Genetic diversity in the lion (panthera leo (Linnaeus 1758)) : unravelling the past and prospects for the future

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Chapter 6

General Discussion

General discussion

In this thesis the phylogeography and levels of genetic diversity are investigated for the lion. A number of genetic approaches is used and resulting data are discussed both in a phylogeographic and a conservation genetics framework. The former gives insight into the evolutionary history of the species, by illustrating which genetic lineages can be identified and how they are related to each other. The latter may contribute to species conservation by translation into recommendations for conservation management. This two-fold approach is using the current genetic makeup to infer scenarios from the past, but also to provide recommendations for the future.

Intraspecific genetic diversity of the lion

In order to obtain a complete overview of genetic diversity within a species, all main populations should be subjected to phylogenetic analyses. In the case of the lion, there is a special interest in the populations from West and Central Africa. Firstly, these populations have rarely been included in genetic analyses, and information regarding their position within the phylogenetic tree of the species is sparse. Secondly, taxonomic and phylogenetic data from other mammals, as well as data on the climatic history of West and Central Africa, suggest that evolutionary forces may have differentiated populations in this part of the continent from population in East and Southern Africa. Thirdly, given the vulnerable position of the small and isolated populations in West and (part of) Central Africa, these populations are in strong need of conservation action if we want to safeguard their future. Therefore, there has been a focus on populations from West and Central Africa in this thesis.

In Chapter 2, two previously used mtDNA markers, cytochrome b and part of the control region, were combined, enabling the integration of newly acquired data with sequences available on GenBank (Dubach *et al.* 2005; Barnett *et al.* 2006b). This was an important extension of existing datasets, since Dubach *et al.* (2005) had not included any population from West or Central Africa, thereby omitting an essential part of the lion range, and the genetic marker used by Barnett *et al.* (2006) gave limited insight into the relationships between genetic lineages. Phylogenetic trees and haplotype networks from Chapter 2 illustrate the close genetic relationship between lions from West and Central Africa and the Asiatic subspecies. The distribution of genetic diversity within the lion implies that the current taxonomy, identifying solely an African and an Asiatic subspecies, does not sufficiently reflect the intraspecific diversity of the lion, and the position of the Asiatic subspecies is questioned.

However, the position of the West African population in the northern branch remained unresolved. Also the positions of the three genetic lineages identified in East and Southern Africa were not completely resolved, and therefore their connection to the northern branch was still unclear. Trees based on exclusively the control region are of limited use, due to the lack of structure, and at this point in time, cytochrome *b* data were missing from East Africa, with the exception of Kenya and two captive populations from Ethiopia and Somalia. A denser sampling and possible identification of intermediate haplotypes may illustrate whether the long lineages identified in Chapter 2, notably in East and Southern Africa, are the result of sampling strategy, or actually reflect the evolutionary history of these populations.

To assess if the pattern discovered in the mtDNA phylogeny is also visible in autosomal data, 15 populations, representing the main genetic lineages identified in Chapter 2, were subjected to microsatellite analyses in Chapter 3. Data from the literature (Driscoll *et al.* 2002; Bruche *et al.* 2012) were combined with five populations from West and Central Africa, which were missing in earlier publications. In addition, a population intermediate to East and Southern Africa, located in Zambia, was added to minimize clustering of sampling locations. Results indicated three clusters in Africa, corresponding to 1) West and Central Africa, 2) East Africa, and 3) Southern Africa, in addition to the Asiatic subspecies. This showed that the intraspecific genetic diversity in the African lion is confirmed by autosomal data.

In Chapter 4, the mtDNA dataset was extended further by more sampling locations, including museum specimen from regions where lions are currently extinct (i.e. North Africa and Middle East) or from areas from which it was not possible to obtain samples from free-ranging lions (e.g. Angola). In addition, cytochrome *b* sequences for previously processed samples by Barnett *et al.* (2006) (Barnett *et al.* 2014), permitted denser sampling, notably in North Africa and the Middle East. Based on a preliminary topology, a total of fourteen samples were selected from the main haplogroups and the complete mitochondrial genomes were sequenced. Phylogenetic trees based on the new, extended dataset display a strongly supported dichotomy, differentiating lions from the northern part of their range (West and Central Africa, and North Africa/Asia), and lions from the southern part of their range (East and Southern Africa). Six main phylogeographic haplogroups are distinguished: 1) West Africa, 2) Central Africa, 3) North Africa/Asia, 4) North East Africa, 5) East/Southern Africa, 6) South West Africa. These results provide a better resolved and more strongly supported phylogenetic tree, and do not contradict the clades identified in other phylogeographic studies on lions, with the exception of the location of individual samples of which the genetic integrity could be questioned (i.e. captive individuals supposedly from Angola or Zimbabwe, included in Antunes et al. (2008)) (Dubach *et al.* 2005, 2013; Barnett *et al.* 2006a; b, 2009, 2014; Antunes *et al.* 2008). This further confirms that current lion taxonomy does not only underestimate intraspecific diversity in the lion, but that the distinction between an African and an Asiatic subspecies is not in line with the most basal split found in the phylogenetic tree of the lion.

Chapter 5 contains phylogenetic trees based on 44,627 SNPs, identified by full genome sequencing of 10 lions, representing the main haplogroups, one leopard and comparison to the reference genome of the tiger (Cho *et al.* 2013). The resulting trees show a hierarchical pattern, an fail to recognize reciprocally monophyletic clades. However, the trees do not contradict previously described patterns based on mtDNA or microsatellite data, and the nested position of the Asiatic subspecies seems to confirm that the current taxonomic distinction is not in line with the evolutionary history of the lion.

Evolutionary history of the lion

Based on the absence of long mtDNA lineages in the northern part of the lions range, it was hypothesized in Chapter 2 that lions in West and Central Africa may represent a relatively recent recolonization of the area after local extinction. Climate data indicate that West and Central Africa was characterized by severe aridity, which may have reduced the number of prey and subsequently the number of lions. The close genetic relationship between West and Central African lions and the Asiatic subspecies suggests that source populations for recolonization may have been in close geographic proximity to North Africa/Asia.

In Chapter 4, the most recent common ancestor of the two major lineages was estimated at ~300 thousand years ago (kya), and major radiation of haplogroups during the last c. 100 thousand years (kyr). In combination with a review of data on the climatic history of the African continent, this enabled the reconstruction of a possible scenario for lion evolution. Two major vegetation zones, dry desert and dense rain forests, representing hydrological extremes, may have contributed to the differentiation between genetic lineages in the lion. Populations may have exhibited contractions to regional refugia when suitable habitat was reduced, and expanded after more favourable changes in the climatic conditions. Last coalescence between the North and South groups coincides with the expansion of dense rain forest along an east-west axis in lower latitude Africa, which may have hampered gene flow between these two major genetic lineages. North-south expansions of the Sahara desert coincided with the major splits in the northern range of the lion, although a connection between North Africa and Central Africa may have persisted during short periods that the monsoon front reached high latitudes, explaining their close genetic relationship.

The haplogroups identified in the lion are further congruent with patterns described in other species, based on taxonomy and/or phylogenetic datasets. For large savannah mammals with a similar range as the lion, numerous species show the distinction between populations in West and Central Africa, and populations in East and Southern Africa (see Table 1 and Figure 1 in Chapter 4). This suggests environmentally driven evolution and possibly common refugia for a range of co-occurring species. Based on phylogeographic patterns in large mammals three major refugial areas have been suggested, being West/Central Africa, East Africa, and Southern Africa (Hewitt 2004; Lorenzen *et al.* 2012). This is in line with main haplogroups described in the lion. A more detailed picture arises from a study using bioclimatic envelope models, describing five possible refugia in sub-Sahara Africa: one in Upper Guinea, one or two in the Cameroon Highlands – Congo Basin, one in the Ethiopian Highlands, one in Angola-Namibia, and one in East/Southern Africa (Levinsky *et al.* 2013). These geographic locations are congruent with the five main groups identified in the lions: West Africa, Central Africa, North East Africa, South West Africa, and East/Southern Africa, respectively (Figure 1). Since delineation of the haplogroups, as shown in Figure 1, is inferred from available sampling locations and current lion range, there may not be a complete overlap with refugial areas as indicated by the model (e.g. in Central Africa). However, as lion range may have shifted through the course of the history, we still conclude that there is a good fit to the proposed refugial areas. The congruence between the lion data presented in this thesis, patterns in other species, and climate data further corroborate the distribution of intraspecific genetic diversity found in the lion.

Genetic diversity and bottlenecks

Due to habitat fragmentation and strong lion population declines in West and (part of) Central Africa, it was hypothesized that these populations would be particularly vulnerable to declines in genetic diversity. Previous studies have shown declines and local extinctions of lion populations in this region (Riggio *et al.* 2012; Henschel *et al.* 2014), a trend also documented in other species (Craigie *et al.* 2010; Bouché *et al.* 2012). However, analyses of 20 microsatellite loci, presented in Chapter 3, did not show significant heterozygote deficiencies, reduced number of alleles or fixed loci in any of the six sampled populations in this region.

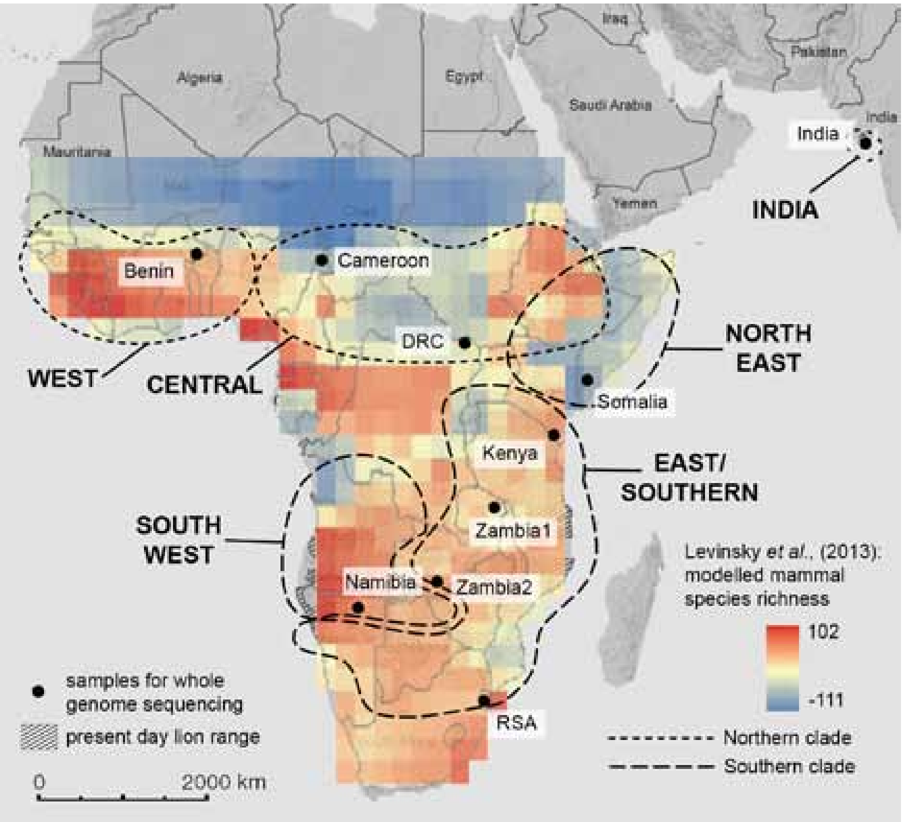

Figure 1. Identified haplogroups in the lion based on mtDNA data, and modelled Last Glacial Maximum species richness for mammals endemic to sub-Saharan Africa, based on Levinsky *et al.* (2013). Lion range data are from IUCN (2014).

Fis values, indicating the inbreeding coefficient of an individual relative to the subpopulation, presented in Chapter 3, were significantly positive in two populations: RSA1 (Kalahari-Gemsbok NP) and Ethiopia1 (Addis Ababa Zoo) (excluding the significantly positive Fis value found in Zambia, possibly due to a Wahlund effect). It was previously shown that RSA1 contains relatively low levels of genetic diversity (Dubach *et al.* 2013), and that the Ethiopia1 is based on a very limited number of founders (Bruche *et al.* 2012). Interestingly, in Chapter 3 significantly negative Fis values were found for the populations Benin (Pendjari NP) and Cameroon 1 (Waza NP), indicating an excess of heterozygotes. A possible explanation would be the mating system, or that these populations have been under pressure, e.g. by hunting in adjacent hunting zones, or due to recent declines (Iongh *et al.* 2009; Riggio *et al.* 2012), and there has been a selection for individuals with high levels of genetic diversity, i.e. heterozygosity. Bottleneck analysis further confirmed the low genetic diversity in RSA1 and Ethiopia1. In addition, Cameroon1 (Waza NP) and Kenya (Amboseli NP) were identified as having experienced a recent population reduction by the program Bottleneck, consistent with observations obtained from monitoring studies (Iongh *et al.* 2009; Riggio *et al.* 2012). In Chapter 5, observed heterozygosity values, based on 18,457 SNPs, showed that ranking of the populations was congruent with previous ranking based on microsatellite data. Although for SNP analyses a single individual was included for each population, this approach follows the "few individuals, several genes" notion, as was mentioned in Chapter 1 (McMahon *et al.* 2014).

Main reason for the absence of clear signs of a reduction of genetic diversity in West and Central African lion populations, might be that range contraction and population declines are too recent to be visible as a genetic signature. In addition, active inbreeding avoidance, such as selection for the most heterozygote individuals as breeders, as was previously shown in wolves (Bensch *et al.* 2006), may contribute to a relatively high diversity in small populations with limited levels of gene flow. However, the significantly negative Fis values may be a first sign of high pressures on these lion populations. Since genetic diversity is rapidly lost in small and isolated populations, caution is warranted and inbreeding may become a problem in the near future. Management interventions may be needed to safeguard these populations against detrimental genetic effects and subsequent declines in fitness in the future.

The genomic complexity of intraspecific genetic diversity in the lion

As different genetic markers represent different evolutionary trajectories (mtDNA vs. autosomal DNA) and due to stochasticity in the coalescence of markers (Edwards 2009; Knowles 2009), species histories should preferably be based on data from multiple, unlinked loci. Many studies use mtDNA markers to make phylogeographic inferences (Arctander *et al.* 1999; Flagstad *et al.* 2001; Moodley & Bruford 2007; Zinner *et al.* 2009; Gaubert *et al.* 2011; Haus *et al.* 2013). Due to the high copy numbers per cell, mtDNA is relatively easy to amplify for sequencing. Therefore it is a useful marker when using samples in which DNA content is low and the DNA is fragmented, such as scat samples or museum specimens. Drawbacks of mtDNA include the fact that it consists of a single locus and that it represents the maternal lineage only. Despite of this, it may provide useful first insight into phylogeographic patterns (Zink & Barrowclough 2008). Since mtDNA is typically inherited through the mother and does not recombine, its effective population size (N_e) is ¼ of the N_e of autosomal markers. Lineage sorting is therefore more rapid, leading to a structure sometimes not (yet) visible in gene trees based on autosomal data.

Autosomal data can be used to complement phylogeographic patterns derived from mtDNA. Microsatellites are useful markers to infer population structure and within-population diversity. Frequency-based approaches (such as STRUCTURE) cluster individuals and provide insight into admixture between the identified clusters. Reticulate events, such as hybridization between clusters or clades, is usually not allowed for in phylogenetic trees, posing a problem when studying intra-specific phylogenetic relationships. However, the phylogenetic relationship between clusters based on microsatellite allele frequencies is difficult to infer. Their mutation pattern, the unit of analysis (typically a pooled sample of individuals, rather than a single haplotype) and difficulties regarding rooting of a microsatellite-based tree, make them less suitable for larger scale phylogeographic questions (Zink & Barrowclough 2008). Despite of this, including microsatellite data may identify cases in which mtDNA and autosomal data show conflicting patterns, e.g. if populations would be linked by nuclear, but not by organellar gene flow (i.e. female gene flow much less than male gene flow). For the reconstruction of evolutionary histories and inferring a phylogenetic tree, genome-wide nuclear markers would be favourable over frequency-based approaches, such as microsatellites.

In this thesis, mtDNA, microsatellites and genome-wide SNP data are included to gain insight into the intraspecific genetic diversity of the lion. Based on mtDNA data, presented in Chapter 4, six reciprocally monophyletic haplogroups can be described: 1) West Africa, 2) Central Africa, 3) North Africa/Asia, 4) North East Africa, 5) Southern/East Africa and 6) South West Africa. Including complete mitochondrial genomes leads to a well supported basal dichotomy, in which the first three haplogroups are placed in the northern clade, and the remaining three haplogroups are placed in a southern clade. The nested position of the Asiatic subspecies in the northern clade led to the notion that the current nomenclature, only recognizing an African and an Asiatic subspecies, does not only underestimate the complete genetic diversity in the species, but also does not follow the most basal genetic differentiation in the species. The only region in which we find evidence for natural admixture based on regional co-occurrence of haplotypes from different haplogroups is Ethiopia, where haplotypes from Central Africa and from North East Africa co-occur. Although the Rift Valley has often been mentioned as a barrier for lion dispersal (Burger *et al.* 2004; Dubach *et al.* 2005; Barnett *et al.* 2006b, 2014), these data indicate that gene flow across the Rift Valley does exist. A mixture of haplotypes is further identified in the Kruger NP/Limpopo area in RSA, likely to be the result of human-mediated translocations as Etosha NP is known to have been a source for several reintroduction projects (Miller *et al.* 2013).

In Chapter 3, microsatellite data were analyzed for fifteen populations, representing the complete geographic range of the lion. Four clusters are recognized: 1) West and Central Africa, 2) East Africa, 3) Southern Africa, and 4) Asia. This illustrates that the genetic structure of the lion within Africa is confirmed by an autosomal marker. However, the relationship of the West and Central African lion to the Asiatic subspecies is difficult to infer from these data. The high level of fixation of alleles in the Asiatic lion is contributing to its distinct status, which is not necessarily a reflection of an ancient evolutionary split, but rather a result of severe bottlenecks and isolation. Although both Ethiopian populations included in the microsatellite analyses, were captive populations, microsatellite data confirm admixture, as was seen based on mtDNA data. The only other population with evident admixture based on microsatellite data, is Zambia. The fact that all included Zambian individuals contain a haplotype from the same haplogroup (East/Southern), suggests that the admixture pattern is the result of male-mediated gene flow. Lions are known to exhibit sex-biased dispersal, in which males leave their natal pride and tend to move further than females (Pusey *et al.* 1987; Spong & Creel 2001). Haplotypes from the East/Southern Africa haplogroup are found from Kenya southwards, across populations in Zimbabwe, Botswana, RSA and Central Namibia, with the exception of the mosaic pattern in the Kruger NP/Limpopo area mentioned above. However, based on microsatellite data, both included RSA populations (Kalahari-Gemsbok NP and Kruger NP) show a strong assignment to the same clusters as Namibia, representing a South West haplotype. This may be the result of the homogenizing effect of male-biased dispersal, whereas the Zambian population may represent the fringe at which admixture in the autosomal data is still evident.

The phylogenetic tree based on 18,457 SNPs, discovered in Chapter 5, shows a hierarchical topology, and fails to identify reciprocally monophyletic clades. The consecution in which the individuals branch off suggests that it reflects continent wide gene flow in a north-south direction. Again, India is nested within the African populations, close to West and Central Africa, thereby provoking the validity of the subspecies status of the Asiatic lion. The loop that is formed between the individuals from DRC, Kenya and Somalia in the PCA, may be an indication of admixture, as we see on a smaller geographic scale in mtDNA and microsatellite data. The position of RSA2 in the PCA and SNP tree should be interpreted with caution, since this is an individual from RSA with a Namibian haplotype, and therefore it is likely that hybridization between two haplogroups has occurred.

Discrepancies between phylogenies based on mtDNA and autosomal data can be explained by lineage sorting times, which is four times shorter for mtDNA. This may lead to discrete, monophyletic groups, which are not retrieved from autosomal data. Therefore, Moritz (1994) notes that requiring reciprocal monophyly for both nuclear and mtDNA markers for the recognition of an ESU seems overly restrictive. However, nuclear allele frequencies should be diverged, to avoid misclassification of populations that are linked by nuclear, but not by organellar gene flow (Moritz 1994). This divergence is visible in the microsatellite data, distinguishing three African clusters. The fact that these clusters are not recognizable in the SNP tree, can be explained by the different mode in which these markers establish their diversity. The mutation rate in multi-allelic microsatellites is much higher than in bi-allelic SNP markers. Therefore the tree based on the SNP data may represent a more ancient pattern of continent-wide gene flow, before populations became isolated in refugia as discussed above. The combination of three types of genetic markers, reveal the underlying complexity of the intra-specific genetic diversity in the lion (Figure 2).

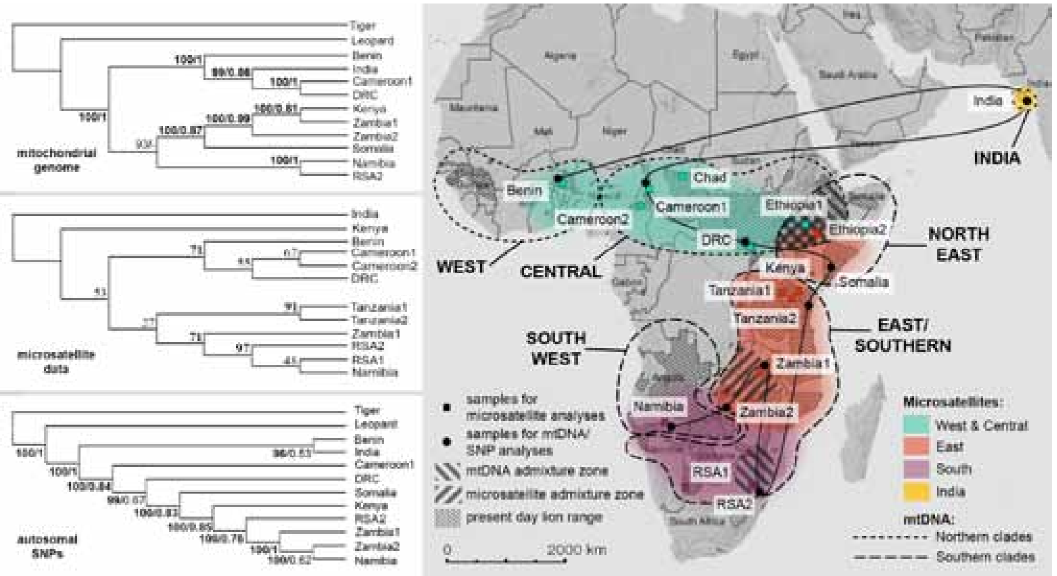

Figure 2. Overview of phylogeographic data from complete mitochondrial genomes, 20 microsatellite loci and 18,457 autosomal SNPs. Support is indicated as posterior probability (Bayesian analysis)/bootstrap support (ML analysis) for mtDNA and SNP trees, the microsatellite tree is based on Da genetic distance and 100 bootstrap replicates. Chad and the Ethiopian populations have not been included in the tree due to missing data, but were added to the map to illustrate admixture patterns. Delineation of haplogroups is based on sampling locations as indicated in Chapter 4. Lion range data are from IUCN (2014).

Implications for taxonomy and management

The classification of populations into subspecies, should ideally be based on the evolutionary history of the species, since this reflects the biological background of the distinguished units. Including phylogeographic data may be a useful tool to assess this. In the case of the lion, the two officially recognized subspecies, the African and the Asiatic lion, do not represent reciprocally monophyletic clades, based on mtDNA or autosomal data. The deepest split in the haplotype tree makes a distinction between lions from the northern part of the range, including the Asiatic subspecies, and lions from the southern part of the range. Admixture between these groups is noticeable in the connecting zone, represented by sampling locations in Ethiopia. Recent hybridization, however, should not be an argument to not recognize these groups as distinct units for conservation, as was previously noted by Moritz (1994). Following the data presented in this thesis, a taxonomic revision would therefore result in two subspecies: a northern subspecies, enclosing West Africa, Central Africa, and India, and a southern subspecies, enclosing North East Africa, East/Southern Africa, and South West Africa.

Propositions for taxonomic revision based on phylogeographic data have also been made by other research groups: Dubach et al. (2013) argues that the limited population size and its unique claim to being Asia's only surviving lion population warrants the distinct subspecies status for the Indian population, in addition to a northern and a southern subspecies. Barnett et al. (2014), however, reasons that subspecies distinctions should follow the recognized phylogeographic groups. This would lead to either 1) elevation of the five African haplogroups to distinct subspecies, or 2) to clustering the Asiatic lion with populations from West and Central Africa as one subspecies, and populations from the southern clade as the second subspecies. Although there may be arguments to retain the subspecies status for the Indian population, based on the ecological and geographic position, this would entail that one of the other subspecies is a paraphyletic group. The alternative, to upgrade all identified haplogroups to subspecies status, is not desirable for two main reasons. Firstly, autosomal data do not show significant divergence of allele frequencies, following the proposed boundaries of the haplogroups, notably in East and Southern Africa. Recognizing the three haplogroups in this area as distinct subspecies may be overly sensitive, as the mtDNA pattern may represent a relic of historic isolation, only sustained by strong female philopatry. Secondly, taxonomic inflation, leading to an increase in (sub)species numbers by splitting existing taxonomic entities, may not benefit conservation (Isaac *et al.* 2004). Therefore it is proposed to follow the deepest split in the haplotype tree for recognizing two lion subspecies, and additional haplogroups for the recognition of ESUs, sensu Moritz (1994). It must be noted that listing activities should be assessed independently from recovery planning, as different scales and units may be appropriate for each of these processes (Mace 2004; Isaac *et al.* 2004).

Comparing the depth of this split with dated splits in other large cats shows that the coalescence age does not provide a clear guideline for taxonomy (Table 1). Notably the coalescence of six subspecies of tigers is relatively recent (Luo *et al.* 2004), whereas in the jaguar no subspecies are recognized but splits are much deeper (Eizirik *et al.* 2001). In leopard, a deep split is found distinguishing African from Asiatic leopards, in which subsequently seven Asiatic subspecies are recognized (Uphyrkina *et al.* 2001). Comparison of these species shows that the depth of the split found in the lion (this thesis) would not be an argument to consider all African lions as a single subspecies.

Table 1. Estimated coalescence times in species from the genus *Panthera*.

Species	Split	Coalesence data	Source
Lion	North (incl. Asiatic subspecies) – South	291,700 ya	This thesis
(*Panthera leo*)		(95% HPD 178,000-417,700)	
Leopard	Africa (incl. South Arabia) – Asia	471,000 ± 102,000 ya	Uphyrkina *et al.* (2001)
(*Panthera pardus*)	7 Asiatic subspecies	169,000 ± 49,000 ya	
Tiger	6 subspecies	72,000 ya	Luo *et al.* (2004)
(*Panthera tigris*)		(95% CI 39,000–104,000)	
Jaguar	1 subspecies, several lineages	280,000–510,000 ya	Eizirik *et al.* (2001)
(*Panthera onca*)		(95% CI 137,000–830,000	

In addition to the distribution of genetic diversity within the species, congruence is found with morphological characters and the historical distinction of subspecies (Hemmer 1974; Mazák 2010) (Figure 3). Up to eight "subspecies" are recognized by some sources (Haas *et al.* 2005), with the Barbary lion (*P. l. leo*) very likely to be extinct and the Cape lion (*P. l. melanochaita*) a possible con(sub) specific with *P. l. krugeri* (Barnett *et al.* 2006a). Hemmer (1974) further suggests to not include *P. l. bleyenberghi* and *P. l. azandica* as distinct subspecies, since differentiation based on morphometric characters is not conclusive. Therefore Hemmer (1974) suggests to follow a basal dichotomy with a distinction between *P. l. senegalensis* and *P. l. persica* in the northern range, and between *P. l. nubica* and *P. l. krugeri* in the southern group. The main discrepancy between genetic data and proposed subspecies delineations in Figure 3, is the border between the West and Central African lion and the East African lion. However, it was not possible to defer information on which this delineation was based, and the illustrations by Hemmer (1974) seem to suggest that the boundary between *P. l. senegalensis* and *P. l. nubica* is located further to the East, more in line with delineation found based on mtDNA haplotypes and miscrosatellite data. The proposed West and Central African identity of individuals in Congo could not be confirmed by an earlier phylogeographic study, in which an included individual from Gabon contained a haplotype widespread in southern Africa (Barnett *et al.* 2006a). In summary, the combination of genetic and morphological data suggest that the current taxonomy, only recognizing the African and the Asiatic lion as distinct subspecies, does not reflect the diversity in the lion. A revision of the nomenclature is suggested in which all populations from the northern part of the range, encompassing West Africa, Central Africa, and India are considered one subspecies, and populations from the southern part of the range, including North East Africa, East/Southern Africa, and South West Africa, are recognized as the second subspecies. The six mentioned lineages should be managed as ESUs, sensu Moritz (1994). We propose the adoption of the names *Panthera leo leo* (Linnaeus, 1758) for the northern subspecies, and *Panthera leo melanochaita* (Smith, 1848) for the southern subspecies, in accordance with the rule of nomenclature (http://iczn.org/iczn/index.jsp). This revision has immediate implications for conservation. Recognizing the northern clade as *P. leo leo*, eliminates separate taxonomic listing for Asiatic lions, but both Asiatic and West African units are considered to be endangered (Breitenmoser *et al.* 2008; Bauer *et al.* 2012; Henschel *et al.* 2014). In West and Central Africa lion numbers are strongly declining (Riggio *et al.* 2012; Henschel *et al.* 2014), along with other species (Craigie *et al.* 2010; Bouché *et al.* 2012). No strongholds (sensu Riggio *et al.* 2012) are identified in this region, whereas several strongholds are recognised within the range of *P. l. melanochaita*. Because West and Central Africa harbour unique genetic lineages, in the lion, as well as in other species, conservation of populations in this region is of utmost importance.

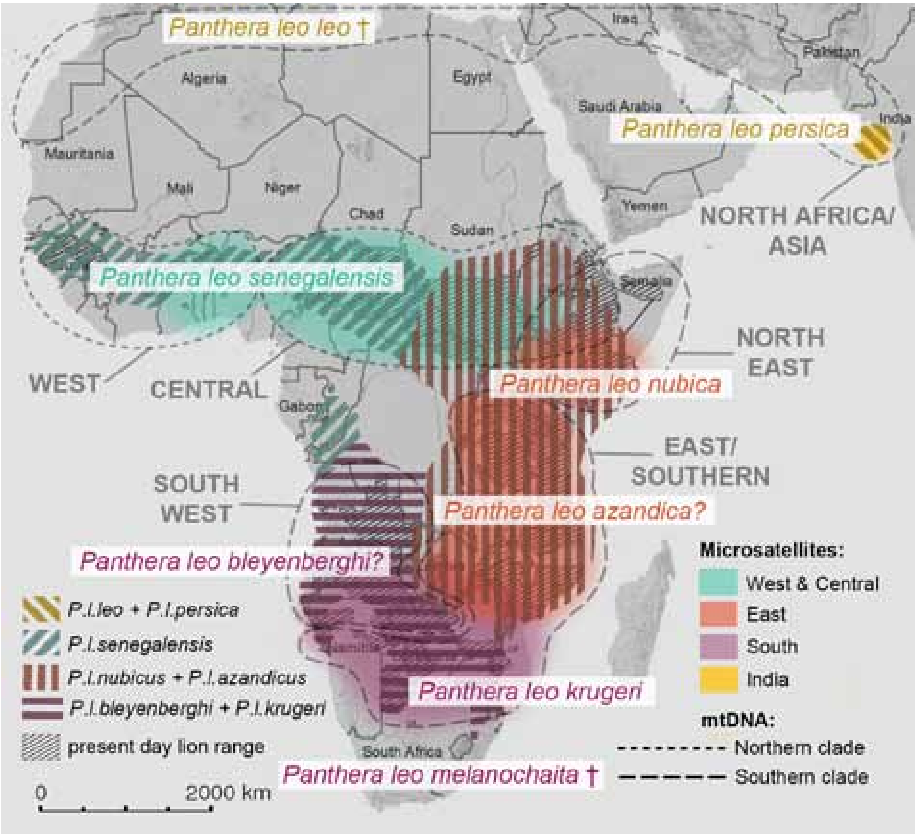

Figure 3. Lion subspecies delineation, based on Haas (2005), with haplogroup delineation and microsatellite clustering indicated by shading. SNP data have been omitted to improve readability. *P. l. azandica* and *P. l. bleyenberghi* are indicated with a '?', following suggestions by Hemmer (1974). Lion range data are from IUCN (2014).

The recognition of units below the subspecies level, such as ESUs and MUs, may be beneficial for management interventions, including translocations, reintroductions and reinforcements of extant populations. Although hybridization of individuals from different phylogenetic lineages may be useful to counteract the effects of inbreeding, it is desirable to maintain the integrity of phylogenetic lineages. Disruption of local adaptation (possibly leading to outbreeding effects) and disturbing natural patterns of genetic diversity within the species by human interference should be avoided, if possible. When considering conservation interventions, it would be advisable to follow the assignment to a haplogroup as a first indication, although admixture, not visible in mtDNA data, may play a role at boundaries of identified haplogroups. For captive-bred lions, often with limited information regarding their free-ranging ancestors and breeding history, assignment to a haplogroup may be less useful, because of the frequent occurrence of admixture in a zoo setting (ISIS 2014) (see 'Captive stocks and implications for breeding'). In general, when assessing source populations for a translocation, it would be advisable to prioritize populations from the same haplogroup, in close proximity to the destination, from which off-take is sustainable and does not disrupt social structures. This is of exceptional importance for countries that harbour lion populations from different haplogroups, e.g. Nigeria and Kenya. In the case of Nigeria, it would not be advisable to translocated individuals between Yankari GR and Kainji Lake NP, but to target populations from the same haplogroup in neighbouring countries as a possible source.

Based on the distribution of genetic diversity and the conservation status of the lion in some regions, certain populations should be prioritized in order to maintain the genetic diversity in the African lion. Following from the vulnerable position of lions in West and Central Africa and the recognition of potential strongholds, sensu Riggio *et al.* (2012), a pragmatic view should be employed. Prioritization of conservation projects in Niokolo-Giunea (Senegal – Guinea Bissau – Guinea – Mali) and W-Arly-Pendjari (Benin – Burkina Faso – Niger) and the Bénoué complex-Gashaka Gumti (Cameroon – Nigeria – Chad), as well as Chad-CAR, may provide a starting point, following recent assessments (Riggio *et al.* 2012; Iongh *et al.* 2014; Henschel *et al.* 2014). Transboundary conservation agreements may be challenging, but also have great potential to involve multiple partners and to address conservation practices on a meso-scale (Sodhi *et al.* 2011).

Although in a few cases reduced genetic diversity is recognized, there is no clear evidence for inbreeding depression in any of the free-ranging lion population included in this study. Despite of this, on the longer term management interventions may be needed to maintain genetic diversity in the small and isolated populations. Assignment of populations to genetic lineages may be helpful to guide these interventions. However, deciding on when a conservation action such as the reinforcement of an existing populations is justified, should best be confirmed by monitoring of demographic factors, such as fecundity and mortality. Certainly, it should be ensured that the pressures, driving the original population to decline or even extinction, are no longer present. In addition, it must be noted that in the consideration of a reintroduction or translocation project, apart from the genetic requirements other biological, socio-economic and cultural aspects need to be taken into account (IUCN SSC 2013).

Captive stocks and implications for breeding

Due to the challenges linked to collecting samples from different source populations, some studies partially relied on captive individuals (Antunes *et al.* 2008; this thesis). Antunes *et al.* (2008) included two captive individuals supposedly from Angola (individuals from the same stock also included in Chapter 2 of this thesis) and one individual supposedly from Zimbabwe. These samples clustered close to Central Africa in their analyses. This is not in line with previously published phylogeographic patterns in lions, leading to questions regarding the origin and breeding history of these individuals. In the case of the Angola lions, a breeding history is published in a study on lens-anomalies in an inbred zoo population (Steinmetz *et al.* 2006). A pure-blooded Angolan origin cannot be confirmed and hybridization with individuals with a Central African haplotype may have occurred. Another well-known example is the recently extinct lion subspecies, the Barbary lion (*P. l. leo*), which was last sighted in 1942 in the western Magreb (Black *et al.* 2013). Several zoos still claim to have a breeding stock, and researchers have called for further captive breeding to avoid definitive extinction (Burger & Hemmer 2005; Black *et al.* 2009). However, a comparison of a part of the mtDNA of five captive Barbary lions to ancient specimen from North Africa held in natural history collections, revealed that none of the captive lions were maternally of Barbary origin (Barnett *et al.* 2006a). Instead, the identified haplotype was also found in lions from Central African Republic (CAR) and Democratic Republic of the Congo (DRC), proposing a Central African origin, at least for the maternal lineage (Barnett *et al.* 2006a).

Comparison of captive populations to an incomplete reference set, may pose another difficulty. Based on the phenotypical distinctiveness of some Ethiopian lions, including the heavily and dark-maned individuals held in the zoo of Addis Ababa, microsatellite and mtDNA data were used to assign these individuals to a reference dataset (Bruche *et al.* 2012). It was concluded that the Addis lions compose a unique, previously unidentified clade. However, the used reference set included solely populations from East and Southern Africa and therefore only contained a part of the genetic diversity found in the African lion. In Chapter 3 and 4, samples of the Addis lions were re-analysed and compared to a larger dataset, including populations from West and Central Africa (Chapter 3 of this thesis). Both mtDNA and microsatellite markers illustrate that the Addis lions are closely related to the West and Central African lion, a signature also found in part of the free-ranging Ethiopian lions.

Assessing genetic diversity, as well as the assignment to genetic lineages, may be helpful for the design of an effective breeding and management program for captive stocks. Current lion holdings (October 2014) show that a total of 2,095 individuals are registered in the International Species Information System (ISIS 2014). Following the putative designation into eight subspecies, 17% of the lions belong to the East and Southern Africa group, 16% represent the Asiatic subspecies, and a mere 4% are West and Central African lions (including the putative Barbary lions, genetically analysed by Barnett *et al.* 2006a). The remaining 63% of the individuals are known hybrids or have an unknown history. Origin and breeding history are often not well-documented or anecdotal, which may mean that the number and purity of genetic lineages present in the captive stocks are severely over-estimated (Bertola *et al.* 2011). In addition, these figures indicate that the West and Central African lion is severely underrepresented in the captive population.

Despite the subspecies designation followed by ISIS, there is no studbook for African lions in captivity. Asiatic zoo lions on the other hand, breed following a subspecies-specific studbook. A Species Survival Plan (SSP) was established in 1981 and was later assessed for its genetic purity (O'Brien *et al.* 1987). It was concluded that two of the seven founder lions used for this stock were of African origin and consequently all captive Asiatic lions are the result of admixture between the African and Asiatic subspecies (O'Brien *et al.* 1987). Even for the free-ranging Asiatic subspecies, the purity of the lineage has been questioned. According to some authors, African mammals were imported to restock royal menageries in India for at least five centuries (Thapar *et al.* 2013). The discussion whether individuals could have escaped their enclosures and populated the local habitat, leading to the "exotic alien" status of the Asiatic lion (and cheetah), or whether they could have hybridized with existing populations is still ongoing (O'Brien 2013; Packer 2013). However, phylogeographic data do not point into the direction of hybridization or replacement of the Asiatic lion by sub-Sahara African individuals (O'Brien 2013; this thesis). Because of the close genetic relationship between the Asiatic lions and the extinct North African/Middle East populations, there may have been gene flow in this region, although this could be both natural as human-mediated.

Different views on the management of a captive stock, poor bookkeeping, and the inclusion of confiscated individuals of unknown origin have led to admixture of different genetic lineages in the vast majority of zoo lions (ISIS 2014). Therefore, it is advisable to not give too much weight to the subspecies designation as found in ISIS. However, even in the absence of pure genetic lineages, the captive population may still be valuable in terms of diversity. The strategy of a breeding programme could be to mimic the genetic diversity of the species, taken into account the available resources to house captive animals. Genotyping the captive stock and identifying a number of "zoo lineages" may contribute to the design of such a breeding programme. When lions are not bred with the ultimate goal to be used in reintroduction programmes, pure genetic lineages are less relevant. Reintroduction projects using captive individuals as a source are still highly controversial, and may not be a suitable conservation strategy for the near future (Hunter & Rabinowitz 2009). Notwithstanding, a certain level of vigilance is needed as several organizations state that they aim to reintroduce captive-bred lions into the wild, although release sites are often fenced areas (ALERT 2014; Vier Voeters 2014).

In the course of this research, five captive lions, suggested for a reintroduction project in Ghana, were analysed for their mtDNA haplotype. Since none of the individuals contained a haplotype from the West African haplogroup (Figure 4), it was advised to exclude them from reintroduction in Ghana. Since past hybridization events cannot be detected by mtDNA analyses, for the confirmation that an individual is genetically suitable for reintroduction in a specific area, mtDNA data should be combined with autosomal data.

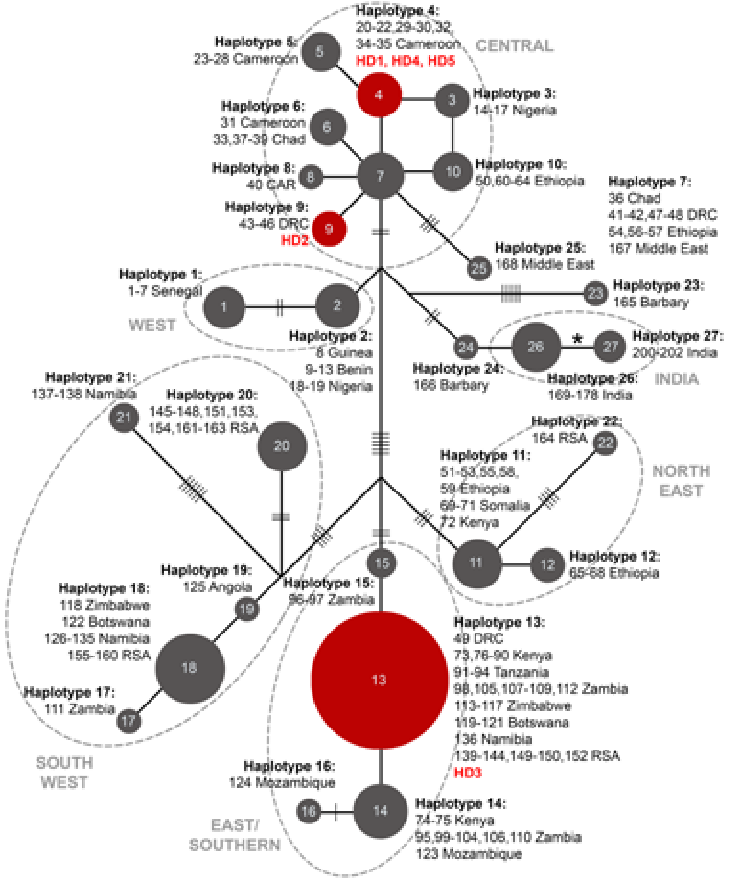

Figure 4. Phylogeographic origin for five captive individuals (HD1-HD5), compared to haplotype data from Chapter 4.

Using a similar forensics approach, as described for the captive individuals above, sources of illegal lion products may be identified, as was previously done for confiscated shipments of elephant tusks (Wasser *et al.* 2008). For a lion skin, confiscated in Libreville, Gabon in 2011, the haplotype was analysed in the course of this project, pointing towards a Central African origin. Although the resulting haplotype is widespread and the country of origin could therefore not be pinpointed, the claim of the arrested trader that the skin was coming from Benin (Henschel, pers. comm.), seems unlikely. As this type of illegal products are likely to be from free-ranging lions, a haplotype may be sufficiently indicative for a source population/region, contrary to analysed individuals in a zoo setting.

Due to frequent hybridization in captive populations, and the absence of a studbook for African lions, it should be considered to genotype existing stocks for assignments to breeding lineages. Upcoming NGS techniques, such as SNP genotyping, provide cost-effective opportunities for large-scale genotyping. Conservation strategies based on maintaining pure genetic lineages should not be overly puristic and it should be noted that management decisions are scale-dependent. Regardless of the ultimate goal of captive breeding, including genetic aspects in the management of captive stocks is of utmost importance, since high degrees of relatedness lead to loss of genetic diversity and, consequently, inbreeding.

Perspectives for the Future

Looking into the future, modelling studies may give insight into the possible scenarios for conservation, although socio-economic and cultural aspects are often not taken into account. Data on population diversity in tigers have been used to model the increase in tiger numbers needed to maintain current levels of genetic diversity in the next 150 years (Bay *et al.* 2014). Also for relatively large populations, considered as tiger strongholds, gene flow between populations is required. More strongly, the authors state that in the absence of gene flow current genetic diversity cannot be maintained, knowing the species' demographic parameters (Bay *et al.* 2014). In addition, the authors test for the effect of a delay in iniation of gene flow between isolated populations, and show that a subsequent increase in the rate of population expansion is needed when implementation of connectivity is postponed (Bay *et al.* 2014). Another study using tiger data, modelled the risk of inbreeding and extinction in populations of different sizes and different levels of gene flow (Kenney *et al.* 2014). Even relatively high levels of gene flow do not safeguard small populations, but significantly lowers the extinction risk in medium to large populations. The relevance of connectivity is stressed to avoid increased extinction risk, however, the future for already small populations looks grim according to the applied model (Kenney *et al.* 2014).

Due to a different population structure, such as social organization in prides (Pusey *et al.* 1987; Gilbert *et al.* 1990; Packer *et al.* 1991a; Spong *et al.* 2002), these models may not be applicable for lions. Although many of the small populations in RSA are fenced, also in unfenced reserves across Africa lions are secluded from gene flow in peripherally isolated populations (Newmark 2008). The presence of private alleles in most studied LCUs in East and Southern Africa and the strong isolation-by-distance (IBD) patterns confirm a general lack of gene flow (Dubach *et al.* 2013). This effect may be even stronger in West and (part of) Central Africa, as these populations are located in a severely fragmented habitat (Riggio *et al.* 2012) and intermediate populations have recently been extirpated

(Henschel *et al.* 2014). Björklund (2003) used deterministic population genetics models and an individual-based stochastic model to assess the risk of inbreeding in the lion in relation to habitat loss. Most influential parameters tested were the number of prides and male dispersal rates. To sustain a large outbred population of lions, the area should sustain a continuous population of at least 50 prides, but preferably 100 prides, and allow for unrestricted male dispersal (Björklund 2003). Assuming an average territory size for a pride of lions of 50 km^2, as it holds true for lions in Selous GR, Tanzania, this would entail that an area of 5000 km^2 is needed to satisfy the requirements of the model (Björklund 2003). Surely other parameters may be more applicable for lions living in a different type of habitat, with different prey densities, and different social structure. However, since gene flow is a prerequisite and only few protected areas contain the area needed according to the model, this illustrates the relevance of including genetic aspects in management of wild lion populations. Further loss of habitat or decrease of suitability is one of the major challenges in lion conservation. A recent study modelled the distribution of the African lion in response to a changing global climate (Peterson *et al.* 2014). Previously, it had already been shown that climatic parameters had a great effect on the variance of demographic parameters (Celesia *et al.* 2009) and home ranges (Tuqa *et al.* 2014). In the face of global climatic changes, it was predicted that few new areas will become suitable for the African lion, however, large areas in West and southern Africa are projected to decrease in suitability. Notably for West Africa, where remaining lion habitat is small and fragmented, this may pose a serious problem.

On a more positive note, management interventions in highly inbred populations have shown that these effects can be reversed by reinforcing the inbred population with new genetic lineages. High percentages of abnormal and immotile sperm were found in the Florida panther (*Puma concolor coryi*), a subspecies which is confined to a single population. As a result of habitat destruction the population was reduced to 30-50 individuals (Roelke *et al.* 1993; Barone *et al.* 1994). This led to severe inbreeding effects, such as an unusually high frequency of kinked tails, cowlicks, low sperm quality and heart defects (Roelke *et al.* 1993). As the population decline continued, these defects increased in frequency. This led to the notion that the only way to preserve the Florida panther was by hybridization with individuals from another subspecies, thereby affecting the “genetic integrity” of the Florida panther. Eight female pumas from Texas (*Puma concolor stanleyana*) were introduced in 1995 to genetically enrich the existing population. The population was monitored intensively and over a course of 12 years the number of individuals had increased threefold, heterozygosity doubled, survival and fitness measures improved and inbreeding correlates declined significantly (Pimm *et al.* 2006; Johnson *et al.* 2010). However, the authors also warned that, although the introduction of new genetic material seemed to benefit the population, this approach does not guard the population for persisting pressures like continued habitat loss.

Data from well known lion bottlenecks, such as in the Ngorongoro Crater and in the Gir forest, illustrate that the lion shows high resilience and is capable of bouncing back after strong bottlenecks (Packer *et al.* 1991b; Driscoll *et al.* 2002). However, it is important to note that a population with low genetic diversity as a result of a bottleneck may be more vulnerable to environmental stochasticity and catastrophes, e.g. disease outbreaks. This suggests that continuous monitoring and managing protected areas as a network with metapopulations is the best way forward.

Final thoughts

The aim of this thesis was to obtain insight into the spatial distribution of genetic diversity in the lion, contributing to the understanding of the evolutionary history of the species, and possible application for guiding conservation decisions. A number of genetic approaches was used to elucidate the underlying complexity of intra-specific genetic diversity. Although one is always limited by the number of sampling locations, and not every single lion population could be included, I believe that the patterns described in this thesis show a relatively complete picture of the intraspecific genetic diversity of the lion.

The development of SNPs for the lion opens up possibilities to directly target these positions by generating a SNP panel, and to analyse a greater number of individuals. This would provide data for a phylogeographic context, but also information on levels of genetic diversity within populations. Scoring SNP data is less error-prone than scoring microsatellites, which especially plays a role when dealing with low quality samples, such as scat or historic samples. Reference samples are not needed, as is the case for the sizing of microsatellite data. In addition, genotyping of large numbers of individuals for SNPs is cost-effective and less labour-intensive, compared to amplifying and sequencing large numbers of PCR products. The use of a SNP panel to study more lion populations, guide breeding and to employ different sample types should be further investigated. Finally, it may be worthwhile to also assess the applicability of the panel for other, related species.

It may be inevitable to follow a pragmatic approach in which certain populations are prioritized. Phylogeographic data can guide conservation decisions and rationalize this prioritization. Insights into the intraspecific genetic diversity, presented in this thesis, will hopefully contribute to the establishment of effective conservation practices, and to safeguarding the lion’s future in its full diversity.

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