This pattern is less explicit after incorporation of these sequences to our data set.

Antunes *et al.* (2008) do not include any West or Central African countries. In the mitochondrial DNA analysis, samples from Angola, Morocco and Zimbabwe fall in one clade, close to the India clade. But all samples in this group were derived from captive individuals, and the Moroccan samples that were included are likely to contain a Central African haplotype as has previously been described by Barnett *et al.* (2006a). The purity of the Angolan lineage in the samples used by Antunes *et al.* (2008) is questionable, considering the pedigree of captive Angolan lions in European zoos (Steinmetz *et al.*, 2006). A similar explanation is hypothesized for the analysed Zimbabwean sample, which was also derived from a zoo. Sequences derived from wild-ranging Zimbabwean lions that were included in our study (control region) cluster with sequences from lions from neighbouring countries, and not with those from West and Central African lions.

In line with the pattern described by Dubach *et al.* (2005) we confirm the distinct position of populations west of the Rift Valley, which were represented in the study of Dubach *et al.* by two sequences from Uganda. The distinct position of some RSA populations is also supported. The cytochrome *b* haplotype networks (MP analysis) show that at least nine point mutations in the cytochrome *b* gene make up the difference between lions from Timbavati and those from other regions in RSA.

We also support the conclusions of Mazák (2010), where one sub-Saharan Africa cluster and one North-Africa/Asia cluster are distinguished. Due to low sample size for West and Central African lions in that study, their taxonomic and phylogenetic position remained largely unresolved. Our data show that lions from this region should be considered to be part of a cluster that also includes North Africa and India.

The risk of extinction is often underestimated, because all populations are considered to belong to a single (sub)species, and are managed as such. Management policies that are based on taxonomic divisions that insufficiently reflect genetic lineages within the taxon may lead to the disappearance of distinct lineages within the species. Therefore it is important to focus on conservation strategies at a different scale, such as ESUs or MUs (Moritz, 1994). These provide a rational basis for prioritizing populations for conservation.

In view of our results, we argue that the existing taxonomy, with the African and Asiatic lion as the only subspecies, does not sufficiently reflect the genetic diversity of this species. Several clades in southern and East Africa show more variety in the studied genetic areas and show less relatedness to the West and Central African lions than to the Asiatic lion. Numerous subspecies are recognized in other African mammals which show this dichotomy (Arctander *et al.*, 1999; Brown & Houlden, 2000; Freeman *et al.*, 2001; Nersting & Arctander, 2001; Eggert *et al.*, 2002; Pitra *et al.*, 2002; Van Hooft *et al.*, 2002; Alpers *et al.*, 2004; Brown *et al.*, 2007).

CONCLUSIONS

In this study, 126 lion sequences were analysed using a number of phylogenetic approaches. The consistent pattern that emerged shows a clear distinction between West and Central African lions (including India) on the one hand, and southern and East African lions on the other. This pattern is most likely to be explained by the climatological history of western Africa and current environmental connections and barriers to lion dispersal. The hyperarid conditions during Holocene glacial periods may have led to the regional extinction of the lion in West and Central Africa, followed by subsequent recolonization from refugia in the Middle East. This would explain why West and Central African lions seem to be closely related to Indian lions, and why they show relatively little genetic diversity. This may indicate that this is an evolutionarily young branch, in comparison to the southern and East African lions, which show much more diversity.

Understanding the geographic pattern of genetic variation within species is critical for conservation management, not only for wild populations but also for breeding of captive stocks. Most zoos only distinguish between accepted subspecies, which do not necessarily reflect the overall genetic diversity of the species. Based on our results, existing management strategies should be reconsidered and West and Central African lions should not only be prioritized based on their current endangered situation, but also based on their genetic distinctness, their different level of genetic variation and their unique evolutionary history. We believe that the evidence presented in this study merits further consideration of the distinct taxonomic status of lions in West and Central Africa.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Appendix S1 Supplementary tables: overview of the lion samples analysed in this study (Table S1); overview of the lion sequences obtained from GenBank that were used to increase the sample size for the phylogenetic analyses (Table S2); primers used for PCR amplification and sequencing (Table S3); and overview of the variable sites of cytochrome *b*, tRNAThr, tRNAPro and the left domain of the control region (Table S4).

Appendix S2 Details of DNA isolation and sequence analysis.

Appendix S3 Graphs derived from the isolation-by-distance analysis, showing the relationship between geographic distance and genetic distance.

BIOSKETCH

Laura D. Bertola is affiliated to the Institute of Environmental Sciences in Leiden, The Netherlands. Her research focuses on genetic variation in, and relationships between, lion populations, both from an evolutionary and a conservation point of view. Her main regions of interest are West and Central Africa.

Author contributions: L.D.B. performed analyses and wrote the manuscript, W.F.v.H. performed analyses and designed the hypothesis, K.V. supervised analyses and wrote the manuscript, D.R.U.d.W. supervised phylogenetic analyses and wrote the manuscript, D.S.Y. and W.A.v.H. performed analyses, H.B., H.H.T.P., P.J.F., H.A.U.d.H. and H.L. assisted with useful advice, E.S. and P.N.T. supplied material and assisted with useful advice, H.H.d.I. supervised analyses, designed the hypothesis and wrote the manuscript. All authors contributed to writing the manuscript.

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