1   **Population and conservation genomics of the world's rarest hyena species, the brown hyena**

2   **(*Parahyena brunnea*)**

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24 **Abstract**

25 With an estimated population size of less than 10,000 individuals worldwide, the brown hyena

26 (*Parahyaena brunnea*) has been listed as 'near threatened' by the IUCN. Despite this rank,

27 studies involving DNA analyses of the brown hyena are limited. Little consideration has been

28 focussed towards population structure within the brown hyena, which could provide valuable

29 insights about its evolutionary history and aid in conservation efforts of the species.

30 Here we report both mitochondrial and nuclear genomes of wild-caught brown hyena individuals

31 from across southern Africa. Mitochondrial DNA shows little to no phylogeographic structure,

32 whereas low-coverage nuclear genomes reveal several potential sub-populations. Moreover, we

33 find that brown hyenas harbour the lowest genetic diversity for a species on both the

34 mitochondrial and nuclear level when compared to a number of mammalian species for which

35 such information is currently available. Our data also reveal that at least on the nuclear DNA

36 level, this low diversity could be the result of a continuous and ongoing decline in effective

37 population size that started about one million years ago and dramatically accelerated towards the

38 end of the Pleistocene. Moreover, our findings also show that the correlation between genetic

39 diversity and the perceived risk of extinction is not particularly strong, since many species with

40 higher genetic diversity than the brown hyena are considered to be at greater risk of extinction.

41 Taken together, our results have important implications for the conservation status and

42 conservation approaches of the brown hyena.

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Introduction:

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48   With the ever-decreasing price of next generation sequencing (NGS), population

49   genomics has become a rapidly evolving field within evolutionary biology (Buerkle and

50   Gompert 2013). First appearing in the late 1990s with the analysis of large-scale datasets on

51   human single-nucleotide-polymorphisms (Hartl and Clark 1997), population genomics analyses

52   have now been implemented in the study of a range of species and has greatly aided in

53   understanding their evolution (Ellegren 2014; Liti et al. 2009; Nielsen et al. 2009). A major

54   argument in favour of the analysis of whole genomic data is that the high number of independent

55   loci available provides both power and accuracy and allows for the separation of real

56   evolutionary effects from noise. This remains true even when considering just a few individuals,

57   making genomic approaches much more versatile than more traditional methods utilising a few

58   loci and large numbers of individuals (McMahon et al. 2014).

59   Population genomic analyses have a wide variety of applications and can be used to

60   accurately gauge population structure and connectivity, taxonomic relationships, genetic

61   diversity, and the demographic history of a species (Shafer et al. 2015; Allendorf et al. 2010;

62   Steiner et al. 2013). Despite the recent rise in the number of genomic sequences obtained, many

63   of these studies are still restricted to already well-studied species such as humans, model

64   organisms and domesticates. However, the use of population genomic analyses is often

65   extremely useful in solving questions about the evolutionary history of less studied, yet

66   evolutionarily important lineages.

67   One little studied but ecologically important lineage is the family Hyaenidae (hyenas and

68   aardwolf). Hyaenidae occupy a species-poor (four extant species) branch within the Feliformia

69   suborder in a sister position to the Felidae family. However, despite its close relationship to the

70   well-studied Felidae family, of which a number of genomes have already been sequenced

71   (Abascal et al. 2016; Dobrynin et al. 2015; Cho et al. 2013), very few genetic studies have been

72   carried out on the Hyaenidae family. Members of Hyaenidae occupy a variety of different niches.

73   The most notable and arguably most important niche being that of the scavenger (Gusset and

74   Burgener 2005; Watts and Holekamp 2007). Scavengers are known to be important for

75   maintaining healthy ecosystem function with profound roles in nutrient cycling and influencing

76   disease dynamics (Benbow et al. 2015).

77   The brown hyena (*Parahyaena brunnea*) is predominantly a scavenger, mainly feeding

78   on large vertebrate carrion (Watts and Holekamp 2007). It is generally found in arid areas across

79   southern Africa and is listed as 'Near Threatened' by the International Union for Conservation of

80   Nature (IUCN). The brown hyena is the rarest of all extant hyena species with estimates of

81   population size being less than 10,000 individuals worldwide (Wiesel 2015). Despite its listing as

82   Near Threatened, brown hyenas continue to be persecuted, often considered as problem animals

83   by farmers or killed for trophy hunting. Incidental and often deliberate poisoning, shooting and

84   trapping of these animals all hamper the survival of this ecologically important species (Kent and

85   Hill 2013).

86   Genetic studies of the brown hyena are very limited but have hinted towards very low

87   genetic diversity within the species (Rohland et al. 2005; Knowles et al. 2009). Species-wide

88   genetic comparisons using a short fragment of the mitochondrial cytochrome b gene found no

89   variability in this region regardless of sample origin (Rohland et al. 2005). Moreover, a study

90   investigating population structure within Namibian brown hyena, utilising microsatellites, also

91   found no detectable population structure (Knowles et al. 2009). The inability of both of these

92   studies to find population structure perhaps stems from very low levels of genetic diversity

93   within the brown hyena. These early indications of low diversity are important to follow up on

94   and investigate. Even though genetic diversity does not necessarily correlate with current day

95   population sizes (Bazin et al. 2006; Leffler et al. 2012), it is still an important factor in

96   understanding past evolutionary events. Importantly, knowledge of the evolutionary processes

97   affecting a species is critical to inform conservation plans aimed at the long term management of

98   its evolutionary potential (Romiguier et al. 2014).

99   Here we present complete mitochondrial and nuclear genomic analyses of brown hyena

100   from populations across its range in Southern Africa (Fig. 1). We find both mitochondrial and

101   nuclear genomic diversity to be extremely low, lower than in any other mammalian species

102   published to date. Our data suggest that this low diversity results from a continuous decrease in

103   effective population size over the last million years. Moreover, our low-coverage genomes reveal

104   a number of potential sub-populations of brown hyena across its range.

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107   **Figure 1 :** Map of the sampling
108   locations of the wild-caught
109   brown hyena included in this
110   study. The red hyena indicates
111   the original area of the South
112   African population prior to
113   translocations in 2003.

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120 **Results:**
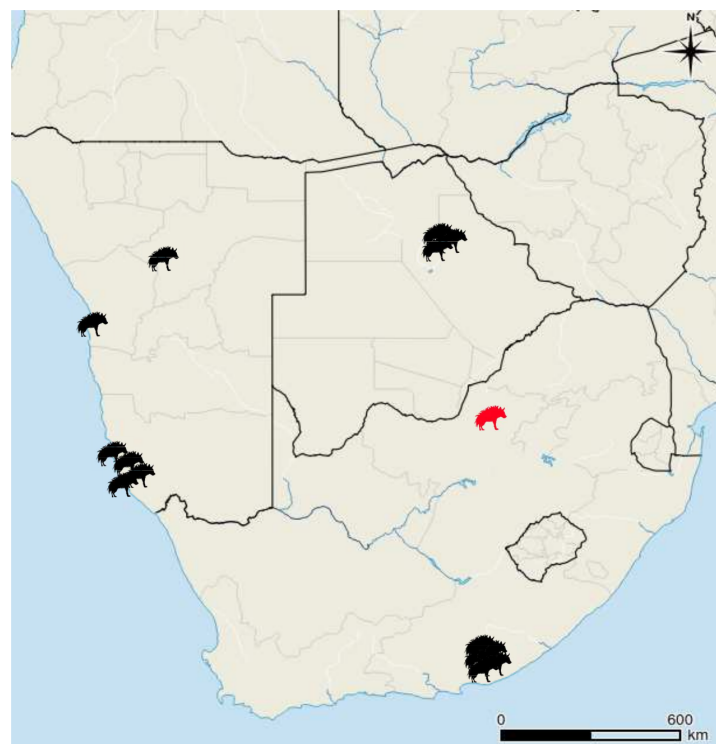
121 **Genome reconstructions**

122   A *de novo* assembly of a striped hyena nuclear genome with a scaffold N50 of ~2Mbp

123 was assembled using Allpaths LG (Butler et al. 2008), default parameters and an additional gap

124 closing step using Gapcloser (Luo et al. 2012) (Supplemental Table S1). BUSCO analyses

125 (Simão et al. 2015) using both the eukaryotic and mammalian databases show high levels of

126 complete BUSCOs (Supplemental Table S2) giving us confidence that our assembly is of good

127 quality and completeness. With this as reference, we successfully mapped low coverage nuclear

128 genomes (2.1 - 3.7x) from 14 wild-caught brown hyena individuals originating from Namibia,

129 South Africa and Botswana and a high coverage nuclear genome from a captive individual

130 (Supplemental Tables S3 and S4).

131   Due to the lack of a published brown hyena mitochondrial genome, we assembled the

132 complete *de novo* mitochondrial genome from a captive individual using MITObim (Hahn et al.

133 2013). We used default parameters apart from mismatch value, where we used zero, and three

134 different bait reference sequences (domestic cat (U20753.1), spotted hyena (JF894377.1) and

135 striped hyena (NC_020669.1)). All three independent MITObim runs produced identical brown

136 hyena mitochondrial sequences, suggesting that our reconstructed mitochondrial genome is

137 correct. We then mapped the remaining wild samples to this sequence (Supplemental Table S4).

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139 **Genetic diversity**

140   Mitochondrial DNA diversity estimates (Fig. 2) using the 14 wild caught mitogenomes

141 from our data set showed that the brown hyena has the lowest diversity when compared to a

142 number of other mammalian mitochondrial genomes as analysed in previous studies (Dobrynin

143   et al. 2015; Miller et al. 2011). All brown hyena individuals shared an average of only three

144   mutational mismatches (k) among them. This is about three times lower than the species with the

145   next lowest level of mitochondrial diversity, the tasmanian devil (*Sarcophilus harrisii*), with a k

146   value of 10, and 30 times lower than the gorilla (consisting of both *Gorilla gorilla* and *Gorilla*

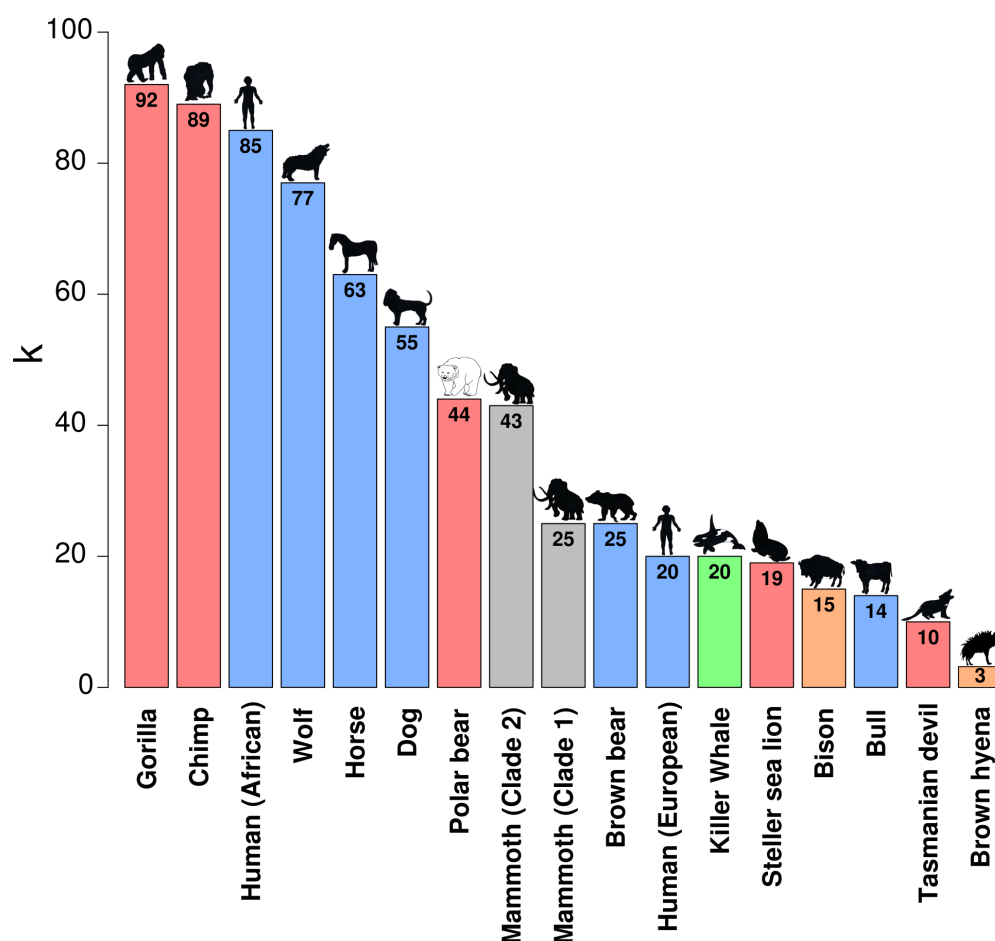147   *beringei*), which has a k value of 92.

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150   **Figure 2:** Mitochondrial diversity comparisons of the brown hyena to a number of other
151   species/populations for which such data are available (Dobrynin et al. 2015; Miller et al. 2011). k
152   represents the average number of substitutions expected between two randomly selected
153   individuals of the same population, bar colours represent conservation status (red - endangered,
154   grey - extinct, orange - near threatened, blue - least concern, green - unavailable) according to
155   the IUCN.

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159   We also calculated genome-wide nuclear heterozygosity estimates of our high coverage

160   captive individual as these are considered a good proxy for nuclear genomic diversity. Results

161   show the brown hyena to have the lowest of any species included in this study, most of which are

162   considered "endangered" (Fig. 3A). These results are consistent with the very low levels of

163   diversity we found within the mitochondrial genome.

164   The brown hyena individual sequenced to high coverage is known to have been born from wild-

165   caught parents, albeit in a captive environment, and should not display large amounts of

166   inbreeding that can be found in captive bred populations. However, to investigate signs of

167   inbreeding, we measured levels of heterozygosity in various window sizes across the brown

168   hyena nuclear genome. The results show no considerable stretches of homozygosity

169   (Supplemental Fig. S1), suggesting that there are no significant signs of inbreeding.

170   We further analysed the distribution of heterozygosity across the genome by randomly selecting

171   1000 non-overlapping windows of 1Mbp in size and comparing the results to the four other

172   species with the lowest heterozygosity included in this study, the orca

173   (*Orcinus orca*), cheetah (*Acinonyx jubatus*), Channel Island fox (*Urocyon littoralis*) and Iberian

174   lynx (*Lynx pardinus*) (Fig. 3B). The brown hyena genome has consistently lower levels of

175   heterozygosity across the genome when compared to the Iberian lynx, orca and cheetah,

176   excluding the windows with the lowest levels of heterozygosity which may indicate higher levels

177   of inbreeding in the other species. However, it can be seen that, while the brown hyena has a

178   lower level of average genome-wide diversity, for the majority of the windows, the Channel

179   Island fox has lower heterozygosity than the brown hyena. Regions of high levels of

180   heterozygosity amongst a sea of low heterozygosity have been previously reported for the

181   Channel Island fox (Robinson et al. 2016). Moreover, it should also be noted that as each

182 estimate only shows the genomic diversity of a single individual and levels are expected to vary

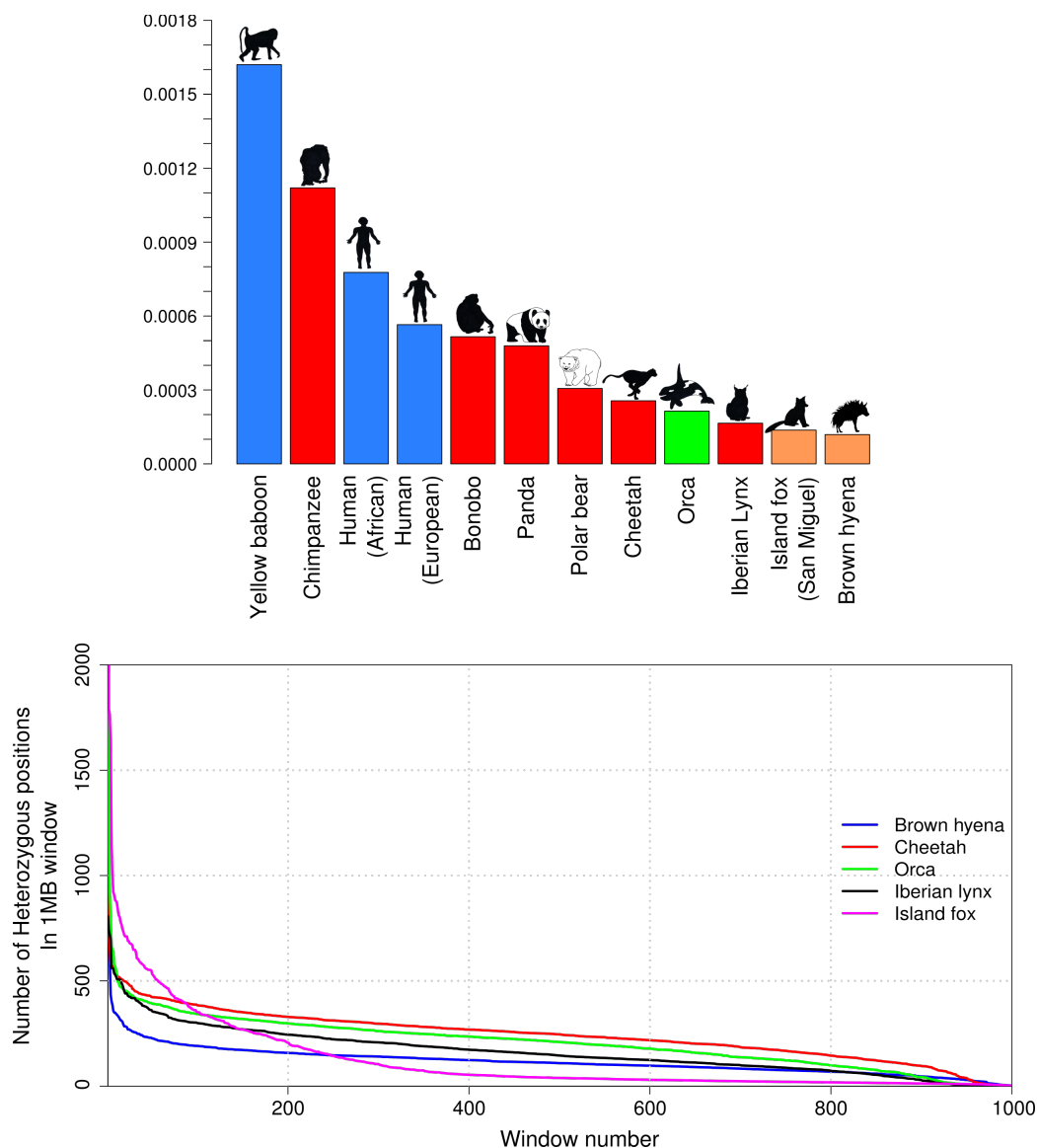183 within natural populations, some caution must be taken when interpreting these results.

Figure 3: Estimated heterozygosity levels in the brown hyena and a comparison to other mammalian species. **A)** Average genome-wide heterozygosity comparisons. Y axis represents the average proportion of sites within the nuclear genome to be heterozygous. Bar colours represent conservation status (red - endangered, orange - near threatened, blue - least concern, green - insufficient data) according to the IUCN. **B)** Heterozygosity density comparisons between the four species with the lowest estimated average genome-wide heterozygosity levels in this study. Y axis represents the number of heterozygous sites within the 1Mbp window. X axis represents the window. Colours represent species (blue - brown hyena, black - Iberian lynx, green - orca, red - cheetah, magenta - Island fox).

Demographic history

As sex chromosomes are known to have a different demographic history than autosomes, we found and removed scaffolds in our assembly related to the X chromosome before running demographic analyses. We aligned our striped hyena assembly by synteny using Satsuma (Grabherr et al. 2010) to the cat X chromosome (126,427,096bp) and found 195 scaffolds (Supplemental Table S5), totaling 117,479,157bp. The alignment was visualised using Circos (Krzywinski et al. 2009) (Supplemental Fig. S2). The Circos alignment shows that the scaffolds found using Satsuma cover the complete cat X chromosome. These scaffolds were then removed before running a Pairwise Sequentially Markovian Coalescent (PSMC) model (Li and Durbin 2011). PSMC analyses using the brown hyena autosomes were consistent with its low genomic diversity (Fig. 4; Supplemental Fig. S3) and revealed a continuous gradual decrease in effective population size over the last one million years. This was then followed by a more rapid recent decrease in effective population size at the end of the Late Pleistocene.
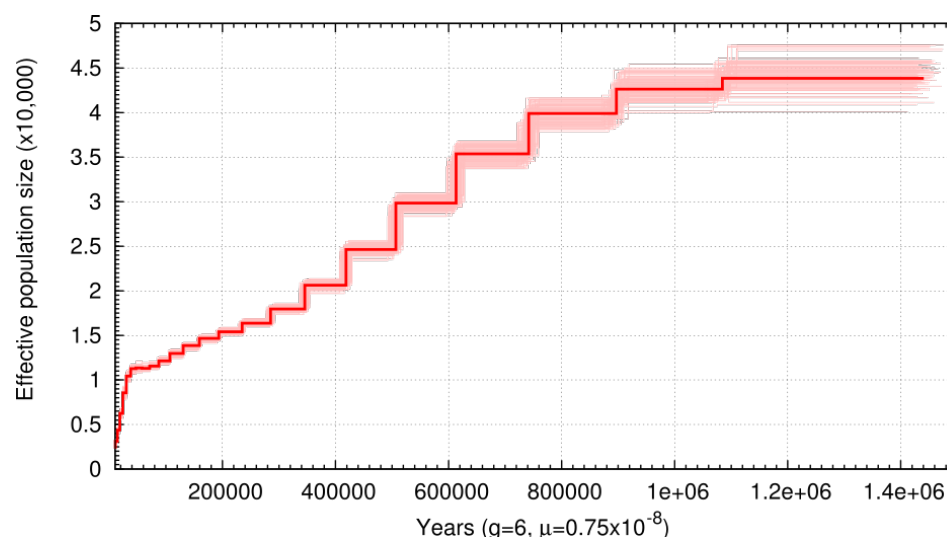

Figure 4: Pairwise Sequentially Markovian Coalescent (PSMC) model plot of the autosomes of one high coverage brown hyena. The Y axis represents effective population size and the X axis represents time in years. Light red bars show bootstrap support values. Calibrated using a generation time (g) of 6 years and a mutation rate (μ) of 7.5x10-9 per generation.

240 **Population structure**

241   In order to infer potential population structure, we reconstructed a haplotype network

242 using the mitochondrial genomes of the wild-caught individuals. This network revealed some

243 phylogeographic structure with all but one haplotype being geographically restricted. This one

244 shared haplotype was shared among individuals from all three sampled countries (Fig. 5A).

245   We then used the mapped genomes of the wild-caught individuals to infer nuclear

246 population structure by carrying out principal component analyses (PCA) and maximum

247 likelihood (ML) phylogenetic analyses. The results showed that clusterings or clades of

248 individuals were in all cases consistent with the geographical origin of the individuals (Fig. 5B;

249 Supplemental Fig. S4). PCA analyses using both single base identity by state (IBS) and genotype

250 likelihoods (GL) produced similar results (Fig. 5B; Supplemental Fig. S5).

251   In contrast to whole nuclear genomic data, both PCA and phylogenetic analyses using

252 single scaffolds did not produce unanimous phylogeographic results. Independent PCA results

253 for the nine longest scaffolds can be seen in Supplemental Fig. S6. Although they do generally

254 support some phylogeographic structure, individuals from different regions partially intermingle

255 in these plots. When running independent per-scaffold ML phylogenetic analyses with scaffolds

256 over 2MB, we find that in 49 out of 333 trees, the South African samples form a monophyletic

257 clade, in 72 out of 333 trees the Botswana samples form a monophyletic clade and in 17 out of

258 333 trees the six Namibian samples form a monophyletic clade. This non-unanimous pattern

259 shows up as clouds surrounding all nodes within the brown hyena lineage when visualising all

260 independent ML trees simultaneously using Densitree (Bouckaert 2010) (Fig. 5C). Although few

261 individual trees support monophyly of either of the three geographical groups, the root canal

262 (consensus tree with highest clade support), defined by Densitree, nevertheless shows a similar

263   topology as the tree constructed using the complete nuclear genome (Fig. 5C; Supplemental Fig.

264   S4). Importantly, it is again fully consistent with the geographical origin of the individuals.
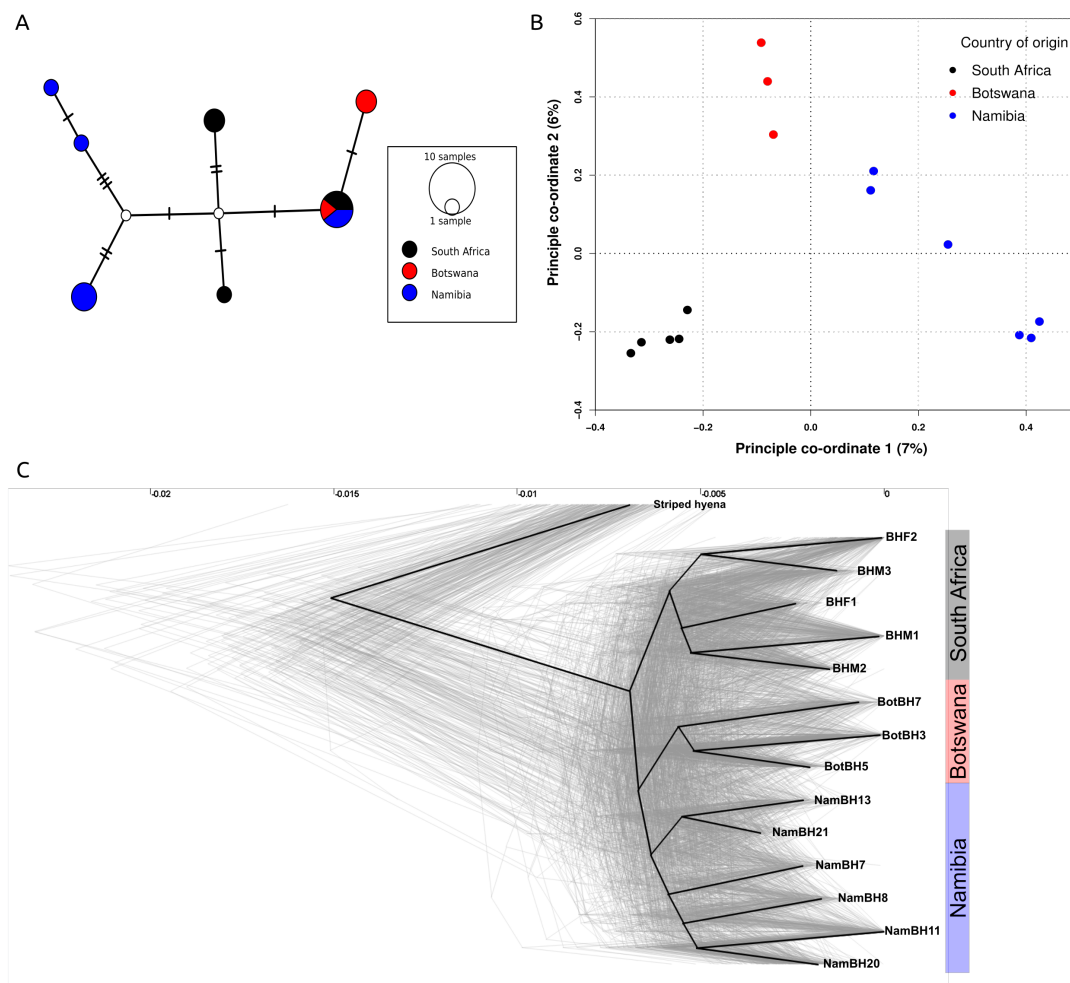
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Figure 5: Genomic diversity analyses **A)** Median joining haplotype network of the 14 wild-caught brown hyena individuals included in this study. Lines on the connecting branches represent single base differences, size of the circle represents number of individuals belonging to a single haplotype and colours represent sampling country (black - South Africa, blue - Namibia, red - Botswana). **B)** Principal components analysis produced using genotype likelihoods for the low coverage genomes of the 14 wild-caught brown hyena individuals in this study. Colours represent country of origin (black - South Africa, blue - Namibia, red - Botswana). Percentages on the X and Y axis represent the percentage of variance explained by each respective component. **C)** Densitree phylogenetic tree. Light grey lines represent phylogenetic trees produced from single scaffolds. The dark black line represents the root canal as defined by Densitree.

289   Admixture and population structure analyses using NGSadmix (Skotte et al. 2013)

290   reached convergence for K values of two to five, indicating that there may be anywhere from two

291   to five different populations inhabiting southern Africa (Fig. 6). When considering a K value

292   most congruent with the number of countries of origin, K3 (Fig. 6B), individuals NamBH13 and

293   NamBH21 stand out as being "admixed" between all three populations. This could be a

294   byproduct of the analysis trying to find a population for them. A K value of four however, shows

295   them as an independent population (Fig. 6C). The latter is the most parsimonious result as it is

296   consistent with their geographic origins (northern Namibia while all other Namibian samples are

297   from the south) and previous findings investigating the distribution patterns of individuals across

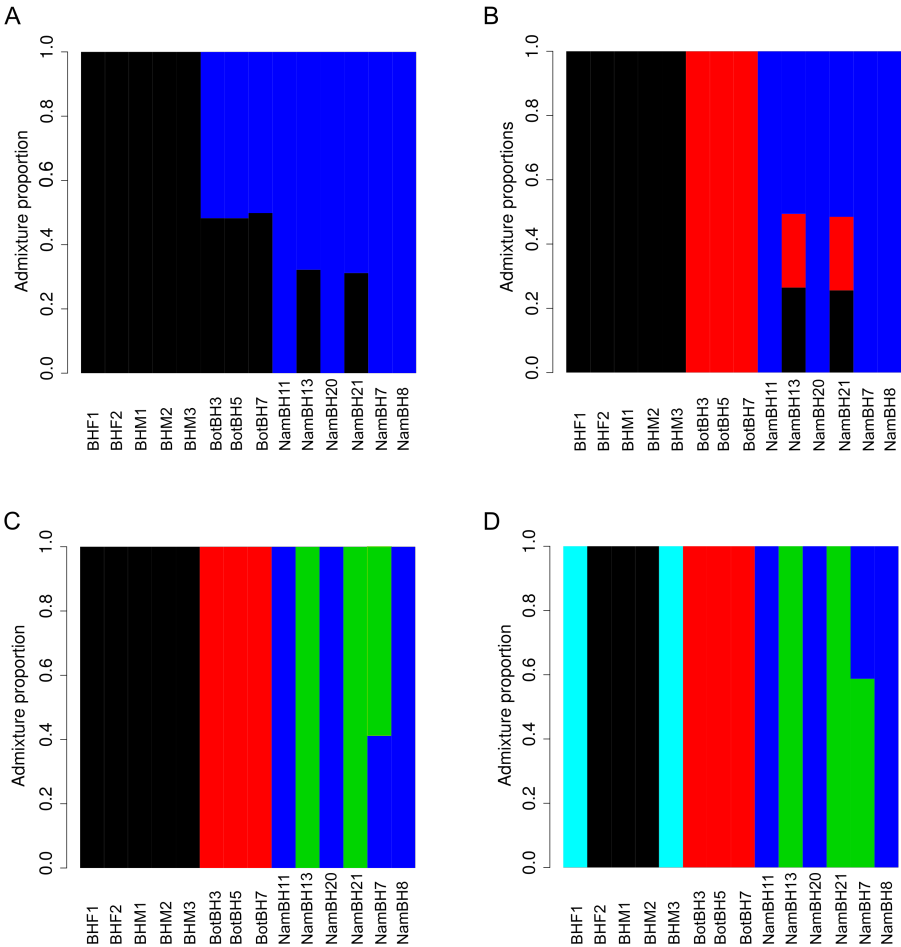298   Namibia (Supplemental Fig. S7).

Figure 6: Admixture plots produced using different K values in NGSAdmix. **A)** K2 **B)** K3 **C)** K4 **D)** K5. Y axis represents the admixture proportion and x axis represents the individual.

321   We carried out D-statistics comparisons testing for population structure (Fig. 7). A high

322   D-value could either represent differential levels of admixture or more recent common ancestry

323   and therefore an incorrect predefined topology. Taking the latter into account, we placed

324   individuals into one of three (defined as: Namibia, Botswana, or South Africa) or one of four

325   (defined as: northern Namibia, southern Namibia, Botswana, or South Africa) predefined

326   populations and compared the D values produced from "correct" topologies, i.e branches H1 and

327   H2 belong within the same population (e.g. Fig. 7A), and "incorrect" topologies, i.e branches H2

328   and H3 belong within the same population (e.g. Fig. 7B). When considering four populations

329   (Fig. 7C) it can consistently be seen that when we break the predefined population structure and

330   therefore topology, we find a higher D value than when individuals in the same predefined

331   population are in the H1 and H2 positions. Assuming that the correct topology is that with the

332   lowest D value and as there is clear separation between D values recovered when breaking and

333   not breaking the predetermined population structure when using four predefined populations, we

334   conclude that there are indeed four populations within our dataset. This pattern is not seen when

335   only three predefined populations (Fig. 7D) are considered as there are many overlapping D

336   values within the Namibian population when "correct" and "incorrect" topologies are tested. This

337   led us to reject a possibility of one single population within Namibia. These results suggest four

338   populations, with a split between northern and southern Namibia. This analysis revealed the

339   same population structure as the NGSadmix analyses, thus corroborating the observation that this

340   dataset consists of four populations rather than three.
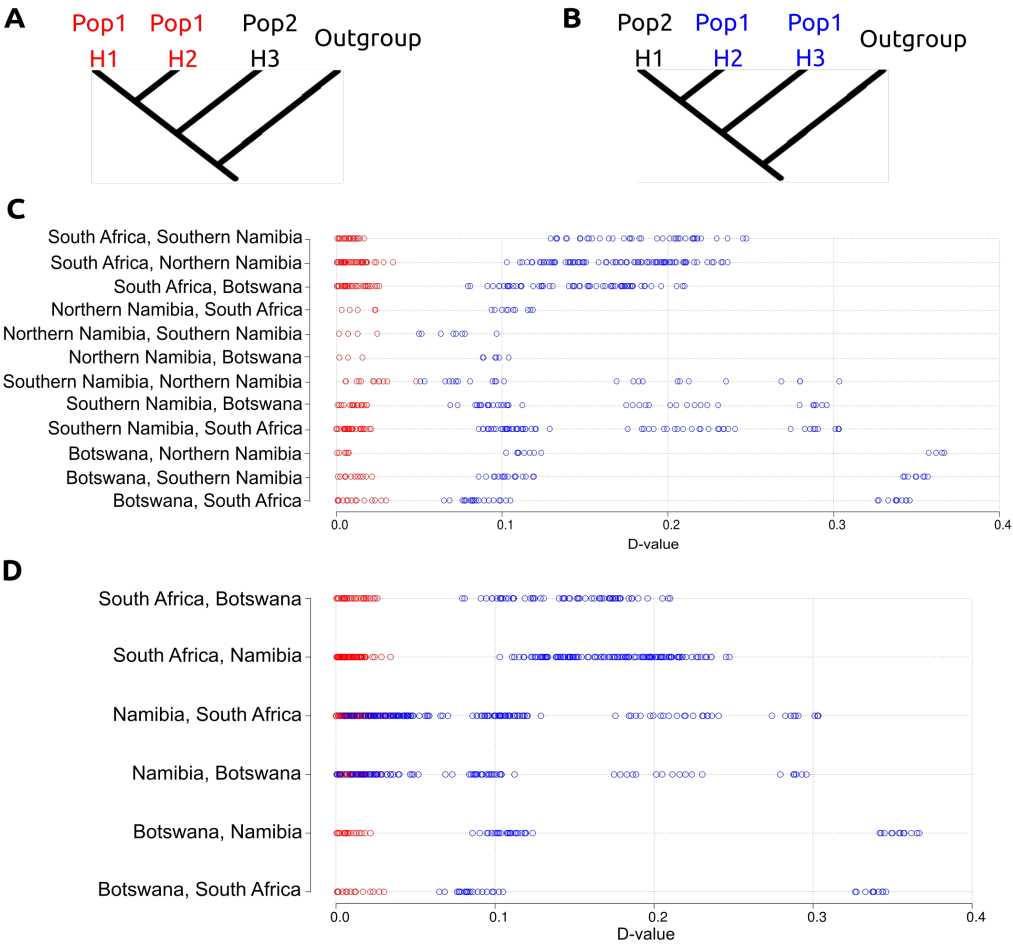
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Figure 7: Topology test using D-statistics. **A)** D-statistic analysis demonstrating the correct predetermined population structure. **B)** D-statistic analysis demonstrating the incorrect predetermined population structure. **C)** D-statistics comparisons when four "populations" are determined a priori. **D)** D-statistics comparisons when three "populations" are determined a priori. Red coloured circles represent comparisons when the predefined population structure is not broken within the comparison. Blue coloured circles represent comparisons when the predefined population structure is broken within the comparison. X axis represents the D-value.

We carried out IBS analyses on a number of other mammalian species for which species-wide low coverage data was available in order to compare these with the population structure within the brown hyena. Each species, including the brown hyena, had 10 representatives randomly selected and pairwise distance comparisons were carried out within each species (Supplemental Figure. S8). For the brown hyena, most pairwise distance values clustered

359 together, representing a relatively high level of shared diversity between different putative

360 populations and suggesting extensive gene flow among these populations. This pattern was not

361 as strong, however, as in some species, such as domesticated sheep which are known to be fairly

362 panmictic (Peter et al. 2007) and still suggests some level of differentiation within the brown

363 hyena. We also found no abnormalities, i.e. potential subspecies, large differences between

364 populations or indications that a number of the individuals were extraordinarily related which

365 could be driving the "population" signal we find in the brown hyena.

366

367 **Discussion:**

368 Hyaenidae occupy a major, albeit species poor branch within Feliformia. The family has

369 a rich fossil history but is now restricted to only four extant species (Koepfli et al. 2006). The

370 abundance of fossil specimens has led to a number of studies focused on the taxonomic

371 relationships within fossil Hyaenidae (Werdelin and Solounias 1991; Turner et al. 2008). Little

372 attention, however, has been focused towards the evolution and population status of the extant

373 species, especially on a molecular level. Despite the brown hyenas rarity, molecular analyses of

374 the brown hyena are currently limited to two studies on microsatellites and short regions of

375 mitochondrial DNA (Rohland et al. 2005; Knowles et al. 2009). Both hinted at low genetic

376 diversity but had very limited results with regard to population structure.

377

378 **Genomic diversity**

379 Using complete nuclear and mitochondrial genomes of 14 wild-caught brown hyena

380 originating from across southern Africa, brown hyenas displayed the lowest level of both

381 mitochondrial and nuclear genomic diversity when compared against other mammalian species

382   for which comparable data were available, including many endangered species (Fig. 2; Fig. 3A).

383   Genomic diversity was even lower than in species famously known to have extremely low levels

384   of genomic diversity, such as the cheetah (Dobrynin et al. 2015) and the Iberian lynx (Abascal et

385   al. 2016), both of which have gained considerable research attention because of this

386   characteristic. In addition to finding very low levels of genomic diversity, demographic analyses

387   of the brown hyena show a gradual, yet steady decline in effective population size over the last

388   million years, with a more rapid decline towards the end of the Late Pleistocene (Fig. 4). The

389   brown hyena is known to have once had a more extensive range, with Middle Pleistocene fossils

390   having been found in Kenya (Werdelin and Barthelme 1997). The continuous decrease in

391   effective population size and low levels of genomic diversity seen today may have occurred with

392   the shrinking of suitable habitats during the Pleistocene (deMenocal 2004) and potentially

393   coincided with the migration of new competitors, such as jackals, into Africa (Koepfli et al.

394   2015).

395   Interestingly, despite very low levels of genetic diversity within both the nuclear and

396   mitochondrial genomes, we found no strong signs of inbreeding in the nuclear genome (Fig. 3B;

397   Supplemental Fig. S1). This was an unexpected result as low genomic diversity is generally

398   expected to arise from high levels of inbreeding (Willoughby et al. 2015). Low genomic

399   diversity has commonly been associated with decreased fitness and higher extinction rates

400   (Spielman et al. 2004; Reed and Frankham 2003). However, the lack of detectable inbreeding in

401   our high coverage individual could mean that this species has evolved into a state of genetic

402   stasis, allowing low genetic diversity to persist across the genome, not strongly influencing the

403   survivability of the species. This genetic stasis could have evolved as a result of the slow but

404   continuous decrease in effective population size seen over the last million years and lack of

405   detectable bottlenecks during that time (Fig. 4). This slow continuous decrease could hamper the

406   influence of genetic drift therefore allowing purifying selection to maintain variability in a

407   number of loci potentially important for the survival of the species. The retention of relatively

408   high levels of potentially important adaptive variation allowing for the retention of evolutionary

409   potential has recently been found in Channel Island foxes (Robinson et al. 2016). Heterozygosity

410   hotspots were shown to be present in a number of genes with high levels of ancestral variation,

411   despite low levels of genome-wide heterozygosity in the Channel Island fox. However, as we see

412   heterozygosity to be fairly evenly distributed across the genome, this doesn't seem to be the case

413   for the brown hyena and it has perhaps been able to persist despite the lack of adaptive variation

414   due to some other factor. Further research would however be required to definitively know

415   whether the brown hyena goes against the commonly perceived notion that high levels of

416   adaptive variation are required to ensure a species survival.

417

418   **Brown hyena population structure**

419   By using a combination of different population structure analyses on the entire nuclear

420   genome we were also able to, for the first time, define population structure within the brown

421   hyena (Fig. 5; Fig. 6 and Fig. 7) despite its very low levels of genetic diversity. Results were in

422   concordance with geographic structuring. Furthermore, by comparing these results to those

423   produced using mitochondrial genomes and single scaffolds, we show that even when using

424   millions of loci (i.e. single scaffolds >2Mbp (Fig. 5C; Supplemental Fig S6)) difficulties with

425   accurately defining population structure can arise. This maybe due to the very low genomic

426   diversity within the brown hyena. This finding reinforces the value of using whole nuclear

427   genomes in population analyses over other approaches such as microsatellites and reduced

428   genomic representation techniques, especially in species suspected to have low genomic

429   diversity.

430

431   **Conservation implications**

432   Our findings provide a greater understanding into the evolution and population structure

433   of the brown hyena, which has wider implications in aiding conservation of this species. Due to

434   the increase in agriculture, many habitable areas for the brown hyena have now been fragmented,

435   and the connectivity between populations has therefore been reduced. The wide geographical

436   range of the brown hyena, coupled with the extremely low levels of genomic diversity, indicates

437   that the brown hyena persists at low population densities, which is in concordance with previous

438   studies analysing natural population densities (Kent and Hill 2013). This demonstrates the

439   importance of connectivity within the species. Given the overall evidence for substantial gene

440   flow between the different populations, well implemented translocations may certainly become

441   important for the survivability of this species in the future. With better definitions of population

442   boundaries, translocations can be implemented in such a way as to avoid outbreeding depression

443   and increase the chances of an animal successfully adapting to its new environment (Allendorf et

444   al. 2010). Moreover, previous studies have shown that translocated brown hyena are able to settle

445   into their new environments and do not return to their original locations (Weise et al. 2015), and

446   that human mediated translocations can lead to the rise of highly dense and successful

447   populations (Welch and Parker 2016). These studies further demonstrate the ability of this

448   species to adapt and accept translocations, and the utility these techniques could have in the

449   future.

450

451 **Methods:**

452

453 **Samples**

454   In total, 15 brown hyena samples were used for this study; five from South Africa, six

455 from Namibia, three from Botswana (Fig. 1) and one from Tierpark Berlin (Supplemental Table

456 S3). One female striped hyena (*Hyaena hyaena*) from Tierpark Berlin was also included to be

457 used for reference based mapping.

458

459 **Striped hyena *de novo* assembly**

460   We extracted DNA from Hyena2069, the Tierpark Berlin striped hyena sample, on a

461 KingFisher Duo robot using the blood DNA extraction kit according to the manufacturer's

462 instructions. The extract was then built into two PCR free Truseq Illumina sequencing libraries,

463 one with 180bp and one with 670bp short inserts. Two Nextera mate-pair libraries were also

464 constructed with sizes of 3kbp and 6kbp. All of the above-mentioned libraries were constructed

465 at the National Genomics Infrastructure (NGI) in Stockholm. All libraries were then sequenced

466 on an Illumina HighseqX using 2x150bp paired-end sequencing at the NGI in Stockholm. The

467 180bp and 670bp insert libraries were sequenced on one lane each, whereas the mate-pair

468 libraries were multiplexed and sequenced together on a single lane.

469   The NGI trimmed Illumina adapter sequences from the raw illumina reads using

470 Trimmomatic (Bolger et al. 2014) and performed a *de novo* assembly using Allpaths LG (Butler

471 et al. 2008) with default parameters. We then performed an additional gap closing step using

472 Gapcloser (Luo et al. 2012). Assembly quality and completeness was assessed using BUSCOv2

473   (Simão et al. 2015) using both the eukaryote and mammalian BUSCO databases (Supplemental

474   Table S2).

475

476   **Captive brown hyena sample**

477   We extracted DNA from a single brown hyena sample from Tierpark Berlin on a

478   KingFisher Duo robot using the cell and tissue DNA extraction kit. The extract was then built

479   into a PCR free Truseq Illumina sequencing library using a 350bp insert size by the NGI in

480   Stockholm. This library was then sequenced on an Illumina Highseq X using 2x150bp paired-

481   end sequencing at the NGI in Stockholm.

482

483   **Wild-caught brown hyena samples**

484   We extracted DNA from the six blood and three tissue samples using a DNeasy blood and

485   tissue extraction kit, following the manufacturer's protocol. We extracted the five hair samples

486   using the DY04 user modified version of the DNeasy kit and protocol. We fragmented DNA

487   extracts using a Covaris sonicator into ~500bp fragments. Fragmented extracts were then

488   constructed into Illumina sequencing libraries using a modified version of the protocol set out by

489   Meyer and Kircher (Meyer and Kircher 2010; Fortes and Paijmans 2015). Library molecules

490   from 400bp to 900bp were selected using a Pippin Prep Instrument (Sage Science) and

491   sequenced on an Illumina Nextseq 500 at Potsdam University, Germany.

492

493   **Raw data treatment**

494   We trimmed Illumina adapter sequences and removed reads shorter than 30bp from the

495   raw reads of the 15 brown hyena samples using Cutadapt v1.8.1 (Martin 2011) and merged

496   overlapping reads using FLASH v1.2.1 (Magoč and Salzberg 2011).

497

498   **Mitochondrial genome reconstruction**

499   As no brown hyena mitochondrial sequence was available, we reconstructed one using

500   the shotgun data from our single high coverage individual. We assembled the mitochondrial

501   genome through iterative mapping using MITObimv1.8 (Hahn et al. 2013) on 40 million

502   trimmed and merged reads, subsampled using seqtk (Li 2012). We removed duplicate reads using

503   prinseq (Schmieder and Edwards 2011). MITObim was performed in three independent runs

504   using three different starting bait reference sequences. The references included the domestic cat

505   (U20753.1), spotted hyena (JF894377.1) and striped hyena (NC_020669.1). We implemented

506   MITObim using default parameters apart from mismatch value where we used zero. Output maf

507   files were converted to sam files and visualized using Geneious v9.0.5 (Kearse et al. 2012).

508   Consensus sequences were constructed in Geneious using a 75% base call consensus threshold,

509   and only sites with over 20x coverage were considered.

510   The reconstructed mitochondrion served as a reference sequence for subsequent

511   mitochondrial mapping analyses. We mapped the trimmed and merged reads from our 14 wild

512   brown hyenas to the reconstructed reference sequence using BWAv0.7.15 (Li and Durbin 2009),

513   using the mem algorithm and default parameters and parsed the mapped files using Samtools

514   v1.3.1 (Li et al. 2009). The consensus sequences were constructed using ANGSDv0.913

515   (Korneliussen et al. 2014), only considering reads and bases of quality scores greater than 25.

516

517 **Mitochondrial analyses**

518 The mitochondrial genomes from the 14 wild-caught brown hyena were aligned using

519 Mafftv7.271 (Katoh and Standley 2013). We constructed a median joining haplotype network of

520 the alignment using Popart (Leigh and Bryant 2015). Nucleotide diversity (pi = 0.000140667)

521 was estimated using Popart. The k value was calculated by multiplying the pi value by the total

522 length of the brown hyena mitochondria. This number was then compared against those from

523 previous studies (Dobrynin et al. 2015; Miller et al. 2011) (Fig. 1B).

524

525 **Low coverage nuclear genome analyses**

526 Trimmed and merged data were mapped to the *de novo* striped hyena assembly using

527 BWA v0.7.15 (Li and Durbin 2009) and parsed using Samtools v1.3.1 (Li and Durbin 2009).

528 We applied the following filtering options for all analyses involving ANGSD (Korneliussen et al.

529 2014): we only considered sites where at least 10 individuals had coverage (-minInd 10), only

530 included sites for which the per-site coverage across all individuals was less than 75. We

531 implemented quality filtering by setting a minimum base quality score of 25 (-minQ 25),

532 minimum mapping quality score of 25 (-minMapQ 25) and only allowed reads that mapped

533 uniquely to one location (-unique_only 1). We also adjusted quality scores around indels (-baq 1)

534 (Li 2011).

535

536 **Brown hyena population structure**

537 Principle component analyses (PCA) were carried out using both single read IBS analyses and

538 GL analyses in ANGSDv0.913 (Korneliussen et al. 2014). IBS analyses were restricted to SNPs

539 occurring in at least two individuals. This was done to remove singletons which could represent

540   sequencing errors. We computed genotype likelihood in ANGSD and converted outputs to a

541   covariance matrix using ngsTools (Fumagalli et al. 2014). Covariance matrices were converted

542   into PCA outputs and visualized using R (R Development Core Team 2008). For the

543   phylogenetic analyses, we performed Maximum likelihood analyses with Raxml v8.2.10

544   (Stamatakis 2014), specifying the striped hyena as outgroup and using the GTR+GAMMA

545   substitution model. We prepared the infile for this by computing consensus sequences using

546   ANSGD with the above-mentioned filters. We then performed genome-wide alignments,

547   removed sites with missing data in three or more individuals, sites where singletons occurred

548   within the brown hyena ingroup and invariant site positions using a custom Perl script.

549   We then repeated the phylogenetic and IBS PCA analyses using single scaffolds. PCA analyses

550   were carried out using nine independent analyses (scaffolds 0-8) and Maximum likelihood

551   analyses were carried out independently for single scaffolds with a length larger than 2MB. We

552   calculated admixture proportions using NGSadmix (Skotte et al. 2013) setting K values from 2-

553   7. We used ANGSD genotype likelihood values as input, only including SNPs with a p value of

554   less than 1e-6. NGSadmix analyses were repeated a maximum of 100 times per K. Only those

555   that converged (produced a consistently identical likelihood score) within these 100 analyses

556   were considered as meaningful. D-statistic analyses were implemented in ANGSDv0.913,

557   sampling a single base per site while specifying the striped hyena as outgroup with default

558   parameters.

559

560   **Comparative population structures**

561   In order to compare the population structure within the brown hyena to those of other species, 10

562   individuals per species were randomly selected for a number of different mammalian species for

563   which such data was publically available (Supplemental Table S6). Comparisons between

564   individuals were performed using single base IBS, only considering sites where at least 7

565   individuals had coverage and SNPs that occurred in at least 2 individuals. Other filtering options

566   included; a minimum base quality score of 25 (-minQ 25), minimum mapping quality score of 25

567   (-minMapQ 25) and reads that mapped uniquely to one location (-unique_only 1). Quality scores

568   around indels were also adjusted for (-baq 1).

569

570   **Species heterozygosity estimates.**

571   High coverage, single individual representatives for a number of species were assessed for

572   heterozygosity levels. Raw data were selected from a range of different species (Supplemental

573   Table S7). Raw reads were all treated comparably, using Cutadapt v1.8.1 (Martin 2011) to trim

574   Illumina adapter sequences and FLASH v1.2.1 (Magoč and Salzberg 2011) to merge overlapping

575   reads. We mapped each species to its respective reference sequence using BWAv0.7.15 (Li and

576   Durbin 2009) and processed the mapped reads further using Samtools v1.3.1 (Li et al. 2009). To

577   adjust for biases introduced by unequal levels of coverage, the resulting bam files were all

578   subsampled to 20x using Samtools (Li et al. 2009). The folded site frequency spectrum and

579   therefore heterozygosity was then calculated from sample allele frequencies, taking genotype

580   likelihoods into account for each species representative calculated using ANGSDv0.913

581   (Korneliussen et al. 2014). For the 1Mbp window analysis, we used the same analysis to estimate

582   heterozygosity as before but performed the analysis in non-overlapping windows of 1MB. We

583   also removed any scaffolds less than 1Mbp in length.

584

585   **Demographic inference**

586 The demographic history of the brown hyena was calculated using PSMC (Li and Durbin 2011),

587 considering only the autosomal chromosomes. Scaffolds representing the X chromosome of the

588 striped hyena were determined through a synteny analysis to the cat X chromosome

589 (CM001396.2) using Satsuma synteny (Grabherr et al. 2010). These scaffolds were then removed

590 along with any scaffold shorter than 1Mbp. A consensus diploid sequence was constructed using

591 Samtools (Li et al. 2009) to be used as input for PSMC. PSMC was implemented using

592 parameters previously shown to be meaningful when considering human data. 100 bootstrap

593 analyses were undertaken. When plotting, we used a generation time of 6 years and a mutation

594 rate of 7.5x10-9 per generation for autosomes.

595 In order to estimate the mutation rate, we carried out a pairwise distance analysis on the striped

596 and brown hyena's autosomes using a consensus base IBS approach in ANGSDv0.913. We then

597 calculated the average per generation mutation rate assuming a divergence date of the two

598 species to be 4.2mya (Koepfli et al. 2006), a genome-wide strict molecular clock and a

599 generation time of 6 years. Additional analyses utilising different mutation rates based on the

600 95% confidence interval of the brown and striped hyena divergence (2.6mya and 6.4mya) can be

601 seen in Supplemental Fig. S3.

602

603 **Data access:**

604 Raw sequencing reads can be found under the accession codes XXXXXX. The striped hyena

605 nuclear genome assembly can be found at XXXXXX and the brown hyena mitochondrial

606 genomes can be found at XXXXXX.

607

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618 **Author Contributions:**

619 The project was conceived by M.W. and M.H. M.W. and L.D. performed lab work; M.W., S.H.,

620 and A.B. performed DNA analyses and interpretation of results. I.W., V.L., R.W, D.M.P., F.S.,

621 and A.L. assisted with locating and sampling of specimens. Final editing and manuscript

622 preparation was coordinated by M.W. All contributing authors read and agreed to the final

623 manuscript.

624 **References:**

625 Abascal F, Corvelo A, Cruz F, Villanueva-Cañas JL, Vlasova A, Marcet-Houben M, Martínez-

626 Cruz B, Cheng JY, Prieto P, Quesada V, et al. 2016. Extreme genomic erosion after recurrent

627 demographic bottlenecks in the highly endangered Iberian lynx. *Genome Biol* **17**: 251.

628 Allendorf FW, Hohenlohe PA, Luikart G. 2010. Genomics and the future of conservation

629 genetics. *Nat Rev Genet* **11**: 697–709.

630   Bazin E, Glémin S, Galtier N. 2006. Population size does not influence mitochondrial genetic

631   diversity in animals. *Science* **312**: 570–572.

632   Benbow ME, Tomberlin JK, Tarone AM. 2015. *Carrion ecology, evolution, and their*

633   *applications*. CRC Press.

634   Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence

635   data. *Bioinformatics* **30**: 2114–2120.

636   Bouckaert RR. 2010. DensiTree: making sense of sets of phylogenetic trees. *Bioinformatics* **26**:

637   1372–1373.

638   Buerkle AC, Gompert Z. 2013. Population genomics based on low coverage sequencing: how

639   low should we go? *Mol Ecol* **22**: 3028–3035.

640   Butler J, MacCallum I, Kleber M, Shlyakhter IA, Belmonte MK, Lander ES, Nusbaum C, Jaffe

641   DB. 2008. ALLPATHS: de novo assembly of whole-genome shotgun microreads. *Genome*

642   *Res* **18**: 810–820.

643   Cho YS, Hu L, Hou H, Lee H, Xu J, Kwon S, Oh S, Kim H-M, Jho S, Kim S, et al. 2013. The

644   tiger genome and comparative analysis with lion and snow leopard genomes. *Nat Commun*

645   **4**: 2433.

646   deMenocal PB. 2004. African climate change and faunal evolution during the Pliocene–

647   Pleistocene. *Earth Planet Sci Lett* **220**: 3–24.

648   Dobrynin P, Liu S, Tamazian G, Xiong Z, Yurchenko AA, Krasheninnikova K, Kliver S,

649   Schmidt-Küntzel A, Koepfli K-P, Johnson W, et al. 2015. Genomic legacy of the African

650   cheetah, *Acinonyx jubatus*. *Genome Biol* **16**: 277.

651   Ellegren H. 2014. Genome sequencing and population genomics in non-model organisms.

652   *Trends Ecol Evol* **29**: 51–63.

653   Fortes GG, Paijmans JLA. 2015. Analysis of Whole Mitogenomes from Ancient Samples.

654   *Methods Mol Biol* **1347**: 179–195.

655   Fumagalli M, Vieira FG, Linderoth T, Nielsen R. 2014. ngsTools: methods for population

656   genetics analyses from next-generation sequencing data. *Bioinformatics* **30**: 1486–1487.

657   Grabherr MG, Russell P, Meyer M, Mauceli E, Alfoldi J, Di Palma F, Lindblad-Toh K. 2010.

658   Genome-wide synteny through highly sensitive sequence alignment: Satsuma.

659   *Bioinformatics* **26**: 1145–1151.

660   Gusset M, Burgener N. 2005. Estimating larger carnivore numbers from track counts and

661   measurements. *Afr J Ecol* **43**: 320–324.

662   Hahn C, Bachmann L, Chevreux B. 2013. Reconstructing mitochondrial genomes directly from

663   genomic next-generation sequencing reads—a baiting and iterative mapping approach.

664   *Nucleic Acids Res* **41**: e129.

665   Hartl DL, Clark AG. 1997. *Principles of population genetics*. Sinauer associates Sunderland.

666   Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7:

667   improvements in performance and usability. *Mol Biol Evol* **30**: 772–780.

668   Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A,

669   Markowitz S, Duran C, et al. 2012. Geneious Basic: an integrated and extendable desktop

670   software platform for the organization and analysis of sequence data. *Bioinformatics* **28**:

671   1647–1649.

672 Kent VT, Hill RA. 2013. The importance of farmland for the conservation of the brown hyaena

673   Parahyaena brunnea. *Oryx* **47**: 431–440.

674 Knowles JC, Van Coeverden de Groot PJ, Wiesei I, Boag PT. 2009. Microsatellite Variation in

675   Namibian Brown Hyenas (*Hyaena brunnea*): Population Structure and Mating System

676   Implications. *J Mammal* **90**: 1381–1391.

677 Koepfli K-P, Jenks SM, Eizirik E, Zahirpour T, Van Valkenburgh B, Wayne RK. 2006. Molecular

678   systematics of the Hyaenidae: relationships of a relictual lineage resolved by a molecular

679   supermatrix. *Mol Phylogenet Evol* **38**: 603–620.

680 Koepfli K-P, Pollinger J, Godinho R, Robinson J, Lea A, Hendricks S, Schweizer RM, Thalmann

681   O, Silva P, Fan Z, et al. 2015. Genome-wide Evidence Reveals that African and Eurasian

682   Golden Jackals Are Distinct Species. *Curr Biol* **25**: 2158–2165.

683 Korneliussen TS, Albrechtsen A, Nielsen R. 2014. ANGSD: Analysis of Next Generation

684   Sequencing Data. *BMC Bioinformatics* **15**: 356.

685 Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA.

686   2009. Circos: an information aesthetic for comparative genomics. *Genome Res* **19**: 1639–

687   1645.

688 Leffler EM, Bullaughey K, Matute DR, Meyer WK, Segurel L, Venkat A, Andolfatto P,

689   Przeworski M. 2012. Revisiting an old riddle: what determines genetic diversity levels

690   within species? *PLoS Biol* **10**: e1001388.

691   Leigh JW, Bryant D. 2015. popart: full-feature software for haplotype network construction.

692   *Methods Ecol Evol* **6**: 1110–1116.

693   Li H. 2011. Improving SNP discovery by base alignment quality. *Bioinformatics* **27**: 1157–1158.

694   Li H. 2012. seqtk Toolkit for processing sequences in FASTA/Q formats.

695   https://github.com/lh3/seqtk.

696   Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows–Wheeler transform.

697   *Bioinformatics* **25**: 1754–1760.

698   Li H, Durbin R. 2011. Inference of human population history from individual whole-genome

699   sequences. *Nature* **475**: 493–496.

700   Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R,

701   1000 Genome Project Data Processing Subgroup. 2009. The Sequence Alignment/Map

702   format and SAMtools. *Bioinformatics* **25**: 2078–2079.

703   Liti G, Carter DM, Moses AM, Warringer J, Parts L, James SA, Davey RP, Roberts IN, Burt A,

704   Koufopanou V, et al. 2009. Population genomics of domestic and wild yeasts. *Nature* **458**:

705   337–341.

706   Luo R, Liu B, Xie Y, Li Z, Huang W, Yuan J, He G, Chen Y, Pan Q, Liu Y, et al. 2012.

707   SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler.

708   *Gigascience* **1**: 18.

709   Magoč T, Salzberg SL. 2011. FLASH: fast length adjustment of short reads to improve genome

710   assemblies. *Bioinformatics* **27**: 2957–2963.

711   Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads.

712   *EMBnet.journal* **17**: 10–12.

713   McMahon BJ, Teeling EC, Höglund J. 2014. How and why should we implement genomics into

714   conservation? *Evol Appl* **7**: 999–1007.

715   Meyer M, Kircher M. 2010. Illumina sequencing library preparation for highly multiplexed

716   target capture and sequencing. *Cold Spring Harb Protoc* **2010**: db.prot5448.

717   Miller W, Hayes VM, Ratan A, Petersen DC, Wittekindt NE, Miller J, Walenz B, Knight J, Qi J,

718   Zhao F, et al. 2011. Genetic diversity and population structure of the endangered marsupial

719   *Sarcophilus harrisii* (Tasmanian devil). *Proc Natl Acad Sci U S A* **108**: 12348–12353.

720   Nielsen EE, Hemmer-Hansen J, Larsen PF, Bekkevold D. 2009. Population genomics of marine

721   fishes: identifying adaptive variation in space and time. *Mol Ecol* **18**: 3128–3150.

722   Peter C, Bruford M, Perez T, Dalamitra S, Hewitt G, Erhardt G. 2007. Genetic diversity and

723   subdivision of 57 European and Middle-Eastern sheep breeds. *Anim Genet* **38**: 37–44.

724   R Development Core Team. 2008. R: A Language and Environment for Statistical Computing.

725   http://www.R-project.org.

726   Reed DH, Frankham R. 2003. Correlation between fitness and genetic diversity. *Conserv Biol*

727   **17**: 230–237.

728   Robinson JA, Ortega-Del Vecchyo D, Fan Z, Kim BY, vonHoldt BM, Marsden CD, Lohmueller

729   KE, Wayne RK. 2016. Genomic Flatlining in the Endangered Island Fox. *Curr Biol* **26**:

730   1183–1189.

731   Rohland N, Pollack JL, Nagel D, Beauval C, Airvaux J, Pääbo S, Hofreiter M. 2005. The

732   population history of extant and extinct hyenas. *Mol Biol Evol* **22**: 2435–2443.

733   Romiguier J, Gayral P, Ballenghien M, Bernard A, Cahais V, Chenuil A, Chiari Y, Dernat R,

734   Duret L, Faivre N, et al. 2014. Comparative population genomics in animals uncovers the

735   determinants of genetic diversity. *Nature* **515**: 261.

736   Schmieder R, Edwards R. 2011. Quality control and preprocessing of metagenomic datasets.

737   *Bioinformatics* **27**: 863–864.

738   Shafer ABA, Wolf JBW, Alves PC, Bergström L, Bruford MW, Brännström I, Colling G, Dalén

739   L, De Meester L, Ekblom R, et al. 2015. Genomics and the challenging translation into

740   conservation practice. *Trends Ecol Evol* **30**: 78–87.

741   Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO:

742   assessing genome assembly and annotation completeness with single-copy orthologs.

743   *Bioinformatics* **31**: 3210–3212.

744   Skotte L, Korneliussen TS, Albrechtsen A. 2013. Estimating individual admixture proportions

745   from next generation sequencing data. *Genetics* **195**: 693–702.

746   Spielman D, Brook BW, Frankham R. 2004. Most species are not driven to extinction before

747   genetic factors impact them. *Proc Natl Acad Sci U S A* **101**: 15261–15264.

748   Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of

749   large phylogenies. *Bioinformatics* **30**: 1312–1313.

750   Steiner CC, Putnam AS, Hoeck PEA, Ryder OA. 2013. Conservation genomics of threatened

751   animal species. *Annu Rev Anim Biosci* **1**: 261–281.

752   Turner A, Antón M, Werdelin L. 2008. Taxonomy and evolutionary patterns in the fossil

753   Hyaenidae of Europe. *Geobios Mem Spec* **41**: 677–687.

754   Watts HE, Holekamp KE. 2007. Hyena societies. *Curr Biol* **17**: R657–60.

755   Weise FJ, Wiesel I, Jr JL, van Vuuren RJ. 2015. Evaluation of a Conflict-Related Brown Hyaena

756   Translocation in Central Namibia. *S Afr J Wildl Res* **45**: 178–186.

757   Welch RJ, Parker DM. 2016. Brown hyaena population explosion: rapid population growth in a

758   small, fenced system. *Wildl Res* **43**: 178–187.

759   Werdelin L, Barthelme J. 1997. Brown hyena (*Parahyaena brunnea*) from the Pleistocene of

760   Kenya. *J Vert Paleontol* **17**: 758–761.

761   Werdelin L, Solounias N. 1991. The Hyaenidae: taxonomy, systematics and evolution. *Fossils*

762   *and Strata* **30**: 1–104.

763   Wiesel I. 2015. *Parahyaena brunnea. The IUCN Red List of Threatened Species 2015:*

764   *eT10276A82344448*. http://dx.doi.org/10.2305/IUCN.UK.2015-

765   4.RLTS.T10276A82344448.en.

766   Willoughby JR, Fernandez NB, Lamb MC, Ivy JA, Lacy RC, DeWoody JA. 2015. The impacts

767   of inbreeding, drift and selection on genetic diversity in captive breeding populations. *Mol*

768   *Ecol* **24**: 98–110.