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SHORT COMMUNICATION

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Urgent call for further breeding of the relic zoo population of the critically endangered Barbary lion (*Panthera leo leo* Linnaeus 1758)

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Abstract The Barbary lion became extinct in the wild around 1942. Since the end of the 19th century, a last purebred captive breeding stock existed at the court of Morocco. The rest of these animals became the core exhibition of the new Rabat Zoo after passing through repeated bottlenecks and possibly some introgression events by foreign lions. This study uses mitochondrial DNA sequencing data to clarify the relationship among these lions and their sub-Saharan and Asian relatives. We analysed mitochondrial cytochrome *b* sequences obtained from a sample from a Barbary lion descended from a young female of the Barbary lion breeding group at the Rabat Zoo and various other members of the genus *Panthera*. In our cytochrome-*b*-based phylogenetic tree, the North African Barbary lion, represented by a biopsy sample from the Neuwied Zoo, joins the Asian lion clade, although it is slightly different from its Asian sister group. However, it is clearly distinct from sub-Saharan lions and can be considered as a genetically defined phylogeographic group of its own. Molecular dating of the extant sub-Saharan and Asian lion groups shows that the split between North African Barbary lions and Asian lions must be considerably more recent than 74–203 kilo years ago.

Keywords *Panthera* · Molecular phylogeny · Lion conservation · Supportive breeding

Abbreviations ky: kilo years · kya: kilo years ago · bp: base pairs · BP: before present

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Introduction

In addition to the sub-Saharan (*Panthera leo senegalensis* group) and the Indian lions (*Panthera leo persica*), the Barbary lion (Atlas lion, *Panthera leo leo* Linnaeus 1758) represents the third major extant phylogeographic lion group (Fig. 1). The distinction between Indian and African lions is clear-cut, especially in skull morphology (Hemmer 1974), and a molecular divergence date of 74–203 kilo years (ky, thousand years) was recently obtained (Burger et al. 2004). However, how close the genetic relationship is between North African and sub-Saharan lions is still the subject of debate. First molecular results based on lions brought from Rabat to the National Zoo in Washington, DC, were published by O'Brien et al. (1987). Working with electrophoretic variation in 46 to 50 allozyme loci, the authors found only low genetic distance estimates among Barbary lions, sub-Saharan African lions and Indian lions. At about the same time, O'Brien observed a protein variation between African lions, including the Barbary zoo population, and Indian lions (oral presentation at the 1986 International Tiger Symposium in Minneapolis). Accordingly, all recent lions were lumped into only two subspecies, an African and an Indian one.

Analysis of the historical record and morphology produces different, even contradictory results. The Barbary lion became extinct in the wild during the second half of the 19th and the first half of the 20th century. Its story probably ended in 1891 in Tunisia and in 1893 in Algeria but not before 1942 in Morocco (Yamaguchi and Haddane 2002). A last purebred captive Barbary lion group was installed at the end of the 19th century in the lions' garden of the sultan of Morocco. Some lions of this then still-flourishing palace breeding group were sold during the sultan's exile between 1953 and 1955. King Hassan II later transferred the remaining animals to the newly founded Moroccan National Zoo (Hemmer 1978a; Yamaguchi and Haddane 2002) where they served as a visitor highlight. Unfortunately, we cannot exclude the possibility of multiple genetic introgressions by lions introduced into this captive breeding group from other sources during the line's

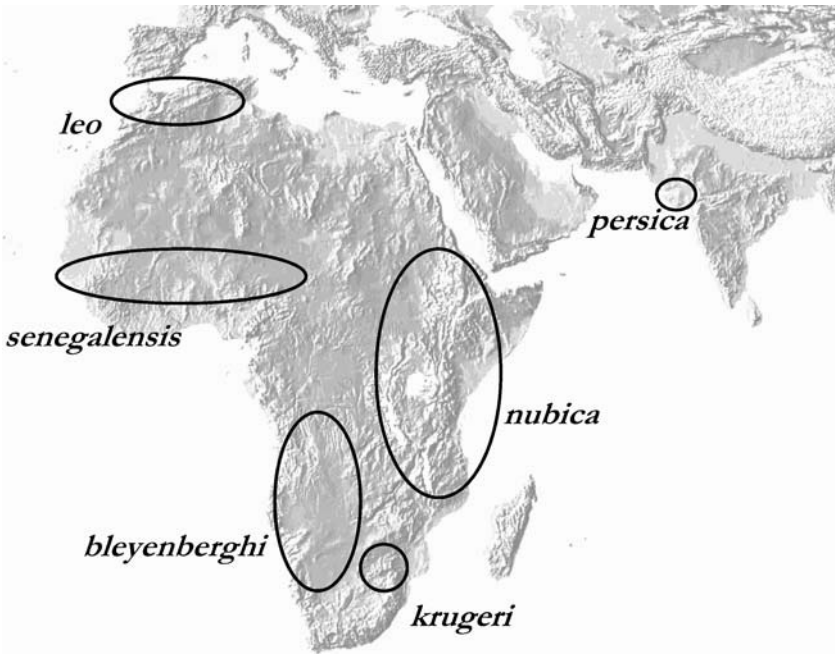

Fig. 1 Approximate geographic distribution of lion subspecies (*P. leo* ssp.) in the 20th century (after Hemmer 1967)

centennial history (Hemmer 1978a). It also comprised repeated bottlenecks at least in 1953 as well as in the late 1960s (Yamaguchi and Haddane 2002).

These lions were studied for morphological and ethological traits by Paul Leyhausen and Helmut Hemmer at the Rabat Zoo in 1974. The resulting goal was to establish an international Barbary lion survival project, which was launched with great expectations over the following years in several European and American zoos (Hemmer 1978a). However, as a consequence of the molecular work reported above, the Barbary lions captive breeding project was finally discredited and several participating zoos abandoned it. Further breeding was limited to a very few zoos. A studbook initiated with the first international diffusion of lions of the Rabat stock was given up at that time of uncertainty. The genetic basis of the project and the remaining reproductively active captive Barbary lion population suffered a dramatic breakdown. In view of this imminent loss of an important natural heritage, Yamaguchi and Haddane (2002) advocated a new and last-minute conservation effort for Barbary lions "even if, in an unlikely event, they were merely phenotypic representatives" of their original population in the wild. They also deplored the lack of DNA-based genetic studies that might help to demonstrate the uniqueness of the last captive animals.

The work presented here is a first step toward further completing the still-fragmentary genetic pedigree of the former free-ranging Barbary lion ancestors and their zoo-bound last descendants, using new knowledge gained from mitochondrial cytochrome *b* sequences.

Material and methods

DNA was extracted from a tissue sample from a Barbary lion cub that died soon after birth (first litter of a young female of the Barbary lion breeding group of Rabat origin in the Zoo at Neuwied, Germany). DNA was extracted from 25 mg of liver tissue using the tissue protocol of the QIAamp DNA minikit (Qiagen). PCR was carried out using primers that cover the cytochrome *b* gene of feline mitochondrial DNA (mtDNA) (CB23u: TGGAATTTA ACCATGACTAATG and CB24l: GGCTGTTGCTTCTTCCTTGAA). PCR products of 1,249 base pairs (bp) were sequenced bidirectionally, and the resulting sequences were compared to various feline DNA sequences. The following sequences were acquired from GenBank: *Felis catus* AB004238; *Panthera leo* AF05 3052, AF384809, AF384810-1, AF384811, AF38 4812, AF384813, AP384814, AF384815, AF384816, AF384817, AF384818, X82300; *Panthera tigris* AF05 3018, AF05 3021, AF053039, AF053048, AF053051, X82301. In addition, the following sequences were produced: *P. leo persica* 1 and 2 (Zoo Frankfurt am Main, Germany), *Panthera pardus* (Zoo Frankfurt am Main), *Panthera leo spelaea* Siegsdorf [47,180+1,190/-1,040 years BP (before present)] and *P. leo spelaea* Kufstein (31,890±300 years BP). The *P. leo spelaea* sequences were produced following rigorous ancient DNA procedures, including replication of sequences from different extracts and extensive cloning and sequencing of overlapping fragments. All pre-PCR work was done in laboratory rooms specifically dedicated to work with fossil DNA as well as following various other authentication criteria as outlined previously (Burger et al. 2004).

The cytochrome *b* sequence alignment consisted of 26 individuals and 1,051 positions. Sequence distance calculations were done using Megalign Software (Lasergene). Neighbour joining (NJ), maximum parsimony (MP) and bootstrap analyses used phylogenetic analysis using parsimony (PAUP*) (Swofford 2001). The bootstrap analyses were set up to perform 1,000 replicates. Molecular clock data were taken from Burger et al. (2004).

Table 1 Divergence values of pairwise cytochrome *b* sequence comparisons among 25 pantherine cats and one domestic cat

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	*F. catus* U20753	–																									
2	*P. tigris corbetti* AF053049	16.2	–																								
3	*P. tigris sumatrae* AF053043	15.9	0.7	–																							
4	*P. tigris altaica* AF053030	16.4	0.6	0.7	–																						
5	*P. tigris tigris* AF053018	16.2	0.8	0.9	0.6	–																					
6	*P. tigris* X82301	15.9	0.7	0.0	0.7	0.9	–																				
7	*P. tigris tigris* AF053025	15.9	0.4	0.5	0.4	0.6	0.5	–																			
8	*P. tigris altaica* AF053033	16.3	0.5	0.6	0.1	0.5	0.6	0.3	–																		
9	*P. leo spelaea* Siegsdorf	16.7	12.1	12.5	12.6	12.4	12.5	12.1	12.5	–																	
10	*P. leo spelaea* Kufstein	16.4	11.9	12.3	12.4	12.2	12.3	11.9	12.3	0.2	–																
11	*P. leo bleyenberghi* Botswana AF384815	16.2	11.4	11.8	11.9	11.4	11.8	11.7	11.8	4.8	4.6	–															
12	*P. leo nubica* Kenya AF384817	16.2	11.7	12.0	12.2	11.7	12.0	11.7	12.0	4.8	4.6	0.6	–														
13	*P. leo bleyenberghi* Namibia AF384813	16.2	11.4	11.8	11.9	11.4	11.8	11.7	11.8	4.8	4.6	0.0	0.6	–													
14	*P. leo bleyenberghi* Namibia AF384814	16.2	11.4	11.8	11.9	11.4	11.8	11.7	11.8	4.8	4.6	0.0	0.6	0.0	–												
15	*P. leo krugeri* Natal AF384818	16.2	11.7	12.0	12.2	11.7	12.0	11.7	12.0	4.8	4.6	0.6	0.0	0.6	0.6	–											
16	*P. leo krugeri* Transvaal AF384816	16.2	11.7	12.0	12.2	11.7	12.0	11.7	12.0	4.8	4.6	0.6	0.0	0.6	0.6	0.0	–										
17	*P. leo* PSS993 AF384810	16.5	11.4	11.8	11.7	11.0	11.8	11.7	11.6	5.0	4.8	0.4	0.8	0.4	0.4	0.8	0.8	–									
18	*P. leo nubica* Uganda AF384809	16.1	11.6	11.9	12.0	11.6	11.9	11.6	11.9	4.9	4.7	0.5	0.3	0.5	0.5	0.3	0.3	0.7	–								
19	*P. leo bleyenberghi* Namibia AF384811	16.1	11.3	11.7	11.8	11.3	11.7	11.6	11.7	4.7	4.5	0.1	0.5	0.1	0.1	0.5	0.5	0.3	0.4	–							
20	*P. leo bleyenberghi* Namibia AF384812	16.2	11.4	11.8	11.9	11.4	11.8	11.7	11.8	4.8	4.6	0.0	0.6	0.0	0.0	0.6	0.6	0.4	0.5	0.1	–						
21	*P. leo* AF053052	16.3	11.8	11.9	12.0	11.5	11.9	11.5	11.9	5.3	5.1	1.4	1.3	1.4	1.4	1.3	1.3	1.6	1.2	1.3	1.4	–					
22	*P. leo* X82300	16.3	11.5	11.9	12.0	11.5	11.9	11.5	11.9	5.1	4.9	1.1	0.9	1.1	1.1	0.9	0.9	1.3	0.8	1.0	1.1	0.8	–				
23	*P. leo persica* 1 Frankfurt	16.4	11.7	12.0	12.2	11.7	12.0	11.7	12.0	5.0	4.8	1.2	1.0	1.2	1.2	1.0	1.0	1.3	0.9	1.1	1.2	0.3	0.5	–			
24	*P. leo persica* 2 Frankfurt	16.4	11.7	12.0	12.2	11.7	12.0	11.7	12.0	5.0	4.8	1.2	1.0	1.2	1.2	1.0	1.0	1.3	0.9	1.1	1.2	0.3	0.5	0.0	–		
25	*P. pardus* Frankfurt	14.6	11.6	11.9	12.1	12.1	11.9	11.6	11.9	9.0	8.9	9.2	9.2	9.2	9.2	9.2	9.2	9.4	9.0	9.0	9.2	9.3	9.3	9.2	9.2	–	
26	*P. leo leo* Neuwied	16.3	11.5	11.9	12.0	11.5	11.9	11.5	11.9	5.1	4.9	1.1	0.9	1.1	1.1	0.9	0.9	1.3	0.8	1.0	1.1	0.8	0.0	0.5	0.5	9.3	–

Results

The average sequence distance was 0.62 and 0.31% among sub-Saharan lions. The two Indian lions and one other sequence identified as belonging to this clade (see below) differed from each other on average by 0.1% and the two cave lions had 0.2% difference. At the sequence level, the Barbary lion differed from tigers by 11.74% (average), from a leopard by 9.3%, from cave lions by 5%, from sub-Saharan lions by approximately 1% and from Asian lions by 0.6% (Table 1). On the amino acid level, there was no difference from Asian lions while cave lions are 2% and sub-Saharan lions are 1.33% apart.

The MP analysis produced 28 equally parsimonious trees. Besides slight differences within the tiger and the African lion clade, all of the MP trees yielded topologies similar to the NJ tree. Figure 2 shows the NJ tree with bootstrap values added at the relevant nodes. Both MP and NJ bootstrap values are given. Five major clades were distinguishable: tigers, leopard, sub-Saharan lions, extinct cave lions and a Barbary *persica* clade. The latter contained two additional sequences from GenBank (accession numbers AF053052 and X82300). One of them was taken from a lioness of unknown descent at the Brookfield Zoo (Cracraft et al. 1998; Jean Dubach and Ann Petric, in correspondence with Wolfgang Frey) and clustered with Asian lions. The origin of the other (Árnason et al. 1995) was not traceable at all. Its cytochrome *b* sequence was identical to that from the Barbary lion from Neuwied Zoo.

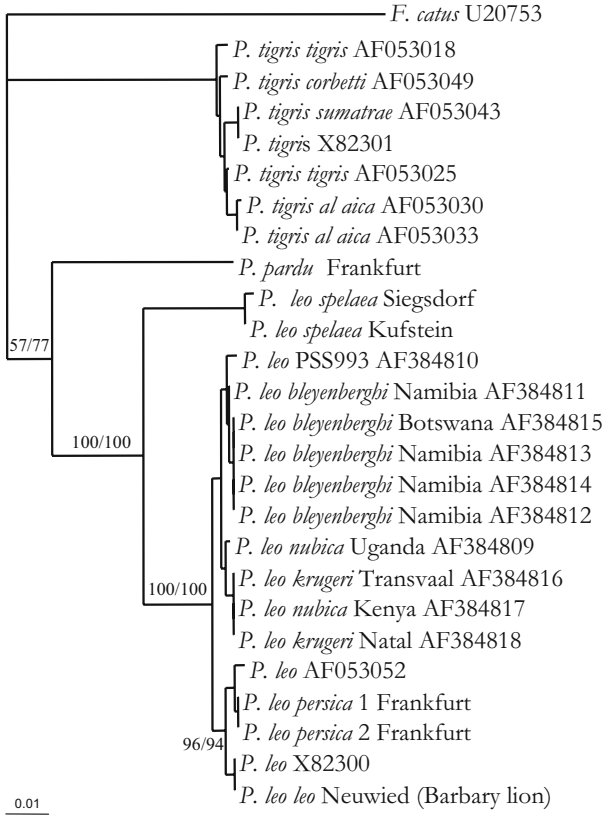

Fig. 2 NJ tree using *F. catus* as out-group. Branch lengths are drawn proportional to estimated change; scale 0.01 substitutions per site. Bootstrap percentages (MP/NJ) are given for relevant nodes. Geographical origin of lions and GenBank accession numbers are noted after the (sub-) species name. For subspecies nomenclature, see Hemmer (1974, 1978b); Burger et al. (2004) and Fig. 1

Discussion

Molecular phylogeny of the genus *Panthera*

Our molecular phylogeny based on cytochrome *b* sequences of 7 tigers, 1 leopard, 17 lions and domestic cat as out-group shows that the tiger branch separates first from the clade of the genus *Panthera*. The age of this split was estimated at 1,428–2,295 ky (Burger et al. 2004). This is followed by the separation of the leopard and the lions (cf. Hemmer 1978b). A later radiation within *P. leo* separates the extinct European cave lions (*Panthera leo fossilis* and *P. leo spelaea*) from recent lions of Africa and India. According to palaeontological data, this separation happened not before 600 kilo years ago [kya, thousand years ago; Garcia Garcia 2001]. The more recent divergence into the extant sub-Saharan and Asian lion subgroups, the *senegalensis* group in Africa and *persica* in Asia, happened according to molecular clock data 74–203 kya (Burger et al. 2004). The North African Barbary lion, *leo*, represented by a biopsy sample from the Neuwied Zoo, joins the Asian lion clade, though with high bootstrap support clearly separated from its Asian sister. However, molecular dating using cytochrome *b* data is not reliable for forms which are that closely related. Nevertheless, the age of the split between North African and Asian lions must have occurred considerably more recently than the molecular date of the preceding split within the *leo* group, i.e. 74–203 kya. Most likely, Asian and North African lions separated not prior to the worldwide ecological upheavals in the course of the last glaciation.

Fate of the Barbary lion

The cytochrome *b* sequence of a specimen originating in the Rabat Zoo Barbary lion breeding stock is not found in sub-Saharan African lions and does not fit in the clade of the latter populations. Rather, the haplotype is closely related to Asian lions. This demonstrates on the mtDNA level the unique nature of the relic zoo population of the Barbary lion. The result of the Barbary lion cytochrome *b* haplotype as a sister taxon to the Asian lion clade (Fig. 2), and far distant from other lions, also corresponds neatly to the earlier morphological finding that Barbary lions are related to Asian lions, as opposed to the sub-Saharan African lions (Hemmer 1974). The detection of a Barbary and an Asian lion haplotype in two zoo lions of unknown origin (see above) is not surprising, as Barbary lions were frequently among the mixture of captive lions used to establish breeding stocks in Europe and North America as early as the 19th century (e.g. Seifert 1978; on the lion stock of the Leipzig Zoo). Due to its splendid mane, male Barbary lions or at least lions with that phenotypic appear-

ance were formerly highly favoured by the zoo community to create a common "European–American zoo lion". Therefore, crossbreeding of Barbary lions and lions of a different provenance was largely a one-way street. Nevertheless, genetic contamination of the Moroccan relic captive Barbary lion stock itself cannot be excluded completely because no records were kept on that stock during its Moroccan captive history. In 1974, Paul Leyhausen and Helmut Hemmer visited the zoo and initiated the international breeding and conservation program. The Rabat lions were subdivided into morphological categories. More than 40% were assessed to be typical Barbary lions, and close to 90% carried more than one of the typical Barbary lion features (Hemmer 1978a). There was also a lioness of known East African origin in the Rabat Zoo, but this animal was not integrated into a breeding group. In recent times, some West African animals were added to the Rabat collection but may not have been crossbred with Barbary lions (Wolfgang Frey, personal communication). Thus, the original breeding program with the goal of Barbary lion conservation started with selected, morphologically "pure" Barbary lions (Hemmer 1978a).

The position of the specimen from the Neuwied Zoo in the cladogram (Fig. 1) is a proof that its mitochondrial lineage is not of sub-Saharan origin and, thus, very likely a descendant of a Barbary lion. As our study refers to mtDNA only, this holds true for the maternal line only. Further molecular work to find nuclear markers is necessary. An earlier study (Janczewski et al. 1995) failed to prove the existence of a unique Barbary lion haplotype, probably because it was based on short or non-variable sequences (289 bp of the cytochrome *b* gene and 358 bp of the 12S RNA gene). This means new conservation measures are beginning late but not too late. Our results should be the basis of an urgent call for new strains within the international zoo community for further breeding of the stock in question, even if the line may have been genetically contaminated to some extent by other lions during its 100 years of captive breeding history. Initial research into a possible future reintroduction into the Moroccan wild have recently received a positive response (Yamaguchi and Haddane 2002).

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