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RESEARCH ARTICLE

Genetic perspectives on "Lion Conservation Units" in Eastern and Southern Africa

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Abstract Current understanding of genetic variation in lions (*Panthera leo*) is inadequate to guide many management decisions necessary for conservation of the species. We studied sequence variation in the mitochondrial cytochrome-*b* (cyt-*b*) gene of 75 lions and nuclear variation at 11 microsatellite loci of 480 lions across 8 range states (Cameroon, Uganda, Kenya, Zambia, Zimbabwe, South Africa, Botswana, and Namibia) and 13 Lion Conservation Units (LCUs) plus two other unassigned sites (Cameroon and Zimbabwe). A total of 11 cyt-*b* haplotypes were found, whose variation follows an isolation-by-distance model. In combination with previously known sequences, the haplotypes document the close relationship, derived position, and limited variability of Asian and West and Central African lions relative to other extant lions. Both phylogenetic analyses and substitution networks identify two clades in Eastern and Southern Africa—one restricted to Namibia and South Africa and the other more widespread across the region. However, these analyses are equivocal on which of these is closest to the ancestor of modern lions. Microsatellite analyses showed high levels of variation within and among populations, subdivision among most LCUs, and evidence of isolation by distance. While rates of gene flow are generally low, admixture among lions in northern Botswana, Caprivi Strip (Namibia) and Zambia is apparent from STRUCTURE analyses. Conservation management plans should incorporate information on genetic variability and gene flow in delimiting management units and in guiding translocations of lions to minimize inbreeding and to control problem animals.

Keywords *Panthera leo* · Lion · Cytochrome-*b* · Haplotype · Microsatellite · Gene flow · Isolation-by-distance

Introduction

Historically, the lion (*Panthera leo*) was distributed over much of Africa, the Middle East, and southwest Asia. Its range extended from the Cape of Good Hope to the Mediterranean, from Senegal to Somalia, and from Greece and Yemen to central India (Barnett et al. 2009). Range collapse from persecution by humans has been rapid and continual. Lions were extirpated from the Cape Region of South Africa in the mid-19th century, from Turkey, Syria, Tunisia, and Algeria by the end of the 19th century, and from Morocco, Pakistan, and Iran between 1922 and 1942 (Guggisberg 1961). Today, only a single population remains in Asia, in the Gir Forest of Gujarat, India. In Africa, lions are mainly restricted to larger parks, reserves, and the remaining wilderness areas in savannas, covering

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no more than 20–25 % of their historic range (IUCN SSC Cat Specialist Group 2006b; Riggio et al. 2012).

Range collapse has been accompanied by plummeting lion numbers. Reliable population estimates for elusive, often nocturnal predators are notoriously difficult, but a variety of estimates converge at roughly 32,000 (Riggio et al. 2012). Rates of decline are alarming, as the number of African lions has fallen 30 % over the past two decades (three lion generations) and perhaps by 48.5 % since 1980 (IUCN 2012). The lion is considered regionally endangered in West Africa, with 480–525 individuals remaining in the entire region (Riggio et al. 2012). Only 7 range states—three in Eastern Africa and four in Southern Africa—are thought to support as many as 1,000 lions each: Ethiopia, Kenya, Tanzania, Zambia, Zimbabwe, Botswana, and South Africa (IUCN SSC Cat Specialist Group 2006b).

Conflicts with people are overwhelmingly responsible for the range and population collapse of lions. Retaliatory killing in response to attacks on livestock and people (Patterson et al. 2004; Packer et al. 2005), native prey depletion through overgrazing and bushmeat harvest (Burton et al. 2011), and loss and fragmentation of habitat (Hunter et al. 2007; Kiffner et al. 2009; Riggio et al. 2012) are the most widely acknowledged causes of lion endangerment (IUCN SSC Cat Specialist Group 2006b; pers. obs.).

Lion conservation units (LCU)

Conserving large predators with extensive, multinational ranges is an international enterprise requiring assessment, coordination, and prioritization. The Cat Specialist group of the World Conservation Union examined lion distribution and status in both West and Central Africa (2006a) and in Eastern and Southern Africa (2006b), modeling their approach on the successful hemispheric strategy for conserving jaguars, *Panthera onca* (Sanderson et al. 2002). They identified 66 areas in Eastern and Southern Africa that cover 61 % of the lion's known and possible range in the region. LCUs were not restricted to or based on protected areas, but many are associated with parks and reserves. Experts assessed viability, limiting factors, and threats for each of the LCUs, which are seen as management units for preserving lions in situ (IUCN SSC Cat Specialist Group 2006b). This region of Africa is thought to support the vast majority of extant lions (Bauer and Van Der Merwe 2004), including all 10 "lion strongholds" (Riggio et al. 2012).

Endangerment in African lions is typically direct, via survivorship, but theoretically there are genetic risks too (Björklund 2003), best exemplified by lions of the Ngorongoro Crater (Packer et al. 1991) and Hluhluwe-Umfolozi (Anderson 1981; Maddock et al. 1996). Range collapse often produces small population size and, despite the lion's impressive capacity for dispersal, a loss of genetic variability (O'Brien et al. 1987). Moreover, fencing parks and reserves to mitigate animal-human conflict interrupts natural patterns of migration and gene flow (Wildt et al. 1987; Hayward and Kerley 2009; Trinkel et al. 2011), which also reduces variability. Reduced genetic variation can have reproductive and other fitness consequences in lions and other large mammals. To safeguard species, it is critical to understand natural corridors to dispersal and gene flow and to identify instances where genetic continuity has been interrupted. Infectious diseases are also likely to move along these corridors.

Genetics and population structure of lions

Although lions are frequently thought to have ranged across Africa, Eurasia, and Beringia during the late Pleistocene, studies by Burger et al. (2004) on mitochondrial cyt-*b* sequences showed that Pleistocene lions belong to a different lineage than extant populations (see also Hemmer 1974). Corrected genetic distances for cyt-*b* of 5–6 % separate Pleistocene lions from modern samples, while the latter typically differ by ca. 1 %. Based on variation in ~358 bp of mt-DNA (hypervariable region 1 and ATPase subunit 8), Barnett et al. (2009) found evidence for three species of lions: modern (African plus Asian) lions, the cave lion of Pleistocene Eurasia and Beringia (both Asia and North America), and the American lion (from late-Pleistocene samples collected in unglaciated regions of southern North America). That study also identified a major dichotomy among modern lions, between samples collected over the lion's historic range in Asia (India, Iran) and North, West, and Central Africa (Barbary, Senegal and Sudan) from those collected in Eastern and Southern Africa (see also Barnett et al. 2006a, 2006b). The genetic distinction of lions in Eastern and Southern Africa from those elsewhere in the species' range was confirmed by studies of Antunes et al. (2008) on mt-DNA (12S and 16S mt-rRNA) and nuclear (*Sry*) sequence and microsatellite variation. Bertola et al. (2011) corroborated that lions in Eastern and Southern Africa were more variable in cyt-*b* and control region haplotypes and were genetically distinct. Regional differences in mt-DNA (cyt-*b* and NADH-5) between lions in Eastern and Southwestern Africa had been previously noted by Dubach et al. (2005).

To date, there has been no systematic attempt to characterize genetically the lions remaining in Africa's reserve systems. Prior appraisals of regional differentiation offer little guidance to various management decisions made continually in lion range states: where should problem animals be translocated? What is the proper source for restocking wild areas or for animals being introduced to

fenced reserves? There is also theoretical interest in the degree to which a large, widespread predator shows regional differentiation and evidence of local adaptation. Although historical interest in this topic has focused on morphological and taxonomic differentiation, current interest extends to the susceptibility and connectedness of these populations to various zoonoses. Accordingly, our goals were to characterize the genetic underpinnings of lion populations in Eastern and Southern Africa, in the heart of their remaining range and over many of Africa's most iconic parks and reserves.

Materials and methods

Sample collection

Samples (blood, biopsy dart, or dried tissue) were collected from as many sites as possible in Eastern and Southern Africa; access to samples and permitting regulations made for a very uneven distribution of sampling effort (See Supplemental Table 1 for site locations). Each lion was sampled by one of the following methods: (1) blood obtained during live-capture, (2) fresh tissue collected via biopsy darting, or (3) a 1 × 1 cm snip of salted and air-dried skin obtained from sport-hunted trophies and lions killed by the wildlife authorities as "problem" animals. Fresh samples were either frozen in liquid nitrogen or placed in an equal volume of long-term storage buffer (LTSB: 100 mM Tris, 100 mM EDTA, 2 %SDS, pH 8.0) for storage and shipment.

Obtaining blood samples from lions was done by first anesthetizing them utilizing a variety of techniques to allow for safe and efficient use of immobilization darts using a Pneudart Projector model 389. Lions were located by tracking, using "call-up" vocalizations, at kills, and using local knowledge of wildlife guides. Once located and a focal animal selected, lions were approached to within 50 meters in a 4WD vehicle. A 2.0 ml aluminum Pneu-Dart projectile with a 1¼″ barbed needle was filled using a combination of teletamine/zolazepam (Telazol) and xylazine (250/200 mg, respectively for males and 167/133 mg, respectively for females) and shot into the deep muscle layers of the shoulder or rump, depending on the animal's orientation. After immobilization and stabilizing anesthesia, animals were examined and biological samples collected. Blood was taken from one of two venapuncture sites: males were bled from the medial femoral vein using the BD Vaccutainer System with 1½″ 18 ga. needles, a plastic collection sleeve and the BD vaccutainer tubes. Females were collected utilizing the same method and equipment, but preferentially from the jugular vein. Blood was drawn into multiple tubes for a variety of testing. Genetic samples utilized 3.0 ml whole blood in either EDTA or heparin mixed 1:1 with LTSB. To increase yield of nucleated cells, we centrifuged 8.0 mls of whole blood at 8,000 rpm for 12 min and then, using sterile plastic, disposable pipettes, harvested the buffy coat and placed it 1:1 in cryovials with LTSB.

Biopsy darts were custom-built for African lions by Pneu-Dart (Williamsport, PA, USA). Biopsy darts were based on a 3 cc drug dart body and were 0.50 caliber, 4½″ in length and incorporated a 1/8″ wide × 3/8″ long cutting ferrule tip which housed a barbed pin. Darts were propelled via a Pneu-Dart Model 389 projector powered by green 0.22 caliber CCI charges. Lions were darted in the shoulder or thigh muscle at distances from 15 to 45 meters. On impact, the biopsy dart's ferrule tip cut and extracted a tiny (ca. 1/8″ × 3/16″ − 5/15″) core of tissue before falling off the animal. Once the dart was recovered, the cutting ferrule was unscrewed from the dart body to reveal the barbed pin containing the tissue sample that was subsequently transferred into a cryovial containing either 95 % EtOH or Tris buffer.

We assembled samples from 480 individual lions residing in eight countries (Cameroon, Uganda, Kenya, Zambia, Zimbabwe, South Africa, Botswana, and Namibia), including 13 LCUs: 15, 20, 31, 32, 33, 36, 38, 44, 45, 46, 48, 49, 50 (sensu IUCN SSC Cat Specialist Group 2006b; see Table 1). In most cases, samples were assigned to the encompassing or a nearby LCU, however a few samples known only as having originated in Botswana and Uganda could not be assigned to LCUs, nor could captives or geo-referenced samples from undesignated areas in Zimbabwe. The Cameroon samples came from an LCU (Bénoué complex-Gashaka-Gumti) identified in the West and Central Africa strategy (IUCN SSC Cat Specialist Group 2006a).

DNA extraction and amplification

DNA extraction and amplification of mitochondrial cyt-b, primer sequences, and PCR conditions followed descriptions in Dubach et al. (2005). We amplified 11 microsatellite loci that map to different chromosomes in the domestic cat (*Felis catus*; lions share the same diploid number, 2n = 38): FCA14, FCA26, FCA30, FCA45, FCA77, FCA94, FCA96, FCA126, FCA132, FCA187, and FCA191 (Menotti-Raymond et al. 1999). Amplification was carried out using 40–90 ng genomic DNA in a 12.5 ul reaction volume containing 0.5 U TAQ polymerase (Promega, Madison, WI), 0.2 mM dNTPs, 1X reaction buffer, 4 pmol each primer, and 1.5 mM $MgCl_2$ (1.1 mM $MgCl_2$ was used for FCA30 and FCA191). The following PCR conditions were used for all loci: initial denaturation at 94 °C for 5 min, 35 cycles (94 °C for 30 s; 48.0–65.0 °C for 45 s; 72 °C for 45 s), followed by a 10 min dwell at

Table 1 Lion Conservation Units (LCUs) evaluated in this study

LCU	Name	Area (km^2)	Category	Percent gazetted	Population size	Trend
15	Laikipia-Samburu	18,910	I	<25	350	Stable
20	Maasai Steppe	125,050	I	25–50	>1,000	Declining
31	North Luangwa	15,166	I	>50	100–250	Stable
32	South Luangwa	18,012	I	>50	250–500	Stable
33	Petauke Corridor	13,681	III	>50	<50	Stable
36	Kafue	39,964	I	>50	250–500	Stable
38	Sioma Ngwezi	4,249	III	>50	<50	Uncertain
44	Etosha-Kunene	48,889	I	>50	315–595	Increasing
45	Khaudum-Caprivi	23,522	II	25–50	100–200	Stable
46	Okavango-Hwange	92,323	I	>50	2,300	Stable
48	Kgalagadi	149,121	I	>50	500–1,000	Stable
49	Greater Limpopo	60,957	I	>50	>2,000	Increasing
50	Hluhluwe-Umfolozi	989	II	>50	80	Stable

Data mainly from IUCN SSC Cat Specialist Group (2006b). LCU categories are: I, viable; II, potentially viable; or III, significant but of doubtful viability. 'Percent gazetted' refers to the proportion under any form of legal protection (national park or reserve, hunting concession, or conservancy). Areal measurements obtained from metadata used in preparing the maps (www.panthera.org)

LCU number (local populations sampled) *15* (Aberdares), *20* (Tsavo West, Tsavo East), *31, 32, 33, 36* (various), *38* (Sioma Ngwezi), *44* (Etosha, Kunene), *45* (Botswana Gate, Kwando, Bushmanland, Caprivi Strip) *46* (various), *48* (Kgalagadi), *49* (Fannie Roberts, Sabi Sands), *50* (Umfolozi)

72 °C in a Bio-rad iCycler (Bio-rad Laboratories, Hercules, CA). Annealing temperature was 48 °C for FCA132; 53 °C for FCA26 and FCA45; 58 °C for FCA14 and FCA30; 65 °C for FCA187; and 50 °C for the remaining five loci. Gradient analysis on the iCycler was used to determine the optimal annealing temperature and magnesium concentration for each primer. All forward primers were labeled with Well-Red dyes for electrophoresis on a Beckman/Coulter CEQ^{TM}8000XL DNA Analysis Genotyping System (Beckman Coulter, Fullerton, CA) and fragments were sized using System Software version 8.0.

Mitochondrial data analysis

Complete cytochrome-*b* sequences for 75 lions and two outgroups (GenBank accession numbers *P. tigris* KC495059 and *P. pardus* EF551002) were obtained and aligned by eye using MacDNASIS v3.2 (Hitachi Software Engineering America, Ltd.), then submitted to Mega 5.0 (Tamura et al. 2011). The lions exhibited 11 different haplotypes (KC495048-KC495058; see Supplemental Table 1).

We obtained 42 additional cyt-*b* sequences for Recent *Panthera leo* from GenBank (GU131164-GU131185, AY781195-AY781210, DQ018993-DQ018996), representing nine additional haplotypes, many of them produced by Bertola et al. (2011). Samples obtained in Morocco, from the Rabat Zoo and supposedly descended from Barbary lions, have a complicated history that includes the possibility that they originated in West or Central Africa instead (Yamaguchi and Haddane 2002; Barnett et al. 2006a). The subroutine CLUSTALW was implemented using default parameters to insure correct alignment; no gaps, insertions or deletions were encountered. Neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) approaches were used to recover hierarchical relationships among the 20 unique lion sequences; transitions and transversions at all three codon positions were examined as were amino acid substitutions. Because ML offers better performance in the face of substitution bias, only those results are presented below. MEGA 5.0 and the Akaike Information Criterion statistics it generated were used to assess models of nucleotide substitution for use in the ML analysis. The stability of the resulting tree was assessed with 10,000 bootstrap replicates.

A haplotype substitution network was constructed using the median-joining option of Network 4.6.1.0 (Bandelt et al. 1999). All 117 lions (75 from our analyses, 42 from GenBank) and two outgroups were used in the network analyses. Following convention, network nodes are either sequences from the dataset or median vectors, and the links are nucleotide differences. A median vector is a hypothetical sequence required by maximum parsimony to connect existing sequences within the network.

Coordinates for all localities were determined with the Geographic Names Server (http://geonames.nga.mil/ggmagaz/) and plotted using ArcMap 9.2 (www.esri.com). The layer for Lion Conservation Units was developed and kindly provided by Panthera (www.panthera.org).

Microsatellite data analysis

Genotype accuracy was verified by random reamplification of approximately 10 % of each population for each locus and sizing several of the same PCR reactions at each capillary change. In addition, allele size and consistency of allele binning over time was monitored graphically by entering the fragment size for each reaction into a Microsoft Excel spreadsheet for each locus.

To measure within-population differentiation, we used Microsatellite Toolkit v 3.1 (Park 2001) to calculate allele frequency, observed and expected heterozygosity and to convert data files for other applications. Observed and expected heterozygosity over all loci per population were also calculated using GENEPOP version 4.0.10 (Raymond and Rousset 1995). Analyses were conducted on populations organized by haplotype groups, in the case of mitochondrial sequences, and by Lion Conservation Units for both sequence and microsatellite data.

Fisher's exact tests of Hardy–Weinberg Equilibrium (HWE) were run across all loci within each population using the Markov chain method (Guo and Thompson 1992) using GENEPOP version 4.0.10 (Raymond and Rousset 1995). Linkage disequilibrium was assessed using GENEPOP (1,000 dememorizations, 100 batches, and 1,000 iterations per batch) with Bonferroni correction ($\alpha = 0.05$; $P = 0.00454$; Rice 1989) to determine if alleles at different loci were randomly assorting (Frankham et al. 2002). FSTAT (Goudet 2001) was used to calculate allelic richness per locus and over all loci per sample via a rarefaction method and to estimate F_{IS} for each population.

To measure genetic differentiation among populations, FSTAT was used to calculate F_{ST}, and the g-test (Goudet 2001) was used to determine the significance of F_{ST} deviations from zero. Significance was adjusted for multiple comparisons using Bonferroni correction (Rice 1989; Sokal and Rohlf 1995). ARLEQUIN version 3.1 (Excoffier et al. 2005) was used to determine significance of genetic variation between populations and geographic groups of neighboring populations using a hierarchical Analysis of Molecular Variation (AMOVA) with 1,000 permutations. Data for both analyses were grouped by mitochondrial clades (three [two arrangements] and five groups) and by LCU (four, six, and eight groups [two arrangements]) to determine if mitochondrial haplotype variation and nuclear microsatellite variation followed similar patterns.

The number of migrants between all pairs of sample sites (Nm) was estimated using the private allele method (Barton and Slatkin 1986) in GENEPOP (Raymond and Rousset 1995). Isolation by distance was analyzed using the program ISOLDE in GENEPOP (Raymond and Rousset 1995) with 1,000 permutations. For these Mantel tests, half-matrices of uncorrected "*P*" values for mitochondrial haplotype groups and geographic distances between group centroids was analyzed. Half-matrices of microsatellite $F_{ST}/(1\text{-}F_{ST})$ values for LCU groups and geographic distances between centroids as ln(distance) were also analyzed. Both sets of data were tested using a Mantel Nonparametric Test Calculator Shareware V. 2.0 with 1,000 permutations implemented in the program, Mantelese (Liedloff 1999) without transformation. Samples from Uganda and LCU 38 were not used in the microsatellite analysis because an F_{ST} value cannot be calculated for a single sample and the provenance of the former was uncertain.

Spearman rank correlations and simple linear regressions were used to assess relationships between pairs of demographic, geographic, and genetic variables across the LCUs. A $P < 0.05$ value was deemed significant. Analyses were implemented in STATISTICA v. 7.1 (StatSoft Inc 2005).

STRUCTURE version 2.2 (Pritchard et al. 2000, 2003) was used to determine the number of incipient subpopulations (K) using the data organized by haplotype groups ($K = 10$; haplotypes j and k were combined because each was represented by a single individual) and by LCU and other regional groups ($K = 15$). This clustering analysis assigns probabilities of each individual's genotype to predetermined numbers of subpopulations; ten repetitions for each value of $K = 1$ to 15 for the LCU arrangements were explored. K was estimated using Markov Chain Monte Carlo (MCMC) with a burn-in of 100,000 repetitions followed by 500,000 iterations. As significant gene flow was expected, the admixture model was used. The true number of clusters was determined by calculating the change in K (ΔK) as described in Evanno et al. (2005). No prior information regarding clustering was used for this test.

Results

Mitochondrial sequences

Complete cytochrome-*b* sequence (1,140 bp) was obtained for 77 individuals, including 75 lions and two *Panthera* species used to root the tree. Overall, we identified 11 haplotypes (*a–k*, Fig. 2), 15 amino acid substitutions, and 9 parsimony-informative substitutions within these sequences. One cluster of haplotypes (*a–d, f*) is widespread, found from Kenya and Uganda south to Namibia, while the other (haplotypes *g–k*) is restricted to Southern Africa. When these haplotypes are combined in analyses with previously documented ones (*l–t*), they represent much of the mitochondrial diversity identified within extant lions. The maximum-likelihood phylogeny (Fig. 1) recovers lions as

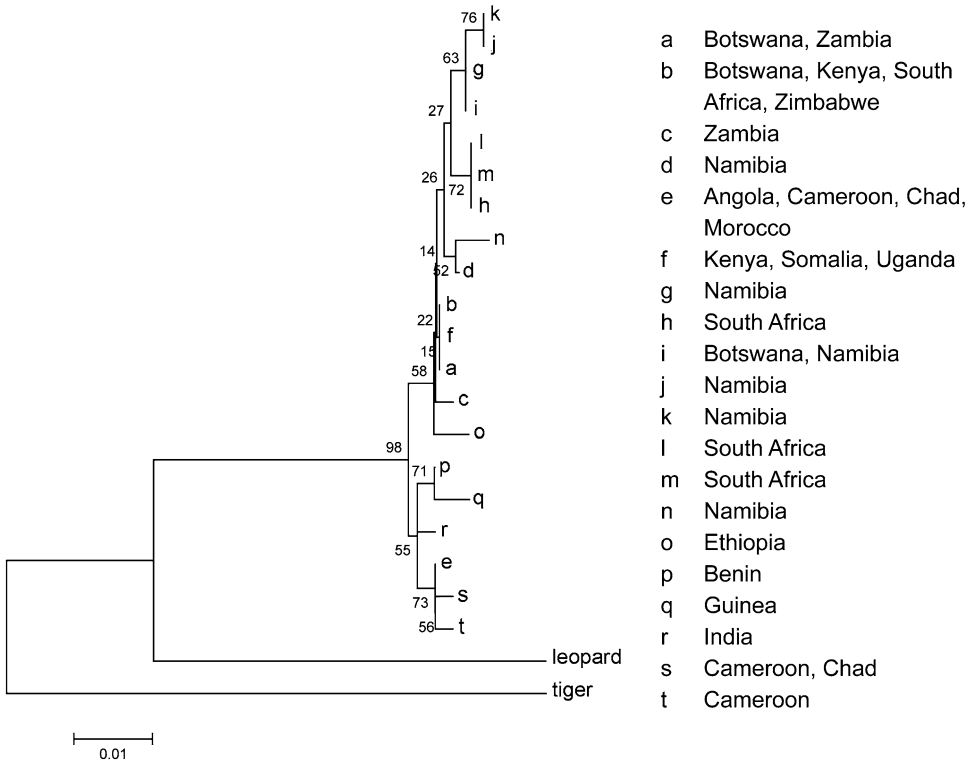

Fig. 1 Maximum-likelihood bootstrap consensus tree (based on 10,000 replicates) for 20 lion haplotypes (*a–t*), using tiger and leopard as outgroups. *Branches* corresponding to partitions represented in less than half the bootstraps are collapsed, and the percentage of replicate trees in which the associated taxa clustered together is shown next to the *branches*. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 453 positions in the final dataset

monophyletic in a sister-group relationship to leopard. Lions are grouped into two well-supported (bootstrap >50 %) clusters: (1) lions from North, West and Central Africa plus India (6 haplotypes), and (2) lions from Eastern and Southern Africa (14 haplotypes). The former group is clearly (bootstrap >50 %) subdivided into three clusters: the Gir Forest sample (*r*), North and Central Africa (*e–s–t*), and West Africa (*p–q*). Lions from Eastern and Southern Africa are more diverse but more weakly clustered. There is a weak partition between Eastern and Southwestern lion populations, with lions from Botswana appearing in both clusters.

Historical relationships of the mitochondrial haplotypes are more clearly evident in the substitution network (Fig. 2). The network is rooted by leopard and tiger, lying 35 and 48 substitutions distant, respectively; each connects to the lion network via a different median vector, and each lies adjacent to a different cluster of lion haplotypes. Haplotypes *g–n* stem from the median vector with tiger, and represent lion populations from Southern Africa (Namibia, Botswana and South Africa). Haplotypes *a–d, f*, and *o* lie adjacent to the leopard vector and span populations throughout Eastern and much of Southern Africa (from Ethiopia and Somalia to Botswana and South Africa). The cluster of haplotypes from India and North, West and Central Africa is recovered as highly derived; *e, s, t, p, q* and *r* all lie within 5 substitutions from the median vector representing their hypothetical common ancestor (Fig. 2).

Pairwise genetic distances among haplotypes, assessed as uncorrected *P*-values, were highest overall for West African *e*, which differed most from South African *h* (LCU 49, Sabi Sands; $P = 0.123$) and least from East African *f* (Uganda, central Kenya, and Somalia; $P = 0.079$). The *h* haplotype also differs greatly ($P = 0.088$) from the *b* haplotype also found in LCU 49 and 50 and is closest ($P = 0.044$) to *g* in northern Namibia (LCU 44). In similar fashion, lions with *d* (LCU 48, Kalahari) were distant from other southwestern populations ($P = 0.09$–0.10 with *j* and *k* in neighboring LCU 44) and much closer genetically ($P = 0.026$) to *b* samples, despite their distribution in Kenya, Zimbabwe, and South Africa.

Microsatellite genotypes

A total of 480 lions were genotyped for 11 microsatellite loci. Deviation from Hardy-Weinburg equilibrium showed no heterozygote excess in any population or locus. After Bonferroni correction, a significant heterozygote deficit was found for two LCUs, 44 and 45, over four loci. Known first-order family groups in Etosha were included in the analysis, which likely bias these results (Marshall et al. 1998; Lyke 2008). When known offspring were removed, only FCA14 showed a significant heterozygote deficit.

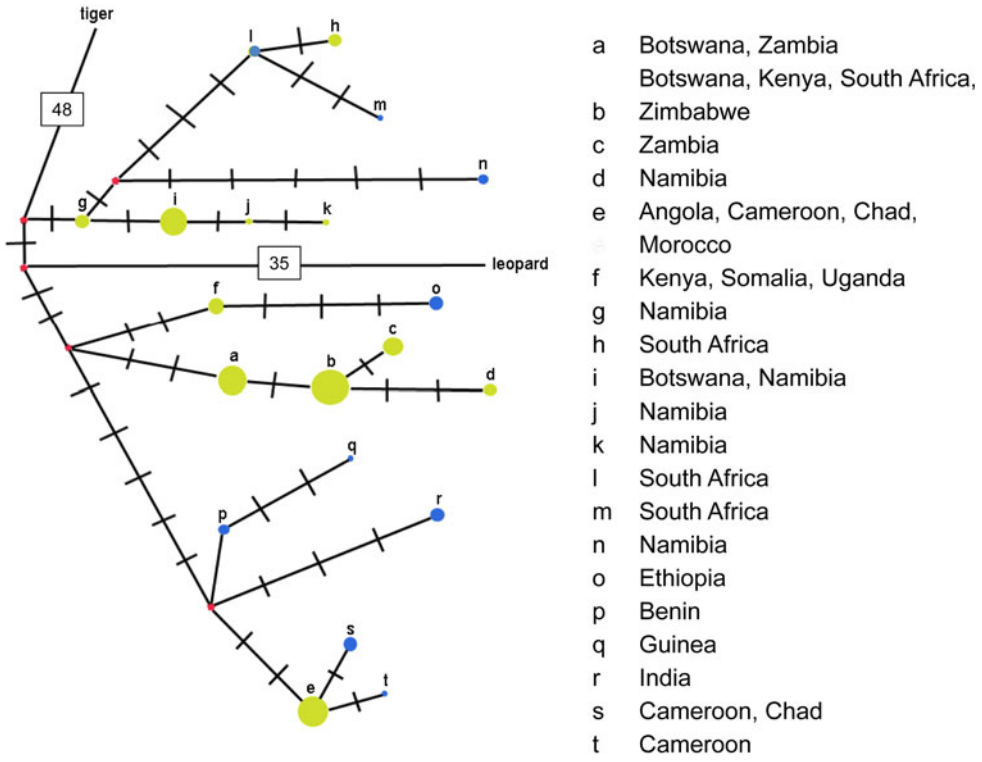

Fig. 2 A substitution network for 120 cyt-*b* gene sequences produced by NETWORK 4.6.1.0. Node *color* corresponds to haplotype source (*yellow*, generated by this study; *blue*, obtained by others; *red*, median vectors or hypothetical states inferred by maximum parsimony), and node size corresponds to haplotype frequency. Each *hatch mark* represents a unique base-pair substitution. Separate nodes connecting this network with leopard and tiger represent unresolved uncertainties at the root of this evolutionary network. See text for discussion. (Color figure online)

There were no deficiencies observed for any locus across all populations.

Significant linkage disequilibrium was observed for several pairs of loci in 9 of 15 groups. However, no pair of loci was significantly linked over all groups, and all loci used here map to different chromosomes in the domestic cat, *Felis catus* (Menotti-Raymond et al. 1999). Consequently, we retained all loci in subsequent analyses.

Genetic variation within LCUs: Heterozygosity levels range from 33 % for lions in LCU 48 (Kgalagadi) to 71 % for lions in LCU 20 (Maasai Steppe; Table 2). Observed and expected heterozygosity levels differed significantly in LCUs 44 and 45. When sampling areas within each (Kunene and Etosha in LCU 44 and Chobe Gate, Kwando, Caprivi Strip, and Bushmanland in LCU 45) were analyzed separately, only the Etosha population showed significant differences. LCU 44 and 45 also had the largest sample sizes (Table 2) and the highest number of alleles and private alleles before adjusting for sample size. After adjustment for sample size, the average number of alleles is similar across LCUs, with LCU 50 (Hluhluwe-Umfolozi) showing the lowest value. Private alleles were observed in all units except LCUs 33, 36 and 38 (Petauke, Kafue, and Sioma Ngwezi, all in Zambia).

Correlating genetic variability with attributes of the LCUs yields few predictable relationships (Table 3). In fact, there is an unexpected *positive* relationship between

Table 2 Analyses of 11 cyt-*b* haplotypes and 11 microsatellite loci data for 13 LCUs plus three other sampling areas (U, Uganda; W, Cameroon; Z, Zimbabwe)

LCU	H	N	A	Ar	Ap	Ho	He	Signif	F_{IS}
15	1	4	3.27	1.64	2	0.614	0.636	ns	0.041
20	1	17	6	1.73	2	0.709	0.728	ns	0.027
31	1	18	5.3	1.73	1	0.702	0.73	ns	0.039
32	1	9	4.7	1.7	1	0.697	0.699	ns	0.004
33	1	6	4.1	1.72	0	0.694	0.721	ns	0.041
36	2	28	5.6	1.68	0	0.685	0.677	ns	−0.011
38	1	1	1.5	1.55	0	0.545	0.545	ns	0
44	4	190	6	1.64	14	0.583	0.641	***	0.092
45	2	76	6.4	1.7	9	0.679	0.704	***	0.035
46	1	24	4.8	1.63	1	0.617	0.626	ns	0.014
48	1	3	2	1.41	6	0.333	0.412	ns	0.228
49	2	12	5	1.73	2	0.682	0.725	ns	0.063
50	1	32	3.1	1.39	1	0.443	0.388	ns	−0.147
U	1	1	1.82	1.64	0	0.818	0.818	ns	0
W	1	5	2.8	1.58	4	0.632	0.582	ns	−0.099
Z		54	5.1	1.66	3	0.633	0.661	ns	0.042

Tabulations include the number of *H* haplotypes observed, *N* sample size, *A* average number of alleles, *Ar* allelic richness, *Ap* number of private alleles, *Ho* observed heterozygosity , *He* expected heterozygosity, *Signif* significance level for deviation from Hardy–Weinberg Equilibrium, *FIS* inbreeding coefficient

Significance: ns = $P > 0.05$, ***$P \leq 0.001$

Table 3 Spearman rank-correlation matrix among physical and genetic attributes of 13 LCUs in Eastern and Southern Africa

	LCU category (I, II, III)	Population size	Population trend	Area (in km^2)	Number of sequences	Number of haplotypes	Hapotypes per sequence	N	A	Ar	Ap	Ho	He
Population size	**−0.764**												
Trend	−0.157	0.266											
Area (in km^2)	**−0.637**	**0.887**	0.219										
Number of sequences	−0.077	0.352	0.193	0.432									
Number of haplotypes	−0.058	0.21	0.439	0.271	**0.803**								
Haplotypes per sample	−0.012	−0.299	0.217	−0.368	**−0.705**	−0.178							
N	0.121	0.138	0.01	0.071	**0.788**	**0.609**	−0.513						
A	−0.215	0.338	0.187	0.386	**0.855**	**0.651**	**−0.642**	**0.725**					
Ar	−0.29	0.083	0.108	0.077	0.22	0.054	−0.368	−0.033	0.512				
Ap	−0.199	0.434	0.276	0.566	0.383	0.416	−0.121	0.302	0.424	0.054			
Ho	−0.29	0.069	−0.061	0.005	0.195	−0.041	−0.407	0.049	0.526	**0.863**	−0.214		
He	−0.29	0.083	0.108	0.077	0.22	0.054	−0.368	−0.033	0.512	1	0.054	**0.863**	
F_{IS}	−0.377	0.287	0.208	0.462	0.084	0.189	0.134	−0.203	0.077	0.275	**0.654**	−0.104	0.275

Boldfaced entries indicate coefficients are significant at the $P < 0.05$ level

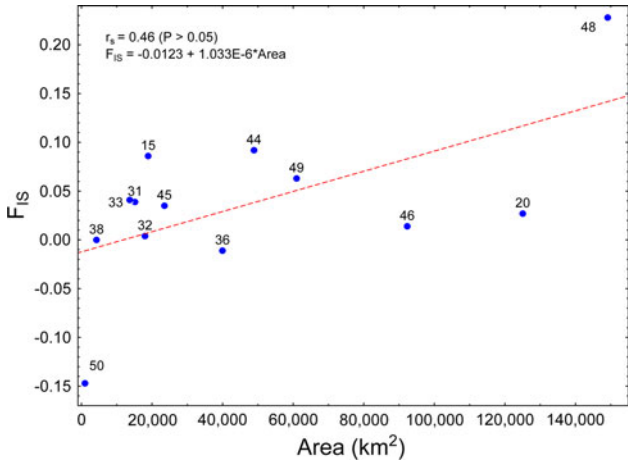

Fig. 3 Relationship between the inbreeding coefficient F_{IS} and LCU area. Points are labeled with LCU numbers. The points are loosely fitted by a line of the form $F_{IS} = -0.0123 + 1.033E - 6$*area

LCU area and F_{IS} ($r_s = 0.65$; $P < 0.05$). As shown by the scatter plot (Fig. 3), this relationship depends importantly on genetic estimates for two LCUs, Kgalagadi (48) and Hluhluwe-Umfolozi (50). The former was represented by only three individuals, while the latter was founded by introduced females and bottlenecked by management (Maddock et al. 1996).

Genetic variation among LCUs: We found significant genetic differentiation among all LCUs tested (Table 4). Virtually all pairwise comparisons were significant, especially for pairs belonging to different haplotype groups (i.e., Eastern vs. Southwestern populations). Lions from LCU 48 (Kgalagadi) differed significantly from lions in LCUs 44, 45 and 46 (Namibia, Botswana and Zimbabwe), but not from LCUs 31, 32, 33, 36, 20, and W lying much farther away (in Zambia, Kenya, and Cameroon). Finally, LCU 33 (Petauke Corridor in Zambia) only differed significantly from LCUs 44, 46, 50, and Z (in Namibia, Botswana, South Africa, and Zimbabwe), suggesting that a major genetic division crosses Zambia.

AMOVA partitions genetic variance among the microsatellites into three categories: within populations, among populations within groups, and among groups. Table 5 shows the variances for each level, grouping samples either by haplotype or by the geographic proximity of LCUs. Several different arrangements were assessed to identify groupings that minimized the among-population/within-group variance and maximized the among-group variation. Only partitions with the highest among-group percent variation for each classification are included. All levels of variation for both cladistic and geographic groupings were significant. When populations are combined into three regional haplotype groupings, the among-group variance explained was high ($F_{CT} = 6.02$; Table 5). However, when the three groupings followed haplotype clades as indicated in Fig. 2 (Haplotype clades: 1 = *a, b, c, d, f;* 2 = *g, h, i, j, k*; 3 = *e*), among-groups variance was lower ($F_{CT} = 4.45$; not shown). Grouping populations by LCUs yielded the highest among-group genetic variance ($F_{CT} = 12.14$). For the LCU arrangements, the higher the number of groups,

Table 4 Genetic differentiation among 12 lion conservation units (LCUs) and two other areas located throughout Africa

LCU	15	20	31	32	33	36	44	45	46	48	49	50	W
20	00.103*												
31	00.118*	0.050***											
32	00.154	0.072**	−0.005										
33	00.133	0.029	0.027	0.033									
36	00.147**	0.089***	0.044***	0.050	0.052								
44	00.210***	0.169***	0.164***	0.160***	0.160***	0.149***							
45	00.145***	0.091***	0.049***	0.064***	0.033	0.029***	0.132***						
46	00.225**	0.142***	0.100***	0.117***	0.077**	0.043***	0.180***	0.030***					
48	00.359	0.245	0.248	0.298	0.258	0.263*	0.268**	0.285*	0.285*				
49	00.131	0.077***	0.075***	0.079**	0.037	0.065***	0.180***	0.060***	0.072***	0.237			
50	00.391**	0.239***	0.296***	0.309***	0.206**	0.224***	0.312***	0.215***	0.265***	0.482*	0.221***		
W	00.272	0.189*	0.190*	0.190	0.170	0.201**	0.251***	0.191***	0.241**	0.435	0.191	0.397**	
Z	00.185***	0.128***	0.130***	0.111***	0.104***	0.109***	0.165***	0.119***	0.157***	0.318**	0.117***	0.186***	0.137***

F_{ST} values and level of significance are given for all pairwise comparisons. LCU38 and Uganda were each represented by a single lion and could not be included in comparisons

Significance: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

Table 5 Hierarchical analysis of molecular variation (AMOVA) in microsatellite loci among three clades of mitochondrial haplotypes and among 13 lion conservation units, combined arbitrarily into eight geographic cluster

Source of Variation	Haplotypes		LCUs	
	3 groups		8 clusters	
	Fixation Index	Percentage Variance	Fixation Index	Percentage Variance
Within LCUs: F_{ST}	0.177	83.23***	0.164	83.58***
Among LCUs within groups: F_{SC}	0.114	10.75***	0.049	4.29***
Among groups: F_{CT}	0.06	6.02*	0.121	12.14***

The fixation index and percent variation is given for three partitions: within populations (V_c and F_{ST}), among populations within groups (V_b and F_{SC}), and among groups (V_a and F_{CT}). Haplotype regions: 1 = *a, c, h, i*; 2 = *b, e, f*; 3 = *g, k, j*

LCU geographic clusters: 1 = 15, 20; 2 = 31, 32, 33, 36; 3 = 38, 45, 46, 49; 4 = 44; 5 = 48; 6 = 50; 7 = W; 8 = Z

Significance: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

the more among-group variance was explained, suggesting that there isn't widespread movement of lions and they are structured by unit. The different results obtained for haplotype and microsatellite groupings may be due to the different time frames, ancient or more recent, that each represents.

STRUCTURE diagrams drawn to reflect groupings by haplotype and by LCU depict genetically distinct populations for each set of partitions, but the plot of ΔK suggests that there are only two groups of lions: lions from Etosha National Park and all other lions (Fig. 5). Regardless of the number of populations or how they are grouped, Etosha lions remain genetically distinct from all others. The highest levels of genetic heterogeneity or admixture can be seen in northern Botswana and eastern Namibia through eastern Zambia (LCUs 33, 36, 38, 45, and 46). Lions in Botswana and Zambia possess the *a* haplotype, grouping with the Eastern clade and haplotypes network, but their microsatellite loci separate them from Eastern lions and ally them in a central area with high levels of admixture (e.g., Chobe Gate in LCU 45). Admixture characterizes most lions in LCU 45, including those from northern Botswana and Khaudum-Caprivi, but lions from Bushmanland form a distinctive cluster (Fig. 5).

Evidence of translocation is apparent in STRUCTURE results for LCU 49 (Greater Limpopo), specifically lions from Sabi Sands (the last six bars). Whereas the mitochondrial haplotype *h* groups these lions with the Southwestern clade, their microsatellites place them with LCU 45 (Khaudum-Caprivi). One lion from LCU 49 (first bar in the group) entered a captive breeding program from the Transvaal region but appears to group instead with Zambian lions. LCU 48 (Kgalagadi) lions, which group with the Eastern clade in terms of haplotypes, have a unique STRUCTURE assignment; without additional samples from this region and areas to the east, it is impossible to

determine the geographic affinities of this population. Finally, the STRUCTURE diagram indicates long-distance movements by lions in Namibia. A lion branded in Etosha National Park (#253, purple bar) moved west to Kunene (also LCU 44), but its microsatellites are shared with LCU 45 (Bushmanland), still farther to the east. More generally, LCU 44 is clearly comprised of two genetically distinctive populations: the Kunene region and Etosha National Park.

Movement among regions: The prevalence of private alleles, the number of migrants (Nm = 0.25 individual/generation), and the isolation-by-distance analyses all suggest that there is little movement among major geographic regions. Isolation-by-distance analyses were significant for both mitochondrial haplotypes ($r_s = 0.512$; $P < 0.005$) and microsatellite loci ($r_s = 0.528$; $P < 0.01$).

Discussion

Phylogeography

A haplotype substitution network constructed by Barnett et al. (2009) from the mt-DNA hypervariable region HVR1 identified haplotype *X* (from Tanzania) at the root of their network for modern lions. Our cyt-*b* network, rooted with both leopard and tiger, is equivocal concerning the ancestral lion haplotype: either the Southwestern clade (haplotypes *g–n*) or the Eastern and Southern clade (*a–d, f, o*) is recovered as basal, depending on whether tiger or leopard is used to root the network. However, both rootings underscore the highly derived position of Asian lions, which are nestled far from the root within a cluster of North, West, and Central African haplotypes. Barnett et al. (2009) used Pleistocene lion sequences in their analysis, which provide a closer outgroup to modern lions and better insights on the polarity of change than more distantly related species of *Panthera*. Although complete cyt-b sequences for *Panthera spelaea* have been analyzed (Burger et al. 2004), those sequences are not publicly accessible.

Other studies (Barnett et al. 2006a, 2006b; Bertola et al. 2011) have also recovered Asian lions amidst North, West, and Central African forms. Bertola et al. (2011) argued that such proximity was attributable to the Pleistocene extinction and subsequent recolonization of North and West Africa from Asian or Middle Eastern refugia. Both outgroup rooting and the limited genetic diversity of that clade suggest that it is younger and derived from lions in Eastern or Southern Africa. But genetic variation offers no more evidence that North and West African lions were derived from Asian ancestors than for the reverse, i.e., that the Asian range was colonized (or re-colonized) by a lion dispersing from North or West Africa. West and Central African lions currently encompass more genetic variation, which favors an Africa-to-Asia polarity, but Indian lions endured a severe late-19th century bottleneck, offering a ready explanation for their reduced variation. The existing variation in West and Central Africa may all have arisen subsequent to back-colonization, which Bertola et al. (2011) hypothesized as late Pleistocene in age. Although Schnitzler (2011) stated that the earliest records for *Panthera leo* in India date to the late Holocene, lions are recorded from the late Pleistocene in Sri Lanka (Deraniyagala 1939; Manamendra-Arahchi et al. 2005).

Taxonomy

Patterns of mitochondrial variation also have implications for lion taxonomy. Two subspecies are currently recognized by IUCN as management units within *Panthera leo*: *P. l. leo* for all extant African populations and *P. l. persica* for extant Asian lions. In conflict with this view, phylogenetic reconstructions robustly recover a major dichotomy within lions that separates lions in Asia and North, West and Central Africa from those in Eastern and Southern Africa. Haplotype substitution networks also confirm the intimate connections among Asian and North, West and Central African lions. Because the name *Panthera leo* (Linnaeus 1758) is based on the Barbary lion, a North African member of this group, the names *leo* and *persica* both apply to the same branch of this dichotomy. The oldest name available for the other branch of this dichotomy is *P. l. melanochaita* (Smith, 1858), originally proposed for the now-extinct Cape lion. Barnett et al. (2006a) showed that Cape lions were likely indistinguishable from other Southern African lions, their extensive manes merely a developmental consequence of temperate climates (Patterson et al. 2006).

Despite recent shared ancestry, Asian lions tend to differ morphologically from those in North, West, and Central Africa. Characteristically, Asian lions have a longitudinal belly fold, less inflated auditory bullae, and more frequently divided infraorbital foramina (Pocock 1931), although these are not qualitative differences (Yamaguchi et al. 2009). These differences may result from relatively recent interruptions to gene flow and effects of small population size in the remaining Asian lions (Todd 1966). Nevertheless, limited population size (<400 individuals) and its unique claim to being Asia's only surviving lion population make it unreasonable to deny taxonomic recognition to *P. l. persica*. Instead, members of this clade from North, West and Central Africa deserve equal recognition as *P. l. leo*, which itself is in desperate need of concerted conservation attention (Bertola et al. 2011).

At present, it is difficult to resolve the taxonomy of lions in Eastern and Southern Africa, which are all regarded here

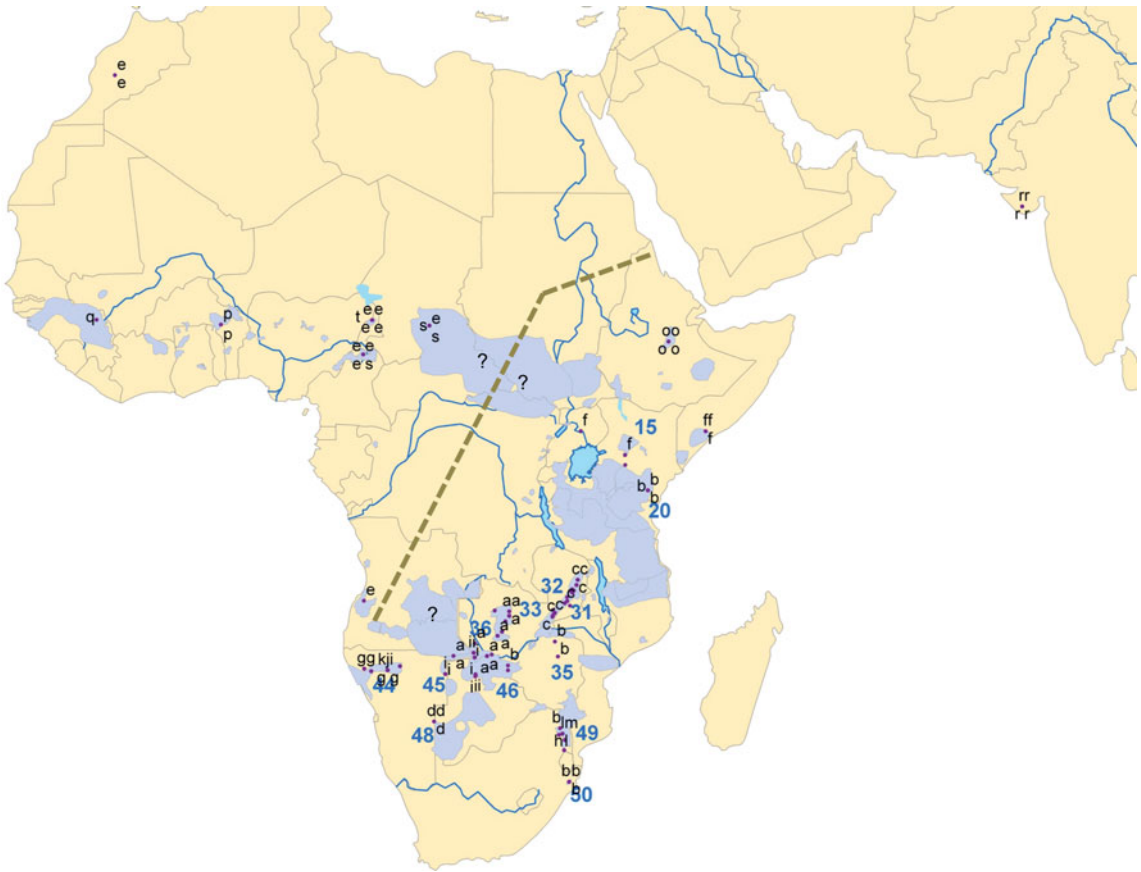

Fig. 4 Map of 20 cyt-*b* haplotypes (*a–t*) for *Panthera leo* across the lion's historic range. The map also shows the positions of 13 Lion Conservation Units (LCUs) across the lion's range in Eastern and Southern Africa. The *dotted line* follows the principal genetic subdivision in extant lions. *Question marks* identify lion conservation areas with unknown affinities

as *P. l. melanochaita*. Despite their greater genetic variability (presenting at least 14 cyt-*b* haplotypes *versus* 6 for all other lions sampled; Fig. 4), the phylogenetic analysis does not reliably identify clades (low bootstrap values in Fig. 1). On the other hand, the haplotype substitution network (Fig. 2) clearly separates haplotypes from east of the Great Rift system (haplotypes *a–d, f,* and *o*) from those in Southern and Southwestern Africa (*g, h–m*), in keeping with earlier phylogeographic appraisals (Dubach et al. 2005). Until additional genetic sampling and accompanying morphological analyses can provide clearer evidence of clades within this group, recognizing additional subspecies in the range of *P. l. melanochaita* seems unwarranted.

Our conclusions regarding the distributions of both *P. l. leo* and *P. l. melanochaita* are ultimately limited by restricted geographic sampling, especially in Central Africa. Barnett et al. (2006a, 2006b) recovered a distinct regional cluster of lions from Democratic Republic of Congo, Central African Republic, Sudan and Ethiopia in their analysis of short (130 bp) control region sequences. The cyt-b haplotype (*o*) recovered from Ethiopian lions clearly falls within the Eastern-Southern clade in our network (Fig. 2), but falls near its base of the maximum-likelihood phylogeny (Fig. 1). Our single sample from Uganda could not be localized, but shared both mitochondrial haplotype and microsatellite loci with lions from the Aberdares in Kenya (LCU 15). Contact between Eastern and Central lineages probably occurs in South Sudan and the Central African Republic, and if multiple genotypes are present there, equivocal relationships would be expected. Our single sample from Angola also shared the Central African haplotype *e* with lions from Cameroon, Chad and, perplexingly, the zoo lions from Morocco (cf. Barnett et al. 2006a). Relatively dense sampling in northern Namibia and the Caprivi Strip suggests that the boundary between the Central clade and both Eastern and Southwestern lineages occurs somewhere in southern Angola and western Zambia.

Management considerations

These results bear on three areas of particular interest to lion conservation. First, genetic variation can be lost in small populations that are managed as isolated entities

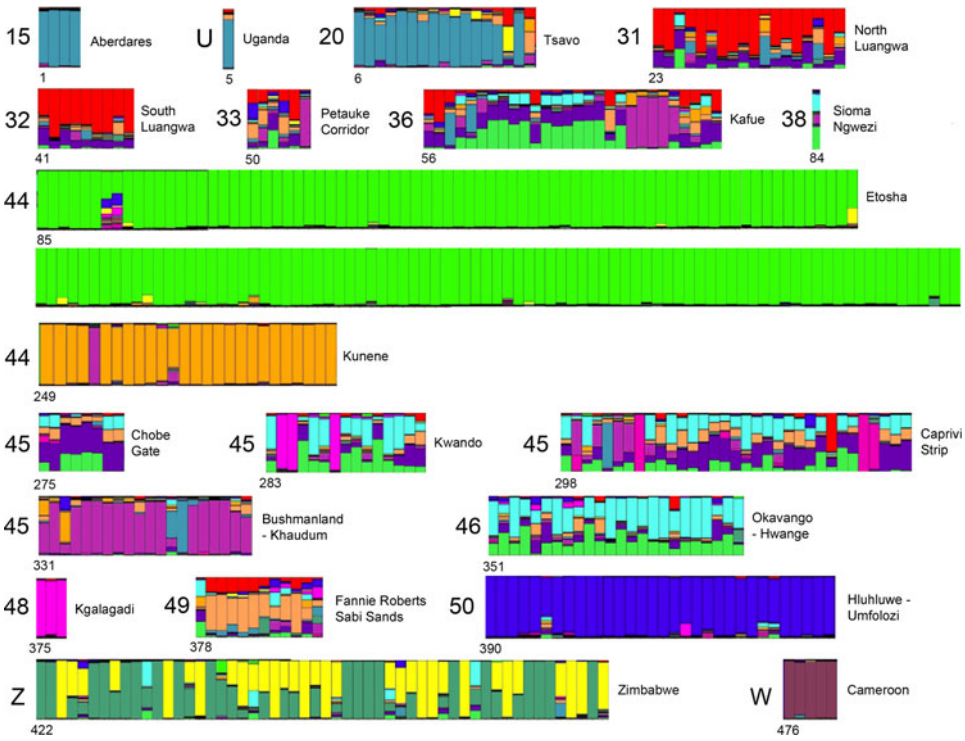

Fig. 5 STRUCTURE classification of 13 LCUs and two other areas ($K = 15$) based on variation of 11 microsatellites among 480 lions. Individual samples are blocked by LCU (*large numbers*) and numbered sequentially within each named sequence (see Supplemental Table 1); sub-populations within these are identified in finer text. The plot reveals relatively homogeneous units (LCUs 15, 50 and W) and highly heterogeneous units (36, 45, and 46) indicative of admixture, as well as obvious composites (LCU 44)

without natural immigration or gene flow, as seen in LCU 50 (Hluhluwe-Umfolozi). For managed populations threatened by inbreeding where natural dispersal cannot be restored, relocations from surrounding areas that simulate gene flow should be considered (Newman and Tallmon 2002). The Hluhluwe-Umfolozi samples used in this study were taken in 1992–1994, prior to a translocation, so that the current status of this population is not represented here. Comparing our baseline with the current genetic status of Hluhluwe-Umfolozi lions would gauge the measurable effects of management-simulated gene flow on the genetics of this population.

Second, some historic lion translocations have clearly brought together different lineages, as is evident in LCU 49. Both the mt-DNA haplotype (*h*) and microsatellite data (Fig. 5) of Sabi Sands lions suggest they originated in the Southwestern region, possibly Botswana. These lions live near typical Eastern lions (the *b* haplotype extends from Hluhluwe-Umfolozi to eastern Kenya), setting up the possibility of human-caused hybridization between lineages. Conceivably, this could lead to erosion of local adaptations and reproductive failures (Storfer 1999; Burke and Arnold 2001), although the capacity of lions for long-distance dispersal must frequently test and/or disrupt such local adaptations. Future restoration efforts that rely on translocations should utilize populations that are most similar genetically, effectively simulating gene flow by introducing compatible genotypes.

The presence of private alleles in most LCUs indicates a general lack of gene flow. The significant pattern of isolation-by-distance for both mt-DNA and microsatellite data corroborate this conclusion. However, some notable exceptions exist. Two sampled LCUs (44 and 45) contained lions with heterogenous genetic characteristics. In LCU 44, the size and range of the western (Kunene) population has recently grown substantially, leading to gene flow with the eastern (Etosha) population. Moreover, a male lion with the genetic attributes of LCU 45 (Bushmanland) was sampled in Kunene and known to breed there; this record appears in the STRUCTURE diagram (Fig. 5) as the purple bar among the orange bars of LCU 44. In the second case, LCU 45 includes the Kwando area in Botswana, Caprivi Strip in Namibia, and Bushmanland in Namibia. Although these areas are in close geographic proximity, lions in all three areas differ genetically. The STRUCTURE chart indicates that lions from Bushmanland are genetically the most distinctive (purple bars in LCU 45; Fig. 5); this genotype also appears present in LCU 36 in Zambia. Conversely, lions from LCUs 31, 32, and 33 in Zambia are not distinguished from one another; all lions we sampled possess the *c* haplotype and similar admixed microsatellite complements. These three reserves lie in close proximity to one another and thus might be amalgamated in future management as they would appear to constitute a single LCU. Likewise, lions from Kwando and Caprivi Strip (LCU 45) share the same haplotypes (*a* and

i) and microsatellites with populations in northeastern Botswana (LCU 46). Genetic distinctions should be considered alongside political, economic, and geographic ones in delimiting and managing LCUs.

Third, both haplotypes and microsatellites show a high level of admixture along the border of Botswana, Namibia, and Zambia (Fig. 5). Individuals from the Caprivi Strip, northern Botswana, and southwestern Zambia possess either the *a* or *b* haplotypes characteristic of the Eastern lineage or the *i* haplotype typical of the Southwestern lineage (Fig. 4). These same individuals are also uncertainly classified to LCU by the STRUCTURE analysis (Fig. 5), in sharp contrast to lions elsewhere (cf. LCU 15, 44, or 50). Both patterns suggest substantial lion movements throughout this area–polymorphism of haplotypes and substantial genetic exchange in the case of nuclear elements. This region appears to be an area of naturally high genetic diversity among adjoining lion populations and one of the few documented contact zones between the Eastern and Southwestern clades. Genetic indications of lion movements across this area are corroborated by direct observations. Radio-and satellite-telemetry studies document trans-boundary movements of lions in this region (L. Hanssen, pers. comm.), sometimes even crossing substantial rivers. We have also documented movements of individual lions or prides between Hwange (Zimbabwe) to Livingstone (Zambia); Mana Pools (Zimbabwe) to the Lower Zambezi (Zambia); Victoria Falls (Zimbabwe) to Livingstone (Zambia); Caprivi (Namibia) to Sioma Ngwezi (Zambia; P. White, pers. comm.), and from Kwando, Botswana into Namibia (M. Briggs, pers. comm.). Given the extent and frequency of these movements, maintaining habitat connectivity in this region should be a conservation priority.

Individual lions can be very wide-ranging, with lions in Waza National Park, Cameroon, having a mean home-range size of 630 km^2 (Bauer and de Iongh 2005) and lions in Kgalagadi Transfrontier Park regularly ranging over as much as 1,450 km^2 (Funston 2011). Despite such movements, both haplotypes and microsatellites show significant isolation-by-distance effects. Contemporary range collapse and fragmentation of lion populations has been too recent to account for this pattern, which is probably a product of the lion's catholic habitat tolerances and its great potential for dispersal.

In summary, regional lion conservation and management plans should consider historic records that document lion range and movement patterns, emphasizing the protection or restoration of landscape connectivity between LCUs. Results from this study may serve as guidelines in identifying and prioritizing potential movement corridors thereby maintaining naturally occurring genetic diversity.

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