

1 **Historical biogeography of the leopard (*Panthera pardus*) and its extinct**

2 **Eurasian populations**

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26 **Abstract**

27 *Background:* Resolving the historical biogeography of the leopard (*Panthera pardus*) is a complex
28 issue, because patterns inferred from fossils and from molecular data lack congruence. Fossil
29 evidence supports an African origin, and suggests that leopards were already present in Eurasia
30 during the Early Pleistocene. Analysis of DNA sequences however, suggests a more recent, Middle
31 Pleistocene shared ancestry of Asian and African leopards. These contrasting patterns led
32 researchers to propose a two-stage hypothesis of leopard dispersal out of Africa: an initial Early
33 Pleistocene colonisation of Asia and a subsequent replacement by a second colonisation wave
34 during the Middle Pleistocene. The status of Late Pleistocene European leopards within this
35 scenario is unclear: were these populations remnants of the first dispersal, or do the last surviving
36 European leopards share more recent ancestry with their African counterparts?

37
38 *Results:* In this study, we generate and analyse mitogenome sequences from historical samples that
39 span the entire modern leopard distribution, as well as from Late Pleistocene remains. We find a
40 deep bifurcation between African and Eurasian mitochondrial lineages (~710 Ka), with the
41 European ancient samples as sister to all Asian lineages (~483 Ka). The modern and historical
42 mainland Asian lineages share a relatively recent common ancestor (~122 Ka), and we find one
43 Javan sample nested within these.

44
45 *Conclusions:* The phylogenetic placement of the ancient European leopard as sister group to Asian
46 leopards suggests that these populations originate from the same out-of-Africa dispersal which
47 founded the Asian lineages. The coalescence time found for the mitochondrial lineages aligns well
48 with the earliest undisputed fossils in Eurasia, and thus encourages a re-evaluation of the
49 identification of the much older putative leopard fossils from the region. The relatively recent
50 ancestry of all mainland Asian leopard lineages suggests that these populations underwent a severe
51 population bottleneck during the Pleistocene. Finally, although only based on a single sample, the
52 unexpected phylogenetic placement of the Javan leopard could be interpreted as evidence for
53 exchange of mitochondrial lineages between Java and mainland Asia, calling for further
54 investigation into the evolutionary history of this subspecies.

55
56 **Keywords:** ancient DNA, hybridisation capture, leopards, mitochondrial genomes, mitogenomes,
57 mtDNA, palaeogenetics, *Panthera pardus*

58
59

60 **Background**

61

62 Achieving a comprehensive understanding of a species' history is important for both evolutionary
63 research and for conservation management. However, this may be impossible using data derived
64 solely from living individuals – particularly for endangered species whose current genetic diversity
65 is depauperate. A potential solution is to study DNA sequences obtained from historical or ancient
66 samples [1], allowing extinct populations and lineages to be investigated and compared with those
67 that exist today. In studies that use such samples, mitochondrial DNA continues to be an important
68 marker as the much higher copy number per cell compared to the nuclear genome generally results
69 in a higher success rate for sequence recovery - particularly for species with low fossil abundance
70 and/or poor biomolecular preservation [e.g. 2–5]. The leopard (*Panthera pardus* Linnaeus, 1758) is
71 an example of a species that is currently distributed across only a fraction of its historical and
72 ancient range [e.g. 6–8]. It is one of only a few large-bodied carnivore species that naturally occurs
73 in a wide variety of habitats; from the Himalayan highlands to the Ethiopian desert, and from the
74 Congo rainforests to the Amur taiga [9]. Human persecution and hunting [e.g. 10–12], habitat
75 destruction [e.g. 13–15] and reduced prey availability [16, 17] have severely impacted the
76 distribution of this elusive predator, and leopards are now extinct in large parts of their historic
77 Asian and African distribution (Fig. 1) [7, 18].

78

79 The fossil record and genetic data from modern and historical samples are generally interpreted as
80 indicative of an African origin of the leopard. The oldest leopard fossils have been recovered in
81 Eastern Africa, and genetic diversity (estimated using both mitochondrial DNA and nuclear
82 microsatellites) in living African populations is higher than in Asian populations, which has been
83 interpreted as evidence for an African origin [19, 20]. The oldest potential evidence for leopards
84 outside of Africa is found in the Early Pleistocene fossil record from South Asia (Pakistan),
85 suggesting an initial out-of-Africa expansion into Eurasia around 2.0 Ma (*Mega annus*; million
86 years ago; [21]). Mitochondrial and microsatellite data, however, suggest that all current Asian
87 populations share a much more recent common ancestor (approximately 622 Ka [*Kilo annus*;
88 thousand years ago]; [19, 22]); more than a million years younger than the oldest fossils found in
89 Asia. The apparent incongruence between the fossil record in Eurasia (which suggests occupation
90 since the Early Pleistocene) and the relatively recent coalescence time of African and Asian modern
91 leopard mitochondrial lineages has been interpreted as indication for two independent out-of-Africa
92 dispersal events, the latter of which founded all modern Asian leopard lineages [19].

93

94 Previous studies that have examined intraspecific variation in leopards have been based on short
95 mitochondrial sequences and microsatellites from recently collected samples [19, 23–29]. In this
96 study, we present near-complete leopard mitogenomes from seven Late Pleistocene specimens (up
97 to 45,000 years old) and from 15 historical samples collected up to 150 years ago, from a range of
98 geographical locations encompassing its entire distribution (Fig. 1). We investigate this
99 mitogenomic data in the context of the proposed evolutionary history and past population dynamics
100 of the leopard, and the role of Pleistocene populations within this scenario. Furthermore, by
101 combining mitochondrial data from previous studies [19, 22, 24, 27] with ancient and historical
102 mitochondrial DNA (mtDNA), we present new insights into ancient and recent population
103 dynamics of one of the most widespread extant felid species.

104

105 **Results**

106

107 Using hybridisation capture and high-throughput sequencing, we retrieved near-complete
108 mitogenomes from 22 leopard samples: one Late Pleistocene Caucasian leopard (~35 Ka), six Late
109 Pleistocene Central European leopards (~45 Ka), and 15 historical leopards from across the entire
110 historical distribution (Fig. 1; Table 1). The mitogenome sequences represent 17 distinct
111 haplotypes, which were analysed in conjunction with three previously published modern leopard
112 mitogenomes. Using a two-step approach (see Methods section for details), we estimated the
113 coalescence times of this dataset in BEAST. Our results support the deep bifurcation between Asian
114 and African leopards, that has previously been proposed based on short mtDNA sequences (Fig. 2;
115 Additional File 1: Fig. S1; [19, 22]). The fossil-calibrated Bayesian analysis of the basal divergence
116 time for all leopard mitochondrial lineages was 710 Ka (95% credibility interval [CI]: 457 - 956
117 Ka), which is also consistent with the previous estimates (932 Ka; Wilting et al 2016, 471 - 825 Ka;
118 [19]). The Pleistocene European sequences form a well-supported clade consisting of three distinct
119 haplotypes, which is sister to a clade containing all mitogenome sequences from Asian leopards.
120 The two clades are estimated to have diverged approximately 483 Ka (95% CI: 305 - 677 Ka). The
121 ancient Caucasian sequence is sister to all modern mainland Asian sequences, with high support
122 (Bayesian posterior probability of 1.0, Fig. 2; Additional File 1: Fig. S1). The estimated coalescence
123 time of mainland Asian mitogenomes, including the ancient Caucasian individual, is 244 Ka (95%
124 CI: 148 - 352 Ka).

125

126 The South-East Asian samples included one specimen collected in Sumatra (PP35), where currently
127 no leopards live and thus is likely to represent a traded specimen imported from elsewhere. A

128 second specimen in this clade had no associated geographical provenance ('East Indies'; PP3).
129 These individuals may represent the Javan leopard lineage, as their mitogenome haplotypes are
130 sister to all Asian leopard sequences (Fig. 2), and they exhibit ND5 sequences identical to
131 previously published sequences from Javan leopards [19, 22]; Additional File 1: Fig. S2). The
132 coalescence time of this lineage and the mainland leopards is estimated at 375 Ka (95% CI: 230 -
133 524 Ka). In contrast to previous studies, our Javan sample (PP32) was placed as a sister lineage to a
134 specimen from Thailand (PP15), nested within all mainland Asian leopards rather than sister to
135 these, with an estimated coalescence time of 64 Ka (95% CI: 32 - 100 Ka; Fig. 2).

136

137 **Discussion**

138

139 Using modern, historical and ancient mitogenomic data from leopard samples from across their
140 current and former geographic range, we provide novel insights into the historical biogeography of
141 the leopard. The resulting data clarify the relationship between current leopard populations and Late
142 Pleistocene fossils, and provide additional evidence for the interpretation of the earliest putative
143 leopard fossils.

144

145 *Origin of the leopard*

146

147 Based on the fossil record, the origin of the leopard has been placed in Eastern Africa. There are
148 fossils that may belong to *P. pardus* dating to about 3.4 - 3.8 Ma from Laetoli (Olduvai; [30]),
149 although some authors suggest that these may be assigned to a different *Panthera* species [31], or
150 some other large-bodied felid [32]. Although the fossil record between 2.0 and 3.8 Ma is sparse,
151 unequivocally identified leopard fossils do confirm their presence in Eastern Africa around 2 Ma
152 [33], which strongly suggests an African origin of the species (Fig. 3).

153

154 We estimate the coalescence time between African and Eurasian leopard mitochondrial lineages to
155 be ~710 Ka, similar to the age found by previous molecular studies [19, 22]. Although the
156 bifurcation between African and Eurasian leopards provides no confirmation for either an African
157 or Eurasian origin for the leopard, the combined fossil and genetic evidence together support Africa
158 as the most likely place of origin. The relatively recent coalescence time between African and
159 Eurasian mitochondrial lineages is also consistent with previous suggestions that the oldest African
160 fossils of 3.8 Ma may not in fact represent *P. pardus* [31, 32]. Within Africa, we found considerable
161 genetic divergence between haplotypes occurring in East Africa. In particular, the haplotype

162 sampled from the Burundi individual forms a divergent sister lineage to all other sampled African
163 haplotypes (95% CI: 368 - 814 Ka), including those from other regions of East Africa. Thus, the
164 mitochondrial divergence occurring within this region of Africa is equivalent to that of the entire
165 African continent, indicating this region as the potential point of origin of the leopard, which has
166 also been suggested based on short mtDNA sequences [23]. Given that, based on the fossil record,
167 leopards were present in East Africa at least around 2 Ma ago [31], this hypothesis requires that
168 mitochondrial lineages established prior to ~600 Ka have either being lost from modern populations
169 through genetic drift or have not yet been sampled. These results do suggest, however, that, as well
170 as for the genus *Homo* and a number of other species [34], East Africa may be the point of origin of
171 modern leopards, although additional sampling of individuals and of nuclear markers is desirable to
172 more robustly test this hypothesis.

173

174 *Out-of-Africa dispersal*

175

176 The first evidence for leopards outside of Africa is ambiguous. In Asia, the earliest occurrence of
177 leopard fossil remains is in the Early Pleistocene of South Asia (Pakistan), suggesting an initial out-
178 of-Africa expansion into Asia around 2.0 Ma [21]. Whether or not these fossils should be assigned
179 to *P. pardus* is subject to discussion, however [31, 32]. These ancient specimens from Pakistan may
180 be better assigned to other medium-sized felids (e.g. Eurasian puma, jaguar or snow leopard), or
181 indicate an earlier dispersal of a leopard-like *Panthera* taxon into Asia (Fig. 3). Unequivocal
182 leopard fossils in Asia are much younger; 0.6 – 0.8 Ma [35, 36]. Furthermore, in Europe, the oldest
183 findings of this species are dated to the early Middle Pleistocene (nearly 0.6 Ma: [37–39]).

184

185 Inferring the timing of colonisation events from the mitochondrial gene tree is challenging, as the
186 population divergence and lineage coalescence will be asynchronous. Lineage coalescence of
187 founding and source populations represents the maximum limit for dispersal time, but could be an
188 overestimate caused by deep coalescence within the source population. Assuming monophyly of
189 founding population lineages, the radiation of the founding population represents the minimum
190 limit on the dispersal time, but this will tend to be an underestimation due to the mutation-lag of the
191 formation of new lineages, incomplete sampling of extant lineages, or by lineage extinction during
192 population bottlenecks. Thus, the true colonisation event lies somewhere along the branch
193 connecting these upper and lower limits. Following this reasoning, the mitogenome data suggest the
194 colonisation of Eurasia (or at least the end of maternal gene flow) some time between 710 and 483
195 Ka ago. This range overlaps with the age of the younger, unequivocal leopard fossils from Asia but

196 not with the older Early Pleistocene Asian fossils whose precise taxonomic assignment has been
197 debated. Achieving a unified biogeographic hypothesis for the colonisation of Asia by leopards thus
198 hinges on the classification of these older fossils. Under the assumption that the first leopard
199 occurrence in Eurasia is represented by the unequivocally identified specimens dating to around 0.6
200 - 0.8 Ma, there is no contradiction between the fossil record and the molecular evidence. If,
201 however, older Early Pleistocene specimens are to be classified as *P. pardus*, then multiple out-of-
202 Africa dispersals and the replacement of all mitochondrial lineages present in Asia from earlier
203 dispersal events is required to explain the observed patterns. Considering the ambiguity of the Early
204 Pleistocene fossil record in Asia, we consider a single out-of-Africa for *P. pardus* more
205 parsimonious given the currently available combined palaeontological and genetic evidence.
206 Clarifying whether the African and Eurasian leopard populations have indeed been separated since
207 this time, or if gene flow continued to some extent, and if the earlier Eurasian leopard-like
208 populations played a role in the evolutionary history of the more recent populations, will require
209 nuclear genomic data with a similar geographical coverage.

210

211 *Ancient Eurasian leopards*

212

213 The origin and classification of the European Pleistocene leopard is equivocal based on fossil
214 remains from the region [e.g. 6, 40–42]. The fossil record suggests that leopards first dispersed into
215 Europe during the Middle Pleistocene, as evidenced by fossils from Western Europe estimated to be
216 around 600 Ka [6, 30, e.g. 35, 37, 38]. Several different forms have been defined in European fossil
217 assemblages based on morphology, which has been interpreted as evidence for several immigration
218 waves from Africa or Central Asia [6]. The Late Pleistocene form, representing the age of the
219 specimens we retrieved mitogenomes from, has been referred to as the subspecies *P. p. spelaea* [6,
220 42]. These populations likely went extinct before the Last Glacial Maximum (LGM), around 25,000
221 years ago [6, 8, 40, 41, 43]. The disappearance of the European leopard populations coincides with
222 the extinction of many other Pleistocene carnivores, such as the cave bear (*Ursus spelaeus*), cave
223 hyena (*Crocota crocuta spelaea*) and *Homotherium* [3, 44–46].

224

225 We recover three distinct haplotypes from the six leopard samples, and as there are no overlapping
226 skeletal elements in the sample set, the number of individuals sampled is uncertain (minimum of
227 three). We find that the Late Pleistocene European leopards share a more recent common ancestor
228 with modern Asian leopards, than either does with African leopards (Fig. 2; Additional File 1: Fig.
229 S1). The estimated coalescence time between the European and Asian mitogenomes is 485 Ka, with

230 a 95% CI of 301 - 675 Ka, which encompasses the age for the oldest fossil records of Europe. This
231 suggests that the European leopards originate from the same out-of-Africa event as the Asian
232 leopard lineages, rather than from an independent migration. The ancient Caucasian leopard lineage
233 is sister to all modern samples from mainland Asia, with an estimated coalescence time of ~244 Ka
234 (95% CI: 148 - 352 Ka).

235

236 Morphological studies have failed to differentiate Pleistocene Eurasian leopard fossils from their
237 modern counterparts [21]. Cranial and postcranial measurements from Middle Pleistocene remains
238 from Poland (estimated to 569 - 125 Ka; [39] and from the Caucasus (less than 350 Ka; [37])
239 overlapped with the variation found in modern leopards, which has been interpreted as evidence for
240 some degree of population continuity between those ancient and modern populations. The dates of
241 these fossils are not older, and actually correspond fairly well to the estimated coalescence time
242 between ancient European mitochondrial lineages and modern leopards (95% CI: 305 - 677 Ka) or
243 between the ancient Caucasian individual and modern leopards (95% CI: 148 - 352 Ka). Thus, some
244 degree of population continuity since the Late Pleistocene, as suggested by the morphology, is not
245 in conflict with our molecular dating. Alleles of Pleistocene European leopards may thus survive in
246 the modern Caucasian population. However, as our dataset does not include modern Caucasian
247 individuals, nor ancient East Asian ones, a direct assessment of population continuity between the
248 Middle Pleistocene and today can only be based on short mtDNA sequences [24, 27]; Additional
249 File 1: Fig. S2). The mtDNA haplotype network has only limited resolution as it is only based on
250 456 bp of mtDNA, but does not support that the ancient Caucasus leopard is particularly diverged
251 from modern Persian leopards (*P. p. saxicolor*; “SAX”; Additional File 1: Fig. S2). The
252 discrepancy between the coalescent times reported for the divergence between Iranian and other
253 Asian leopards (16 – 270 Ka; [24]), and that between our Caucasian sample and other lineages (148
254 – 352 Ka) could suggest that the ancient Caucasian leopard represents a distinct lineage that
255 diverged from other Asian leopards prior to the Iranian leopards, although it should be noted that
256 the credibility intervals overlap considerably. Tracing the potential contribution of the European
257 leopard to modern Caucasian or other modern leopard populations will require the analysis of
258 nuclear DNA from Pleistocene leopards. Unfortunately, the Pleistocene samples analysed in the
259 present study were not suitable for the analysis of nuclear DNA due to their low endogenous DNA
260 content, despite multiple re-sampling attempts [47].

261

262 *Java and the Asian leopard*

263

264 The Javan leopard has been assigned to the subspecies *P. p. melas* (“MEL”), based on both
265 morphological and genetic distinctiveness of these island leopards from mainland Asian populations
266 [19, 22, 36]. Previous studies found Javan leopards to represent a mitochondrial lineage that is sister
267 to the entire diversity of mainland Asian leopards [19, 22]. Due to this position as sister lineage to
268 all Asian leopards, it has been suggested that the Javan leopard represents a relict population
269 founded from a Middle Pleistocene leopard population from the South East Asian mainland, after
270 which it became isolated on the Sundaic islands (Uphyrkina et al., 2001; Wilting et al., 2016). Deep
271 divergence between mainland and Sundaic species or populations has also been found in a range of
272 other animals including terrestrial mammals [48] such as tigers [49] and leopard cats [50], but also
273 birds (Hughes et al., 2003), bats (Hughes et al., 2011), and frogs (Inger and Voris, 2001).

274
275 Under the assumption that both of our samples with unclear provenance (‘Sumatra’ and ‘South East
276 Asia’, sample codes PP3 and PP35) do in fact represent the Javan population, the molecular dating
277 performed with our mitogenome dataset suggests that the Javan leopards became established on the
278 island some time after 375 Ka (95% CI: 230 - 524 Ka). This is in line with previous estimates (393 -
279 886 Ka; [22]). Low sea levels during glacial periods exposed portions of the Sunda shelf, creating
280 land bridges connecting the mainland with the Sundaic islands [51], which would have allowed the
281 colonisation of and subsequent isolation on the South East Asian islands [22, 36]. If there was any
282 more recent gene flow between the islands and the mainland during Late Pleistocene periods of low
283 sea levels [52], it is not expected to have been at high frequency, as there has been no evidence
284 found in the mitochondrial data investigated until now. However, the sample in our dataset that was
285 reported to be of Javan origin (sample PP32) was placed as a sister lineage to the mitogenome
286 recovered from an individual from Thailand. Investigation of short mtDNA sequences confirmed
287 that the mitochondrial haplotype from this putative Javan leopard is closely related to *P. p.*
288 *delacouri* (“DEL”), the mainland South-East Asian leopard subspecies (Additional File 1: Fig. S2).
289 Trade routes between the South East Asian islands and the mainland have been reported to exist for
290 almost 2000 years [53], so leopards could have been moved to Java via such human-mitigated
291 routes – either by trading live animals or as hunting trophies. Alternatively, our results also fit a
292 scenario in which leopards migrated to the South-East Asian islands from the mainland during the
293 late Pleistocene, supplementing the original locally surviving Javan mitochondrial haplotypes with
294 mainland Asian haplotypes; the latter of which could have been lost during the drastic population
295 declines during the past century [7]. The coalescence time of this putative Javan leopard and the
296 Thai individual is estimated to be 64 Ka (95% CI: 32 - 100 Ka), which could be consistent with a
297 dispersal from the mainland to the Sundaic islands some time (e.g. during the LGM) after the Toba

298 supervolcanic eruption that occurred 73 Ka [54]. Additional sampling and/or the investigation of
299 nuclear data is needed to further clarify the origin and relatedness between mainland and Javan
300 leopards.

301

302 Our dataset further included a number of South and East Asian leopards; two Indian leopards (*P. p.*
303 *fusca*), one Indochinese *P. p. delacouri* and three published North-Chinese leopards, *P. p.*
304 *japonensis* (Table 1; “FUS”, “DEL” and “JAP”, respectively). The estimated coalescence time for
305 these individuals is relatively recent (122 Ka; 95% CI: 73 - 178 Ka). This recent divergence time
306 could point towards extensive population reductions on the Eurasian mainland during the
307 Pleistocene, leading to a bottleneck and loss of divergent lineages for all Eurasian leopards except
308 for Java and Europe. A loss of genetic diversity during the Pleistocene as suggested by recent
309 mitogenome coalescence times has also been found for other carnivores (e.g. 151 Ka for leopard
310 cats, 95% CI: 87 - 215; [50]), suggesting that this pattern may be detectable across other Asian
311 carnivores. Considering the deeper coalescence time of all modern African mtDNA lineages (600
312 Ka), the African leopards are not likely to have experienced a bottleneck to the same extent as
313 Asian leopards.

314

315

316 **Conclusions**

317

318 This study represents an example of how genetic data from ancient DNA can inform on the
319 evolutionary history of species and thereby provide additional clues on identification of fossils,
320 including those from which no genetic data can be recovered because they are beyond the expected
321 range of DNA survival, such as the 2 Ma year old fossils from Pakistan. The molecular dating of
322 mitochondrial lineages is in line with the age of the earliest unequivocal leopard fossils from
323 Eurasia, and thus it encourages a re-evaluation of the older leopard-like fossils. Our results are
324 therefore compatible with previous suggestions that older fossils should be assigned to other large-
325 bodied felids. Furthermore, similar to many other Eurasian carnivores, our data suggests that with
326 the extinction of European populations and the proposed Pleistocene population bottleneck in
327 mainland Asia, leopards experienced not only a reduction of their geographical distribution but also
328 a loss of mitochondrial lineages that, to date, have not been detected in the modern gene pool.
329 Leopard dispersal is generally driven by males, and thus may not be detectable using mitochondrial
330 DNA alone. Finally, although we do not observe any evidence in our data to suggest that ancient
331 European mitochondrial lineages persist in modern Asian populations, it is possible that at least part

332 of the genetic legacy of Pleistocene leopards survives in the modern nuclear genome, as was
333 recently shown to be the case for archaic hominins [55, 56] and the extinct cave bear [57].

334

335

336 **Methods**

337 *DNA extraction, library preparation and capture*

338 All pre-PCR steps for ancient and historical bone samples were performed in dedicated cleanroom
339 facilities of the Evolutionary Adaptive Genomics group at Potsdam University, with all standard
340 ancient DNA precautions (e.g. decontamination procedures for all reagents and materials, negative
341 controls for both extraction and library preparation). Extraction was performed following a protocol
342 optimised for the retrieval of short DNA fragments [58]. Illumina sequencing libraries were
343 constructed following a double-stranded library preparation protocol for the historical samples [59,
344 60] and a single-stranded library protocol for the ancient samples, using UDG/Endonuclease VIII to
345 excise uracils from the molecules [61]. Optimal amplification cycle numbers for each library were
346 estimated using qPCR and then used for dual-indexing PCR. Four historical samples yielded high
347 endogenous content, allowing the mitogenome sequence to be recovered using shotgun sequencing
348 (Table 1; Additional File 1: Table S1). For all remaining samples, mitogenome enrichment was
349 performed using an in-solution capture approach with synthetic baits. A bait-set targeting felid
350 mitochondrial DNA was designed by placing 52-mer probes (1 bp tiling) across the mitogenomes of
351 five felids: leopard (*Panthera pardus*, EF551002 (RefSeq: NC_010641), lion (*Panthera leo*,
352 KF776494), domestic cat (*Felis catus*, NC_001700), bobcat (*Lynx rufus*, NC_014456.1) and
353 cheetah (*Acinonyx jubatus*, NC_005212). Bait sequences containing simple repeats longer than 24
354 bp were removed [62]. Baits were synthesized, amplified and converted into biotinylated single-
355 stranded DNA probes as described elsewhere [63]. Capture was performed following the protocol in
356 Horn (2012). For ancient samples, two serial captures were performed to improve the enrichment
357 rates. For historical samples, either one or two captures were used (Table 1). Sequencing was
358 performed on the Illumina NextSeq platform using 75bp paired-end sequencing, using custom
359 sequencing primers for the single-stranded libraries [61, 64].

360

361 *Sequence processing*

362 Raw sequences were trimmed and merged using SeqPrep with default parameters and a minimum
363 length of 30bp (available from <https://github.com/jstjohn/SeqPrep>). The trimmed and merged reads
364 were then aligned to a leopard mitogenome sequence available from GenBank (Acc. Nr.

365 KP202265) using the Burrows-Wheeler Aligner (BWA aln) v0.7.8 and samtools v1.19 [65, 66] with
366 default parameters. Reads with low mapping quality (<Q30) were removed. Duplications were
367 marked and removed taking both mapping coordinates into consideration, using
368 MarkDupsByStartEnd.jar (<http://github.com/dariober/Java-cafe/tree/master/MarkDupsByStartEnd>).
369 Summary statistics can be found in Additional File 1: Table S1. Mitogenome consensus sequences
370 were retrieved using Geneious v7.0 [67], using a minimum sequence depth of 3x and a strict 90%
371 majority rule for base calling. The resulting consensus sequences for each sample were combined
372 with three mitogenome sequences available from GenBank at the time of analysis, and aligned
373 using ClustalW with default parameters ([68]; as implemented in Geneious). The control region, as
374 well as any positions in the alignment that contained missing or ambiguous data, were removed to
375 avoid any adverse influence of misalignments or numts. The resulting alignment (13,688 bp in
376 length) was manually annotated in Geneious using the published sequence (*Panthera pardus*;
377 Genbank Acc. Nr. KP202265) as reference.

378

379 *Phylogenetic analyses*

380 PartitionFinder v1.1.1 [69] was used to identify an optimal partitioning scheme from all possible
381 combinations of tRNAs, ribosomal RNA genes and protein-coding genes, considering all
382 substitution models available in BEAUti, using the Bayesian Information Criterion (BIC). The
383 partitioned maximum likelihood tree was calculated using RaxML-HPC v8.2.4 [70] on the CIPRES
384 black box version, with default substitution models for each partition, on the CIPRES Science
385 Gateway ([71]; Additional File 1: Fig. S1). Molecular dating was performed using BEAST v1.8.2
386 [72]. We adopted a two step strategy for the calibrated analyses: first, we generated a fossil-
387 calibrated phylogeny using a total of seven fossil dates (Additional File 1: Table S2) under a Yule
388 speciation model for 15 sequences from various Felidae species, including two of the most
389 divergent leopard mitogenomes. The divergence estimates recovered for the two leopard
390 mitogenomes (mean 0.81 Ma, standard deviation 0.12 Ma) was then applied as normal prior on the
391 root height for the intraspecific analyses, and were run with a Bayesian Skyline coalescent
392 population model. Preliminary analysis using a lognormal relaxed clock model failed to reject zero
393 variation in substitution rates across branches of the phylogeny, and so a strict clock model was
394 employed with an open uniform prior on the mean per-branch substitution rate of 0 to 20% per
395 million years. The MCMC chain was run for a sufficient number of generations to achieve
396 convergence (to a maximum of 10 million, sampling every 10,000 states) and adequate posterior
397 sampling of all parameters (ESS >200), checked using Tracer v1.5 (available from
398 <http://www.beast.bio.ed.ac.uk/Tracer>). TreeAnnotator v1.8.2 was then used to remove the first 25%

399 of trees as burnin (corresponding to 2,500 trees) and extract the Maximum Clade Credibility (MCC)
400 tree with nodes scaled to the median heights recovered by the posterior sample. The minimum-
401 spanning network for the short mtDNA alignment (267 individuals, 456 bp in length) was generated
402 using Popart (Additional File 1: Fig. S2) [73].

403

404 **List of abbreviations**

405

406 Ma – *Mega annus*; million years ago

407 Ka – *Kilo annus*; thousand years ago

408 mtDNA – mitochondrial DNA

409 CI – Credibility Interval

410 LGM – Last Glacial Maximum

411

412 **Declarations**

413

414 *Ethics approval and consent to participate*

415 Not applicable

416

417 *Consent for publication*

418 Not applicable

419

420 *Availability of data and material*

421 Leopard mitogenome consensus sequences will be made available on GenBank (NCBI Accession

422 Numbers MH588611-MH588632). The alignment used for phylogenetic inference and the BEAST

423 XML input file used for fossil calibration analyses will be made available upon request.

424

425 *Competing interests*

426 The authors declare that they have no competing interests.

427

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432 interpretation, or writing the manuscript.

433

434 *Authors' contributions*

435 JLAP, MH conceived the project; JLAP, MM designed the experiments; JLAP, KH, BN performed
436 the experiments; JLAP, AB analysed the data; GFB, RWH, DN, UJ, WR, contributed samples and
437 provided interpretation of their context, JLAP, MH, AB, DWF, GFB participated in fundamental
438 discussion and interpretation of the results; JLAP and AB wrote the paper with input from all
439 authors. All authors have read and approved the manuscript.

440

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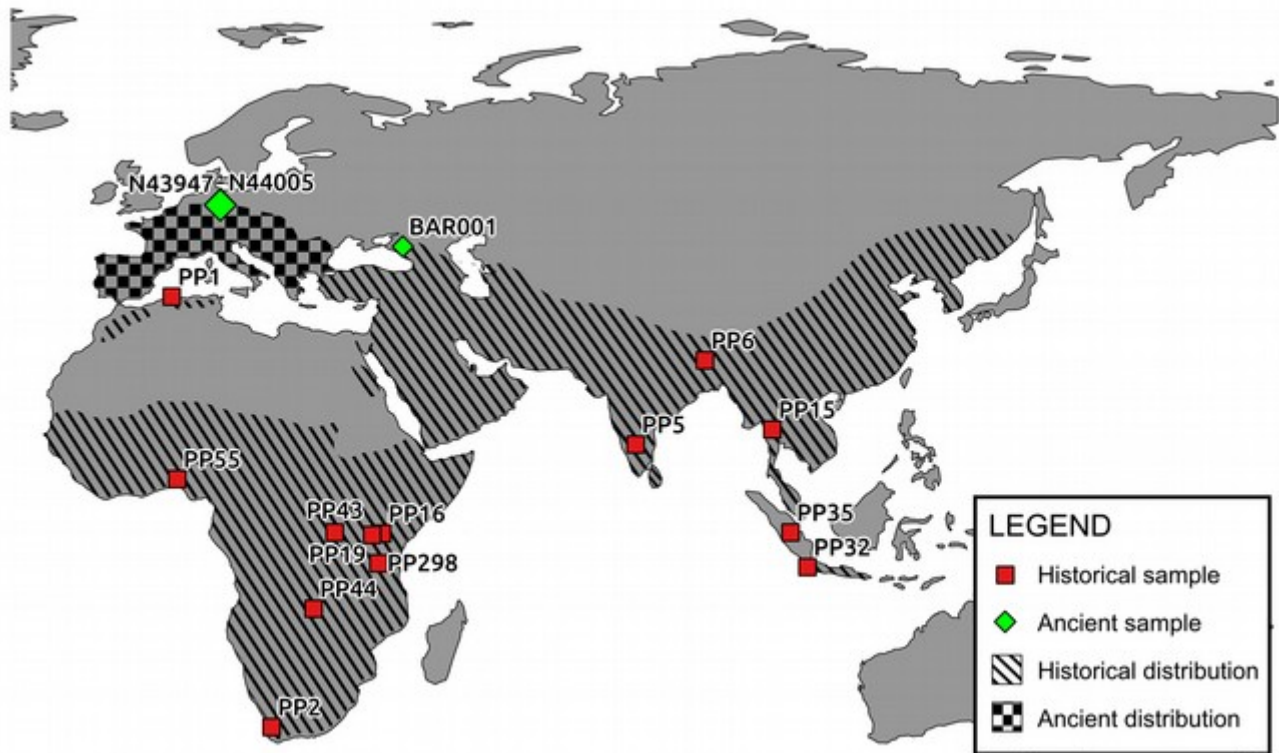
442 We would like to acknowledge Peter Rask Møller and Hans Baagøe from the Natural History
443 Museum of Denmark for providing access to samples from their zoological collection. We thank
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446

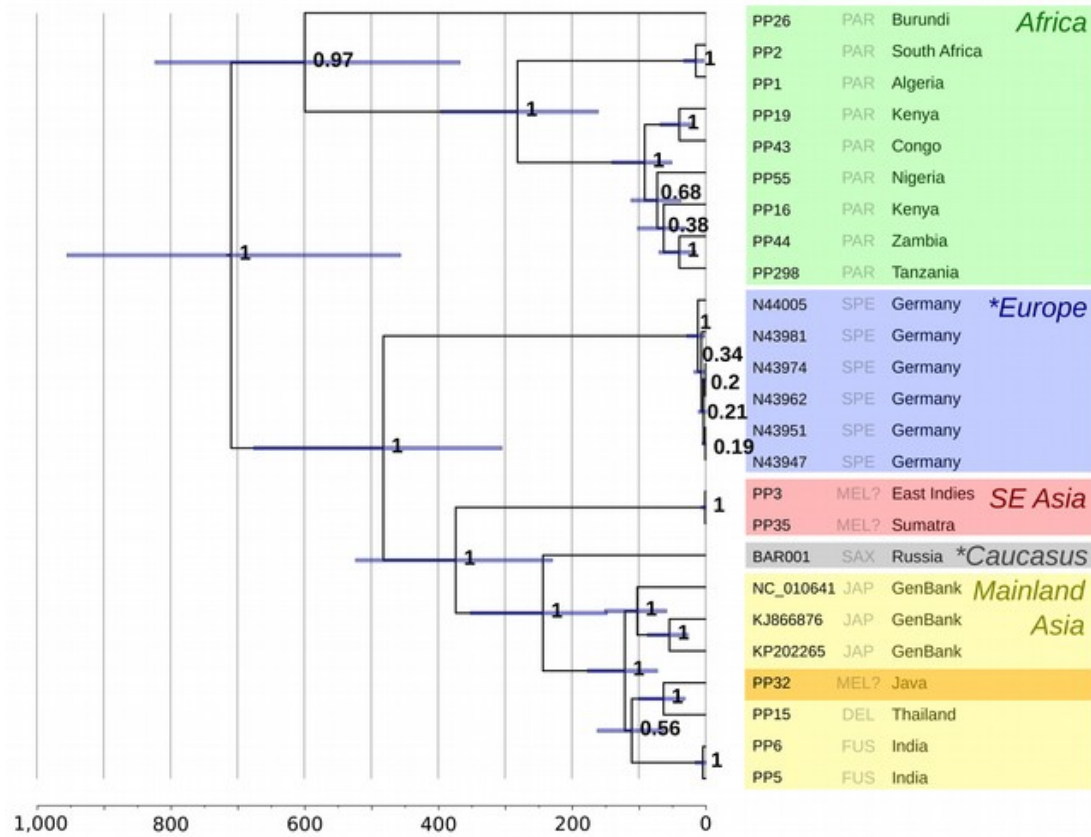
447

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448 **Figures and Tables**

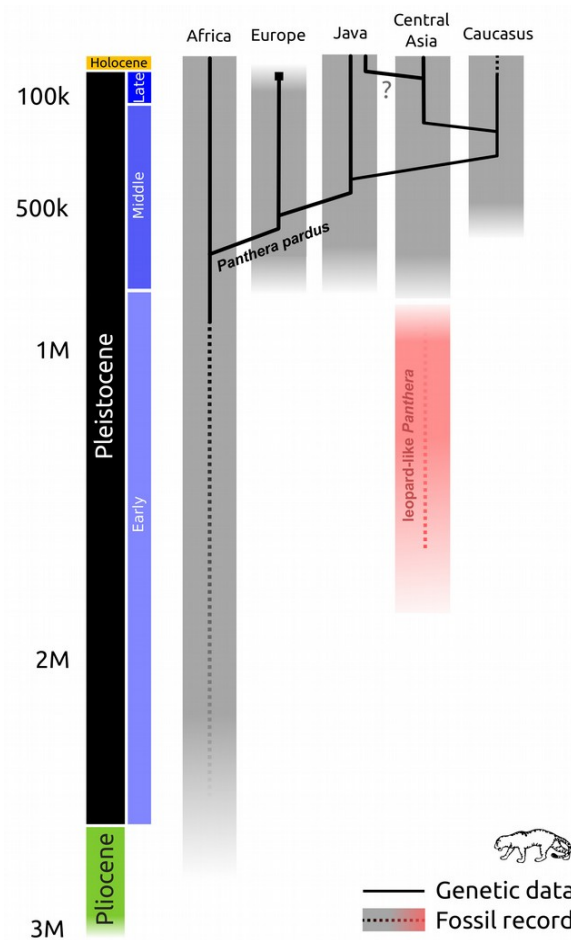


450 **Fig. 1:** Map indicating the location of the samples included in our study, with the approximate
451 historical and ancient (Pleistocene) distribution of the leopard (adapted from Uphyrkina et al., 2001;
452 Diedrich 2013). The current distribution of leopards is severely reduced compared to the historical
453 range, and highly fragmented (Jacobson et al. 2016). Sample PP3 is not displayed due to its
454 ambiguous provenance (“East Indies”).
455



458 **Fig. 2:** Calibrated mitogenomic phylogeny of 25 leopard mitogenome sequences. Node support is
 459 indicated by Bayesian Posterior Probabilities, blue node bars indicate the 95% credibility interval of
 460 divergence times. The lower axis shows the estimated coalescence times in thousands of years.
 461 Colours indicate the locality of the samples; the unexpected placement of the Javan leopard is
 462 highlighted dark yellow. The three-letter code corresponds to the putative subspecies for each
 463 individual, following Miththapala *et al.* 1996, Uphyrkina *et al.*, 2001; Diedrich 2013. Asterisks
 464 indicate the Late Pleistocene samples. The RaXML maximum likelihood phylogeny can be found in
 465 Additional File 1: Fig. S1.

466



469 **Fig. 3:** Proposed phylogeographical history for the leopard (*Panthera pardus*), comparing the
470 genetic data with the fossil record. Shaded areas indicate the age of the fossil record for *P. pardus*
471 (grey) for each region, and the disputed Asian leopard-like *Panthera* fossils (red). The solid lines
472 indicate the relationships between the mitogenome lineages recovered in this study.

473
474

475

HISTORICAL						
Museum accession number	Sample code	Origin	Date	mtDNA recovery	Collection	GenBank accession number
ZMUC 24	PP1	Algeria (North Africa)	1850	2x serial capture	Natural History Museum of Denmark	MH588618
ZMUC 25/26/206	PP2	South Africa (Cape South Africa)	Unknown	2x serial capture	Natural History Museum of Denmark	MH588619
ZMUC 27/14	PP3	East Indies (South-East Asia)	1836	1x capture	Natural History Museum of Denmark	MH588620
ZMUC 29	PP5	India (Central India)	1839	Shotgun sequencing	Natural History Museum of Denmark	MH588621
ZMUC 775	PP6	India (East India)	1884	1x capture	Natural History Museum of Denmark	MH588622
ZMUC 1967	PP15	Thailand (South-East Asia)	1924	1x capture	Natural History Museum of Denmark	MH588623
ZMUC 2115	PP16	Kenya (East Africa)	1928	2x serial capture	Natural History Museum of Denmark	MH588624
ZMUC 3490	PP19	Kenya (East Africa)	1945	Shotgun sequencing	Natural History Museum of Denmark	MH588625
ZMUC 3980	PP26	Burundi (Central-East Africa)	1880	1x capture	Natural History Museum of Denmark	MH588626
ZMUC 31	PP32	Indonesia (Java)	1848	Shotgun sequencing	Natural History Museum of Denmark	MH588627
ZMUC 34	PP35	Indonesia (Sumatra)	1866	2x serial capture	Natural History Museum of Denmark	MH588628
ZMUC 3739	PP43	DRC (Central Africa)	1951	2x serial capture	Natural History Museum of Denmark	MH588629
ZMUC 4446	PP44	Zambia (Central Africa)	1960	Shotgun sequencing	Natural History Museum of Denmark	MH588630
ZMUC 5719	PP55	Nigeria (West Africa)	1958	1x capture	Natural History Museum of Denmark	MH588631
ZMUC SC007	PP298	Tanzania (Eastern Arc)	2013	2x serial capture	Natural History Museum of Denmark	MH588632
ANCIENT						
Museum accession number	Sample code	Origin	Date	mtDNA recovery	Collection	GenBank accession number
-	BAR001	Mezmaiskaya Cave (Russia, Northern Caucasus)	> 35 Ka	2x serial capture	Zoological Institute, St. Petersburg	MH588611
N43947	N43947	Baumannshöhle (Rübeland, Germany) (Joger & Rosendahl, 2012)	~ 40 Ka	2x serial capture	Natural History Museum Braunschweig	MH588612
N43951	N43951	Baumannshöhle (Rübeland, Germany) (Joger & Rosendahl, 2012)	~ 40 Ka	2x serial capture	Natural History Museum Braunschweig	MH588613
N43962	N43962	Baumannshöhle (Rübeland, Germany) (Joger & Rosendahl, 2012)	44710+/-630*	2x serial capture	Natural History Museum Braunschweig	MH588614
N43974	N43974	Baumannshöhle (Rübeland, Germany) (Joger & Rosendahl, 2012)	~ 40 Ka	2x serial capture	Natural History Museum Braunschweig	MH588615
N43981	N43981	Baumannshöhle (Rübeland, Germany) (Joger & Rosendahl, 2012)	37880+/-300*	2x serial capture	Natural History Museum Braunschweig	MH588616
N44005	N44005	Baumannshöhle (Rübeland, Germany) (Joger & Rosendahl, 2012)	40470+/-410*	2x serial capture	Natural History Museum Braunschweig	MH588617
PUBLISHED						
NCBI GenBank accession number	Sample code	Origin		Reference		
NC_010641	NC_010641	China		Lei et al. 2011		
KJ866876	KJ866876	North-Chinese leopard		Dou et al. 2016		
KP202265	KP202265	North-Chinese leopard		Li et al. 2016		

477 **Table 1:** Samples from which a (almost) complete mitogenome was reconstructed or recovered
 478 from GenBank. Samples indicated with an asterisk are C14 dated, the date is provided in
 479 uncalibrated years before present (additional dating information is included in Additional File 1:
 480 Table S3).

481

482

483 **Additional File 1:**

484 **Fig. S1:** Maximum likelihood phylogeny, with different *Panthera* species as outgroup.

485

486 **Fig. S2:** Minimum-spanning network of short (456 bp) mtDNA sequences (ND5 gene), including
487 previously published data (based on 267 sequences in total). Number of substitutions are indicated
488 as tick-marks on the branches connecting the haplotypes. Colours indicate the subspecies [19,
489 following 25].

490

491 **Table S1:** Summarised sequence statistics for samples included in our study.

492

493 **Table S2:** Fossil constraints and calibration priors used in the time-calibrated BEAST analysis
494 performed for Felidae alignment. The resulting root age was then applied as calibration for the
495 leopards-only phylogeny.

496

497 **Table S3:** Radiocarbon dating (^{14}C) information.

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