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1 **This submission is intended as an ARTICLE in the Discoveries section.**

2
3 **Full Title:** Interspecific gene flow and the evolution of specialisation in black and white rhinoceros.

4
5 **Short title:** Pliocene gene flow in African Rhinoceros

6
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28

29 **Abstract**

30

31 Africa's black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros are closely related
32 sister-taxa that evolved highly divergent obligate browsing and grazing feeding strategies.
33 Although their precursor species *D. praecox* and *C. mauritanicum* appear in the fossil record ~5.2
34 million years ago (Ma), by 4 Ma both were still mixed feeders, and were even spatio-temporally
35 sympatric at several Pliocene sites in what is today Africa's Rift Valley. Here, we ask whether or
36 not *D. praecox* and *C. mauritanicum* were reproductively isolated when they came into Pliocene
37 secondary contact. We sequenced and *de novo* assembled the first annotated black rhinoceros
38 reference genome, and compared it with available genomes of other black and white rhinoceros.
39 We show that ancestral gene flow between *D. praecox* and *C. mauritanicum* ceased sometime
40 between 3.3 and 4.1 Ma, despite conventional methods for the detection of gene flow from whole
41 genome data returning false positive signatures of recent interspecific migration due to incomplete
42 lineage sorting. We propose that ongoing Pliocene genetic exchange, for up to 2 million years
43 after initial divergence, could have potentially hindered the development of obligate feeding
44 strategies until both species were fully reproductively isolated, but that the more severe and
45 shifting palaeoclimate of the early Pleistocene was likely the ultimate driver of ecological
46 specialisation in African rhinoceros.

47

48 **Keywords:** Reproductive isolation, ancestral gene flow, incomplete lineage sorting, rhinoceros,
49 Pliocene, genomes

50 Introduction

51

52 Although the age of Pleistocene mammalian megaherbivores is largely over, Africa is the
53 only continent to still harbour significant wild populations of its late-tertiary megafauna. Africa's
54 black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros are relics of this bygone
55 "golden age" of large mammals, yet, because of long-term historical demand for their products,
56 man has succeeded in driving this iconic group to the brink of extinction across the world (Hillman
57 1980, Chilvers 1990). Although rhinoceros are among the world's most endangered mammals,
58 the two African species have fared slightly better than their three Asian counterparts, owing mainly
59 to intensive conservation interventions during the second half of the 20th Century, resulting in a
60 global population of approximately 20,000 white and 5,000 black rhinoceros (Emslie *et al.* 2016).
61 However, the unfortunate consequence of these population gains is that the most recent poaching
62 epidemic, driven by increasing demand for rhinoceros horn in East and South-East Asia (Milliken
63 and Shaw 2012, Kennaugh 2015), is targeting the more common African species.

64 A rich fossil record shows that rhinoceros species have endured a series of severe Plio-
65 Pleistocene climatic and tectonic upheavals, to which the majority of their megafaunal
66 contemporaries succumbed (Barnosky *et al.* 2004). In Africa, the paleoclimate during this time
67 fluctuated between warmer, wetter, forest-rich interglacial periods that favoured browsers, and
68 cooler, drier grassland-rich glacial periods that benefited grazers. Although black and white
69 rhinoceros are closely related, they have evolved divergent feeding strategies. The black
70 rhinoceros is the smaller of the two species and primarily a browser, holding its head horizontally
71 to the ground in order to feed on leaves and twigs using a hooked upper lip. In contrast, the white
72 rhinoceros is an obligate grazer with hypsodont teeth, a heavy, elongated skull that is held
73 vertically and lower to the ground, with squared-off lips to enable efficient grazing. Both feeding
74 strategies evolved within the last 6-7 million years (Ma), from about the end of the warm Miocene
75 epoch, as global CO₂ levels decreased, leading to the more arid, seasonal and shifting
76 paleoclimates of the Plio-Pleistocene (WoldeGabriel *et al.* 2001, deMenocal 2004). Although
77 grazing rhinoceros such as *Teleoceras* had already evolved during the Miocene, the stem lineage
78 leading to modern African rhinoceros was a mixed-feeder, represented by the morphologically
79 intermediate *C. neumayri*, which inhabited late Miocene southern Europe from the Balkans to Iran
80 approximately 7-9 Ma (Zeuner 1934, Geraads 2005, Geraads and Spassov 2009). This stem
81 lineage diverged in Africa into *D. praecox* and *C. mauritanicum*, the direct ancestors of black and
82 white rhinoceros respectively (Geraads 2005, Geraads 2017). The earliest fossil appearance of
83 *D. praecox* is at Kuseralee in the Middle Awash Valley of Ethiopia (Giaourtsakis *et al.* 2009,
84 Geraads 2017) and the *Ceratotherium* lineage at Langebaanweg in South Africa (Hooijer *et al.*
85 1972, Geraads 2005), both sites dating to about 5.2 Ma. The initial divergence between *D.*
86 *praecox* and *C. mauritanicum* must therefore have occurred no later than around the Mio-Pliocene
87 boundary about 5.3 Ma.

88 While changing paleoclimates provide a means for the evolution and fixation of different
89 adaptations, they may also eventually bring speciating populations into secondary contact, where
90 gene flow might bring their diverging evolutionary trajectories back into line (Mayr 1942) and/or
91 promote the introgression of adaptive features between populations (Pardo-Diaz *et al.* 2012,
92 Dasmahapatra *et al.* 2012, Racimo *et al.* 2015). Secondary contact between diverging precursor

93 species could have taken place at several mid-late Pliocene sites (3.0-4.3 Ma) in what is today
94 Africa's Rift Valley, where both *D. praecox* and *C. mauritanicum* fossils co-occur within the same
95 horizon (Geraads 2010, red-grey squares Figure 1). One particularly rhinoceros-rich site is
96 Kanapoi in north-western Kenya, where middle Pliocene (4 Ma) *D. praecox* and *C. mauritanicum*
97 fossils show evidence that they had already evolved some of the adaptations to browsing and
98 grazing respectively. However, cranial morphology and stable $\delta^{13}\text{C}$ isotope ratios of these
99 precursor species from Kanapoi and other mid-Pliocene sites confirm that both precursor species
100 were still mixed feeders relative to their modern descendants (Geraads 2017).

101 The transition between these mixed feeding precursors and specialized modern species
102 occurred in East Africa in the late Pliocene or early Pleistocene, as the first fossil emergence of
103 the black rhinoceros was at Koobi Fora about 2.5 Ma, and white rhinoceros at Olduvai around 1.8
104 Ma (Hooijer 1969, Harris 1983, Geraads 2010). The Pleistocene distribution of the black
105 rhinoceros appears to have been widespread across sub-Saharan Africa, outside dense Central
106 and West African rainforests, and is similar to its Holocene distribution (Figure 1), but with a strong
107 genetic discontinuity on either side of the Zambezi River in South-Central Africa (Moodley *et al.*
108 2017). Given that observed levels of microsatellite and mitochondrial genetic diversity were much
109 higher to the north of the Zambezi, we hypothesise that black rhinoceros expanding from East
110 Africa, crossed into southern Africa prior to the existence of the river's present day course, and
111 were then restricted to the subregion by a river capture event 125-150 Ka (Moore and Larkin
112 2001) with limited gene flow connecting eastern and southern populations. In contrast, the
113 Pleistocene white rhinoceros ranged more widely than the black rhinoceros, occurring from South
114 Africa to as far north as Libya (Geraads 2010). However, this range contracted significantly into
115 two genetically distinct populations during the Holocene, with the northern white rhinoceros
116 inhabiting central African grasslands west of the Nile River, and the southern white rhinoceros
117 restricted to grasslands south of the Zambezi. Although their Holocene ranges are discontinuous,
118 microsatellite data suggest that the two white rhinoceros populations may have come into
119 secondary contact sometime during the last glacial period (14-106 Ka) when grasslands were
120 continuous between eastern and southern Africa (Moodley *et al.* 2018).

121 In this study we ask whether *D. praecox* and *C. mauritanicum* were reproductively isolated
122 when they came into secondary contact at Kanapoi and other mid-Pliocene sites, and whether
123 ongoing genetic exchange between the two precursor species could have delayed the evolution
124 of their obligate modern day feeding strategies. We attempt to answer this by estimating the time
125 at which the black and the white rhinoceros became fully reproductively isolated. Furthermore,
126 we date the divergences within each species and contrast these with the times at which
127 populations last came into secondary contact. Given their specialised feeding roles, we also
128 predicted a strong influence of fluctuating Pleistocene paleoclimates on the demographic history
129 of each species.

130 Until the advent of evolutionary genomics methods, these ideas were largely untestable,
131 mainly because of the limited resolution of Pliocene evolutionary events from the handful of
132 previously available molecular markers (Groves *et al.* 2006, Moodley *et al.* 2018). In contrast,
133 data from millions of polymorphic loci from whole genomes now offer the opportunity to
134 reconstruct patterns of genome wide diversity, divergence, and demographic history over much
135 deeper time periods. Therefore, to shed light on these questions, we established the first high-

136 coverage *de novo* black rhinoceros genome assembly and, together with the previously generated
137 white rhinoceros assembly and two further resequenced rhinoceros genomes, we carried out
138 comparative analyses of the evolution of both African species.

139

140

141 **Results**

142

143 *De novo assembly and annotation of the black rhinoceros reference genome*

144 We present a high quality reference genome for the black rhinoceros (SAMN14912225)
145 from an ear tissue sample of an individual sampled in KwaZulu-Natal, South Africa, at the
146 southern end of the species range. We reconstructed the assembly using a combination of paired-
147 end, mate paired, and chromatin-based sequencing libraries. First, we generated a baseline
148 assembly using a combination of short and long insert libraries using the *de novo* assembler
149 Allpaths-LG (Gnerre *et al.* 2011). We subsequently carried out super-scaffolding using chromatin-
150 based Chicago libraries (Putnam *et al.* 2016) and the Hi-Rise pipeline. This resulted in a 34.6-fold
151 coverage genome with a total assembly length of 2.33GB, with a scaffold N50 of 28.5MB and
152 4,264 scaffolds. A BUSCO assessment (Simão *et al.* 2015) of the gene content of the assembly
153 revealed only 13 (0.3%) duplicated, 88 (2.1%) fragmented and 116 (2.8%) missing mammalian
154 single copy orthologs. We then annotated the assembly using *ab initio* gene prediction and
155 homology-based gene identification, which resulted in 19,914 transcripts.

156 To unravel the evolutionary history between black and white rhinoceros, and to capture
157 as much of the variation within each species, we analysed our newly sequenced genome together
158 with three other African rhinoceros genomes. The black rhinoceros was represented by genomes
159 from its southern and eastern (SAMN14911588, 16-fold coverage) populations, and the white
160 rhinoceros by its southern (35-fold coverage) and northern (SAMN14911569, 16-fold coverage)
161 populations.

162

163 *Genome-wide heterozygosity*

164 The proportion of heterozygous sites in African rhinoceros genomes varied both within
165 and between species. The highest values were from populations in the northern range of both
166 species, with the eastern black rhinoceros and northern white rhinoceros returning the highest
167 diversity values at 0.00075 (S.D. 0.00071-0.00079) and 0.00045 (S.D. 0.00036-0.00054)
168 heterozygous sites per base respectively. The southern populations of both species revealed
169 lower values with southern black rhinoceros at 0.00031 (S.D. 0.00022-0.00040) and the southern
170 white rhinoceros at 0.00027 (S.D. 0.00022-0.00033) heterozygous sites.

171

172 *Divergence and mutation rates*

173 Based on the autosomal sequences, we calculated an average pairwise divergence of
174 0.0093 between the two species. Within each species, lower divergences were estimated
175 between eastern and southern black rhinoceros (0.0011) and northern and southern white
176 rhinoceros (0.0010). Using a conservative estimate (latest possible occurrence) for the split, or
177 end of panmixia, between black and white rhinoceros lineages at the Mio-Pliocene boundary
178 ~5.3Ma (Geraads 2005, 2017), these pairwise distances were translated into approximate within-

179 species divergence times of 641 and 578 thousand years ago (Ka) for the black and white
180 rhinoceros, respectively (Figure 2A). Using the pairwise distance between the white and black
181 rhinoceros, we calculated an autosomal mutation rate for African rhinoceros of 8.8×10^{-10}
182 substitutions per year, which is only slightly lower than the commonly implemented human
183 mutation rate of 1×10^{-9} (Li and Durbin 2011), and refutes the commonly held view that evolutionary
184 rates in rhinoceros genomes are substantially lower than the mammalian average (Gissi *et al.*
185 2000). Furthermore, we calculated the per generation mutation rate for each rhinoceros species
186 independently, assuming a generation time of 24 years for the black rhinoceros (Moodley *et al.*
187 2017), giving a mutation rate of 2.1×10^{-8} , and a generation time of 27 years for the white
188 rhinoceros (Moodley *et al.* 2018), giving a mutation rate of 2.4×10^{-8} .

189

190 *Demographic reconstruction*

191 We reconstructed the demographic histories of both African rhinoceros species over the
192 second half of the Pleistocene (<1.4 Ma) using a pairwise sequentially Markovian coalescent
193 (PSMC) model. Both species show a gradual reduction in effective population size (N_e) to less
194 than half their original size until about 520-540 Ka ago in black rhinoceros and 440-460 Ka in
195 white rhinoceros (yellow stars, Figure 2B). The demographic trajectories of both species also
196 diverged at this low point, indicating the approximate times at which the ancestral populations of
197 black rhinoceros and white rhinoceros divided, signalling the end of panmixia within each species.
198 After this point, all four genomes then appear to follow independent Middle Pleistocene population
199 expansions. Interestingly, the southern populations of both species reach their highest size at
200 about 230 Ka, earlier than their northern counterparts the eastern black rhinoceros at 200 Ka and
201 the northern white rhinoceros at 180 Ka. All four populations then contract to Holocene levels of
202 N_e below 5,000, although both southern African populations show a secondary but minor
203 population expansion at about 50 Ka for the southern white rhinoceros and within the last 20 Ka
204 for the southern black rhinoceros. It is important to note that PSMC-inferred demographic
205 trajectories are often difficult to interpret literally (Beichman *et al.* 2017). We also investigated
206 whether different sequencing depths, especially in the case of the northern white and eastern
207 black rhinoceros (16-fold coverage) may have influenced the observed demographic trajectories.
208 Based on a comparison between PSMC trajectories reconstructed using our newly sequenced
209 ~35-fold coverage southern black rhinoceros genome, and the same genome downsampled to
210 16-fold coverage (Supplementary Figure S1), we deduced that the differences caused by
211 differential coverage was negligible.

212

213 *Post-divergence gene flow*

214 Signatures of post-divergence gene flow between the two African rhinoceros lineages
215 were inferred through a variety of approaches relying on the known topology of the African
216 rhinoceros species tree (Figure 2A). We first implemented the four-taxon ABBA/BABA or D-
217 statistic analysis (Durand *et al.* 2011), which showed evidence for significant levels of post-
218 divergence interspecific gene flow between southern white rhinoceros and both black rhinoceros,
219 as well as between eastern black rhinoceros and both white rhinoceros (Supplementary Table
220 S1, Supplementary Figure S2). This result was unexpected as it did not follow a geographically
221 mediated pattern, as one would expect gene flow between geographically close lineages to be

222 the most probable, that is, between northern white and eastern black, and between southern white
223 and southern black rhinoceros (see Figure 1). Then, to add further levels of information, such as
224 the direction of gene flow and whether gene flow occurred between ancestral lineages, we
225 performed the complementary five-taxon Dfoil analysis (Pease and Hahn 2015) which utilises a
226 system of four D-statistics to distinguish introgressions in a symmetric five-taxon phylogeny, using
227 the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) as outgroup. This analysis also indicated
228 several instances of gene flow (Supplementary Table S2), although most of these were at a very
229 low frequency. Dfoil analysis did, however, suggest similar levels of high frequency gene flow
230 between the ancestral white rhinoceros lineage and both black rhinoceros. We then extracted
231 regions consistently showing evidence for admixture, regardless of window size, from the Dfoil
232 results and cross referenced these putatively introgressed genomic segments against the white
233 rhinoceros annotation, revealing an exchange of 47 protein coding genes, the majority of which
234 had no human analogue (Supplementary Table S3). Using the recovered gene codes, we ran a
235 gene ontology (GO) enrichment test with GOrilla (Eden *et al.* 2009) to investigate whether certain
236 biological processes may have been selectively retained from past introgression events. We
237 found no significantly enriched GO terms. Finally, we investigated the length of contiguous
238 introgressed windows to understand the relative timing of introgression. We found the vast
239 majority of introgressed windows to be singletons with only very few consecutive windows
240 detected (Supplementary Table S4).

241 As both D-statistics and D-foil analyses rely on the D-statistic to infer gene flow, they can
242 both be confounded by similar caveats and biases based on the data. Therefore, we computed
243 the D3-statistic (Hahn and Hibbins, 2019), which is a three-sample test for introgression that uses
244 pairwise distances to estimate the presence of admixture in a triplet taxa ((A, B), C). D3 bypasses
245 the need for an outgroup genome to polarise ancestral and derived alleles, so should be more
246 robust than D-statistics when no suitable closely related outgroup is available. To test for
247 significance, we ran the D3 analysis using both 100 KB, and 1 MB, non-overlapping sliding
248 windows. Results showed no significant levels of differential gene flow between any of the African
249 rhinoceros triplets and were consistent regardless of window size (Supplementary Table S5). To
250 further test for admixture we also implemented Treemix (Pickrell and Pritchard, 2012) and the F3-
251 and F4-statistics (Reich *et al.* 2009, Keinan *et al.* 2007). These analyses neither confirmed nor
252 excluded the possibility of post-divergence interspecific gene flow suggested by D-statistics, but
253 we include their details in Supplementary methods, Figures S3-S6 and Tables S6 and S7.

254 *Reproductive isolation and the cessation of post-divergence gene flow*

255 To ascertain when admixture between the speciating African rhinoceros lineages may
256 have ceased, we conducted multiple F1 hybrid pairwise sequentially Markovian coalescent model
257 (hPSMC) analyses using pseudo-diploidised African rhinoceros genomes, and intermediate
258 mutation rates and generation times. This analysis is based on the premise that a pseudo F1
259 hybrid genome cannot coalesce more recently than the speciation event of the two parental
260 species (Cahill *et al.* 2016). This point of coalescence is represented by a transition from an infinite
261 population size to the population size of the shared ancestral lineage prior to divergence, thus
262 allowing the determination of the latest time for the development of reproductive isolation between
263 the two species. However, as hPSMC utilises PSMC, and PSMC is known to portray rapid
264

265 changes in ancestral N_e as gradual transitions, one cannot apply a purely qualitative approach to
266 estimating divergence times. Therefore, we ran simulations specifying various divergence times
267 between the individuals of interest. Simulations were run using the `hPSMC_quantify_split_time.py`
268 python script from the hPSMC tool suite specifying pre-divergence N_e , time windows for
269 divergence, and default parameters. Results from the real data as well as simulations based on
270 N_e 's calculated before its exponential increase to infinity, indicated that reproductive isolation
271 between black and white rhinoceros lineages occurred between 3.3 and 4.1 Ma (Figure 3A), much
272 more recently than the initial divergence time of the two species at ~5.3 Ma or earlier (Geraads
273 2005, 2017). This result was the same regardless of which of the two genomes of each species
274 were compared and which species was used as the mapping reference.

275 When applying the same hPSMC and simulation analyses within each species, we found
276 gene flow to have also continued long after the initial divergence of and cessation of panmixia
277 within the lineages. We found that white rhinoceros last experienced north-south gene flow
278 approximately 200-300 Ka after the species diverged into northern and southern populations, at
279 some point in time between 100-220 Ka (Figure 3B). Gene flow between eastern and southern
280 populations continued for even longer after divergence in the black rhinoceros (~400-500 Ka),
281 until ceasing more recently between 30-130 Ka (Figure 3C).

282

283 *Evaluating D-statistics in the presence of ancestral gene flow*

284 To further evaluate our seemingly unlikely D-statistics results, we ran simulations in 1MB
285 blocks based on a simple model specifying ancestral gene flow between the ancestral black and
286 white rhinoceros lineages prior to their divergence into their respective subspecies (Figure 4) and
287 ran D-statistics on these simulations. Although results differed based on specified ancestral
288 migration rates (Supplementary Tables S8-S10), we found significant Z-scores indicating post-
289 divergence gene flow between the southern black rhinoceros and both white rhinoceros
290 subspecies as well as between the northern white and both black rhinoceros subspecies, even
291 though we did not model subspecies-level gene flow.

292

293

294 **Discussion**

295

296 In this study we generated the first reference genome assembly for the critically
297 endangered black rhinoceros, from an individual belonging to the species' southern-most
298 population. We analysed this southern black rhinoceros reference genome, together with nuclear
299 genomes from eastern black (Kenya), southern white (South Africa), and northern white (South
300 Sudan) rhinoceros, to uncover the evolutionary history of and relationships between the two
301 species of African rhinoceros.

302

303 *Genomic diversity, Pleistocene declines and expansion*

304 Levels of genome wide heterozygosity support recent population histories of
305 anthropogenically-mediated decline in the black and white rhinoceros, as shown previously
306 (Moodley *et al.* 2017, Tunstall *et al.* 2018, Moodley *et al.* 2018). All four rhinoceros genomes
307 showed a mid-Pleistocene decline, which may have been associated with a gradual cooling of

308 the earth at the beginning of the Pliocene. However, a subsequent population expansion of all
309 genomes is not consistent with the paleoclimatic record, since this was just after the time when
310 glacial cycles became more severe (<800 Ka, Figure 2C). Rather, the increase in effective
311 population size occurred at the point at which panmixia in both species ended (Figure 2). It is
312 possible that the 520 Ka expansion in black rhinoceros was associated with an interglacial cycle,
313 whereas the 440 Ka white rhinoceros expansion could be associated with an interglacial, but both
314 species effective sizes were inferred to have expanded, regardless of subsequent glacial cycles,
315 until about 240 Ka. Alternatively, the early evolution of additional genetic substructure within
316 diverging regional populations of each species, followed by their isolation during unfavourable
317 climatic periods, could also have inflated effective population sizes, even if census sizes remained
318 stable (Mazet *et al.* 2015, Mazet *et al.* 2016). Although most mtDNA lineages to have evolved in
319 the white rhinoceros were already extinct by the Holocene (Moodley *et al.* 2018), in the black
320 rhinoceros, mtDNA is highly structured with both eastern and southern lineages (Moodley *et al.*
321 2017), lending weight to this interpretation.

322 At approximately 240 Ka, southern black and white rhinoceros underwent a population
323 decline, followed by northern white and eastern black rhinoceros at 180 Ka. Until now, only the
324 genomes of bonobos, Nigeria-Cameroon chimpanzees, the spectacled bear and east African
325 baboons show a similar Middle Pleistocene decline around 150-200 Ka (Prado-Martinez *et al.*
326 2013, Kumar *et al.* 2017, Rogers *et al.* 2019). It is interesting that these declines in both African
327 rhinoceros coincide with the emergence of modern humans. A similarly sharp demographic
328 decline was also inferred for the Sumatran rhinoceros, but more recently at about 100 Ka (Mays
329 *et al.* 2018), coinciding with the appearance of humans in Asia. It is also intriguing that southern
330 populations of both rhinoceros species decline before populations in the north and east, as it could
331 imply differential levels of population pressure across Africa.

332
333 *Divergence, the end of panmixia and the cessation of gene flow within each species*

334 Within-species autosomal divergence times for black (641 Ka) and white (578 Ka)
335 rhinoceros were consistently about 100 Ka older than the times at which panmixia is inferred to
336 have ceased for each species using PSMC. Although divergence times do not account for
337 demographic events or gene flow, values are remarkably similar considering their different
338 methods of inference. The reported divergence times are also within the confidence limits of
339 mtDNA data for white rhinoceros (Harley *et al.* 2016, Moodley *et al.* 2018), but not for black
340 rhinoceros, where mtDNA divergence between southern and eastern black rhinoceros was
341 inferred to be significantly more ancient (920-3,575 Ka, Moodley *et al.* 2017). It is possible that
342 values for the end of panmixia may have been downwardly biased as populations of both species
343 underwent very similar demographic expansion trajectories after their PSMC curves became
344 dissociated (Figure 2B). However, divergence and the end of panmixia occurred long before the
345 final cessation of gene flow within both species (Figure 3B/C), indicating ongoing secondary
346 contact, potentially during phases of demographic expansion during the last 400,000 years. While
347 the two white rhinoceros populations appear to have come into secondary contact less recently
348 (100-220 Ka), this estimate is still consistent with gene flow during the last glacial period, as
349 recently inferred from microsatellite data (Moodley *et al.* 2018). The eastern and southern black
350 rhinoceros on the other hand, appear to have come into more recent genetic contact across the

351 Zambezi valley (Figure 3C). The Zambezi's paleo-upper and -lower reaches were joined by river
352 capture between 125–150 Ka (Moore and Larkin 2001), and while our results appear to contrast
353 with a strong mtDNA and microsatellite discontinuity on either side of this river (Moodley *et al.*
354 2017), at least one East African mtDNA haplotype was sampled on the southern bank of the
355 Zambezi, and one southern African haplotype was sampled north of the Zambezi, hinting that
356 although the river may have acted to maintain the genetic integrity of populations to its north and
357 south, it was also periodically fordable for black rhinoceros. In summary, these results suggest
358 that the period required between divergence and the end of panmixia to the cessation of gene
359 flow is dependent on how frequently climatic changes were able to bring diverging populations
360 into secondary contact, and in Africa, the expansion and contraction of habitats with glacial cycles
361 appears to have maintained gene flow long after population divergence. Although post-divergence
362 gene flow was observed previously in other taxa, including rhinoceros (Wang *et al.* 1997, Won
363 and Hey 2005, Lee and Edwards 2008, Moodley *et al.* 2018), our results provide yet another
364 cautionary note in evolutionary and conservation inference, that estimated times of divergence do
365 not necessarily correlate with the cessation of genetic contact.

366

367 *Gene flow between African rhinoceros species*

368 Both D-statistics and Dfoil suggested gene flow between the two African rhinoceros at the
369 subspecies level, that is, within the 63 Ka gap after the divergence of eastern and southern black
370 rhinoceros, but before the divergence of northern and southern white rhinoceros. While this
371 scenario might be plausible, it is highly unlikely that diverging eastern and southern black
372 rhinoceros populations both came into secondary contact with the ancestral white rhinoceros
373 population within this short space of time. These putatively unrealistic results may have arisen
374 due to caveats of the D-statistics analysis itself, our dataset, the biology of the individuals involved,
375 or a combination of all three. Possible explanations for false positive signs of introgression could
376 include ancestral population structure, which produce deviations from expectations based solely
377 on incomplete lineage sorting (ILS, Slatkin and Pollack 2008), introgression from unsampled or
378 extinct “ghost” lineages, differences in relative population sizes of the lineages or in the timing of
379 gene flow events, or different evolutionary rates or sequencing errors between the H1 and H2
380 individuals and therefore differential divergence of the extant lineages from their common
381 ancestor (Zheng and Janke, 2018). We also considered whether our taxa (both ingroup and
382 outgroup) were too divergent from one another, which would lead to evolutionary signals being
383 overwhelmed by noise caused by multiple substitutions and substitution saturation, although
384 previous studies have shown D-statistics to be robust to these factors (Zheng and Janke, 2018).
385 Owing to these uncertainties in interpreting our D-statistics and Dfoil results, we performed a
386 number of additional analyses to infer gene flow including; D3, F3-statistics, F4-statistics,
387 Treemix, and hPSMC. While each method has its own caveats, the combination of all methods
388 provides us with a suite of information to aid in the interpretation of our results. Unlike D-statistics
389 and Dfoil, D3 and F3-statistics found no evidence for recent, subspecies-level gene flow. F4-
390 statistics suggested gene flow had occurred between the four African lineages but could not be

391 used to determine which lineages were involved, and Treemix produced ambiguous results that
392 may reflect its unsuitability for our dataset.

393 One potential explanation for these contradictory results was uncovered via hPSMC
394 analyses. hPSMC showed that gene flow between the two species ceased relatively early, during
395 the mid-late Pliocene between 3.3 and 4.1 Ma, long before divergence into subspecies lineages.
396 This result was consistent between genomes and regardless of which reference (black or white
397 rhinoceros) was used for mapping. The lack of contiguous regions of gene flow inferred by Dfoil
398 also suggest an absence of recent interspecific migration, as recent secondary contact between
399 black and white rhinoceros subspecies would have resulted in larger and more continuous tracts
400 of introgression (Pool and Nielsen, 2009). Instead, recombination and ILS appears to have broken
401 up such tracts into mainly singleton windows, indicating that the last gene flow event(s) between
402 the two species must have occurred prior to the divergence of subspecies lineages, thus
403 corroborating hPSMC results, which suggest the development of reproductive isolation during the
404 Pliocene.

405 Finally, we tested the idea that Pliocene gene flow between the ancestral black and
406 ancestral white rhinoceros lineages, could have resulted in false positive signatures of recent
407 gene flow. Although the jackknifing significance test should be robust to such a case, we
408 addressed this possibility by running D-statistics on simulated data, generated using a simple
409 model and various levels of ancestral gene flow, followed by subspecies divergence. We found
410 significant Z-scores for subspecies level gene flow, even though the only migration events
411 simulated were those between the ancestral lineages (Supplementary Tables S8-S10). These
412 simulation results, together with hPSMC and a lack of contiguous gene flow tracts strongly
413 suggest that gene flow between the black and white rhinoceros lineages ceased during the
414 Pliocene, long before the divergence of their subspecies (Figure 4), and that genetic signatures
415 of this ancient introgression are differentially present in our sampled African rhinoceros genomes
416 due to ILS. We caution that future studies which infer recent interspecific gene flow employ a suite
417 of independent analyses, including simulations, to rule out the possibility that gene flow occurred
418 between ancestral lineages, with subsequent random genetic drift leading to ILS.

419 420 *The evolution of specialisation*

421 Our results suggest that the African rhinoceros precursors *D. praecox* and *C.*
422 *mauritanicum* may still have been able to exchange genes with each other when they came into
423 secondary contact at Kanapoi in Kenya 4 Ma (Figure 4). This was supported by the hPSMC
424 analysis, which shows that gene flow ceased up to two million years after the initial divergence of
425 ancestral *Diceros* and *Ceratotherium* lineages. On the other hand, if gene flow was not possible
426 at Kanapoi, it was likely because reproductive isolation had only just become fully developed
427 between the two species.

428 The evolutionary consequences of this ancestral gene flow are intriguing. We analysed
429 the segments of DNA inferred to be exchanged between the two species, but did not find any
430 significantly enriched GO terms, leading us to hypothesise that there was little to no evidence for
431 adaptive introgression, as observed in other recent studies for example, Pardo-Diaz *et al.* (2012)
432 and Dasmahapatra *et al.* (2012). Perhaps this is not surprising since a classical view (Mayr 1942)
433 would predict that periods of secondary contact and ongoing gene flow between *D. praecox* and

434 *C. mauritanicum* prior to 4 Ma may have continually undermined the diverging evolutionary trends
435 of both lineages towards browsing and grazing, respectively. The rhinoceros-rich fossil record of
436 the mid-late Pliocene provides some evidence to support this view, because despite over a million
437 years since their initial divergence, both species maintained their ancestral mixed feeding state
438 throughout most of the Pliocene (Geraads 2017). So phenotypically similar were *D. praecox* and
439 *C. mauritanicum* during this period, that palaeontologists often misidentified one species for the
440 other (Geraads 2005, Geraads 2010). On the other hand, there is also extensive theoretical
441 (Barton 1979, Barton 1987) and empirical (McCracken *et al.* 2009, Hohenlohe *et al.* 2012,
442 Poelstra *et al.* 2014) evidence that adaptation can occur even in the face of gene flow, when
443 introgressing alleles confer a selective advantage, with advantageous loci often in tight linkage
444 disequilibrium, or if hybrid fitness is low (Barton and Hewitt 1985). The fact that both *D. praecox*
445 and *C. mauritanicum* had developed some level of specialisation prior to the onset of reproductive
446 isolation, suggests that adaptation may have been occurring despite Pliocene gene flow.
447 However, eventual reproductive isolation between the two species likely resulted from an
448 accumulation of larger numbers of loci under selection (Barton and Hewitt, 1989). The evolution
449 of fully specialised browsing and grazing African rhinoceros species could only have occurred
450 during the critical phase after reproductive isolation between them was established (3.3-4.1 Ma),
451 but before the internal splits within each species (500-600 Ka, Figure 4). The fossil emergence of
452 phenotypically modern black (2.5 Ma) and white (1.8 Ma) rhinoceros falls exactly within this
453 interval. The timing of these fossil emergences suggests that the more severe and shifting
454 paleoclimates of the Pleistocene provided the heterogeneity of environments that ultimately drove
455 the evolution of obligate feeding strategies in African rhinoceros species.
456

457 **Methods**

458

459 *Establishing the black rhinoceros reference assembly*

460 To ensure a straightforward assembly of our reference genome, we undertook to sample
461 from a more genetically depauperate black rhinoceros population where heterozygous sites are
462 likely to be more sparsely distributed across the genome. Of the five remaining aboriginal stocks
463 in Africa, KwaZulu-Natal (South Africa) contains the lowest levels of genetic diversity (Anderson-
464 Lederer 2011, Moodley *et al.* 2017) owing to an early 20th Century population collapse. The
465 KwaZulu population has since recovered to over 2,000 individuals (Emslie *et al.* 2016). We
466 obtained ear notches taken during routine management of a male and female black rhinoceros
467 (*D. b. minor*) from the Zululand Rhino Reserve, near the town of Mkhuze in KwaZulu-Natal, South
468 Africa. Both samples were taken by a veterinarian under an ordinary permit (OP 4368/2015) from
469 the provincial authority Ezemvelo KZN Wildlife and preserved in 99% alcohol. The samples were
470 then couriered to the Naturhistoriska riksmuseet under CITES permit number 51491-15 where
471 DNA was extracted with a Kingfisher Duo (ThermoFisher Scientific) using the Cell and Tissue DNA
472 Kit. The best quality sample, a male individual (SAMN14912225), was selected for genome
473 sequencing. We employed an exhaustive sequencing strategy, establishing two short insert DNA
474 libraries of 180bp and 650bp as well as three mate-pair DNA libraries of 3 KB, 5 KB and 20 KB
475 fragment size. The libraries were sequenced on the Illumina HiSeq X platform, with one lane for
476 each of the short-insert libraries and one lane for a pool of the three mate-pair libraries. We then
477 *de novo* assembled these reads using Allpaths-LG v52485 (Gnerre *et al.* 2011) according to the
478 method described by Pujolar *et al.* (2018).

479 We further improved our reference assembly by generating three Chicago libraries
480 (Putnam *et al.* 2016) from the reference sample at Dovetail Genomics (Santa Cruz, CA). This
481 method uses *in vitro* reconstituted chromatin to achieve 3D folding of the DNA. The folded DNA
482 is then cut using an endonuclease and subsequently ligated back together. The advantage of this
483 method is that some links are made between regions of the same DNA strand up to hundreds of
484 KB apart, due to their close proximity in the 3D folding. The Chicago libraries were assembled
485 with Dovetail's Hi-Rise scaffolding pipeline. To assess the gene-content of the assembly, BUSCO
486 v3.0.2 was run using its set of 4,104 single-copy mammalian orthologs (Simão *et al.* 2015).

487

488 *Genome annotation*

489 Next, we carried out repeat and gene annotation. To do so, we first masked repeats in the
490 genome using a combination of *ab initio* repeat finding and homology-based repeat annotation
491 using RepeatModeler (<http://www.repeatmasker.org>) and RepeatMasker ([http://www.repeat](http://www.repeatmasker.org)
492 [masker.org](http://www.repeatmasker.org)), respectively. For homology-based repeat annotation we used the mammal repeat
493 consensus sequences from Repbase (Bao, *et al.* 2015). For the gene annotation, we did not mask
494 simple repeats beforehand to improve mapping during the homology-based annotation
495 implemented in Maker2 (Holt and Yandell 2011). The gene annotation was performed using a
496 combination of *ab-initio* gene prediction (using SNAP (Korf 2004) and Augustus (Stanke and
497 Waack 2003)) and homology-based gene annotation using Maker2. We used protein annotations
498 of the horse (EquCab2.0; GCF_000002305.2), the white rhinoceros (CerSimSim1.0;

499 GCF_000283155.1) and human (GRCh38; GCA_000001405.37) for the homology-based gene
500 annotation step. This resulted in the annotation of 19,914 genes.

501
502 *Raw data processing and mapping*

503 To investigate the evolutionary history of African rhinoceros, we analysed the South
504 African black rhinoceros reference assembly together with the Broad Institute's white rhinoceros
505 reference genome (CerSimSim1.0), obtained from a female southern white rhinoceros (*C. s.*
506 *simum*, iMfolozi, Studbook# 159) which was wild caught in 1963 at iMfolozi Game Reserve, South
507 Africa. To include as much of the variation within each species as possible, we further
508 resequenced the genomes of a female East African black rhinoceros (*D. b. michaeli*, Sally,
509 Studbook# 78, SAMN14911588), wild caught in 1950 in the Kibwezi District, southern Kenya; and
510 a female northern white rhinoceros (*C. s. cottoni*, Nola, Studbook# 374, SAMN14911569), wild
511 caught in 1974 in the Shambe Region of Sudan, now South Sudan. Both Kenyan and South
512 Sudanese samples were obtained from the San Diego Zoo, and sequencing to 16-fold coverage
513 was carried out at the Broad Institute. As an outgroup genome, we used the recently sequenced
514 Sumatran rhinoceros (*Dicerorhinus sumatrensis*, Mays *et al.* 2018), which diverged from the clade
515 containing African rhinoceros about 18 million years ago (Margaryan *et al.* 2020).

516 Raw reads were all treated comparably before being mapped to a specific reference
517 genome. We used Cutadapt v1.8.1 (Martin 2011) to trim Illumina adapter sequences from the
518 ends of reads and remove reads shorter than 30bp. We then merged overlapping read pairs using
519 FLASH v1.2.1 (Magoč and Salzberg 2011). We mapped the resultant reads of the five individuals
520 used in the study to their respective reference sequences, unless otherwise specified, using
521 BWA v0.7.15 (Li and Durbin 2009) and processed the mapped reads further using SAMtools
522 v1.3.1 (Li *et al.* 2009). We mapped both the East African black rhinoceros and South African black
523 rhinoceros to the newly assembled black rhinoceros genome, both the northern white rhinoceros
524 and the southern white rhinoceros to the published southern white rhinoceros genome, and the
525 Sumatran rhinoceros to the Sumatran rhinoceros genome (GCA_002844835.1).

526
527 *Genetic variation*

528 We estimated autosomal heterozygosity from each of the four African rhinoceros
529 individuals. To determine which scaffolds were most likely autosomal in origin, we found putative
530 sex chromosome scaffolds for each of the rhinoceros reference genomes and removed them from
531 future analyses. We found putative sex chromosome scaffolds through synteny by aligning the
532 rhinoceros reference genomes to the Horse X (Genbank accession: CM000408.2) and Human Y
533 (Genbank accession: NC_000024.10) chromosomes. Alignments were performed using satsuma
534 synteny (Grabherr *et al.* 2010) and utilising default parameters. To adjust for biases in
535 heterozygosity levels that could arise due to different global coverages between the genomes of
536 the individuals being investigated, we subsampled all of the resultant alignments down to that of
537 the lowest coverage individual, 16-fold, using SAMtools. We then estimated the autosomal
538 heterozygosity from all scaffolds above 100 KB in length, using sample allele frequencies in
539 ANGSD v0.913 (Korneliussen *et al.* 2014), taking genotype likelihoods into account and specifying
540 the following filters -minq 25 -minmapq 25 -uniqueOnly 1 -baq 1 -remove_bads 1. We calculated
541 the standard deviation for each of the heterozygosity estimates by performing the realSFS

542 function in the ANGSD package in independent 20MB windows of covered bases (-nSites
543 20,000,000).

544

545 *Genome divergence and mutation rate*

546 To estimate the mutation rate per generation for each species, we computed pairwise
547 distances between the black and white rhinoceros autosomes twice independently: once with all
548 four African rhinoceros mapped to the black rhinoceros reference genome, and again with the four
549 genomes mapped to the white rhinoceros reference, and we took the average of the results. That
550 is, the average distance between the eastern black + southern white, eastern black + northern
551 white, southern black + southern white, southern black + northern white. We computed pairwise
552 distances using a consensus base IBS approach (-doIBS 2) in ANGSD and applying the filters -
553 minQ 25 -minmapq 25 -uniqueonly 1 -remove_bads 1. Using this information, we then computed
554 the mutation rate per generation assuming a genome-wide strict molecular clock and using the
555 following equation: mutation rate = pairwise distance x generation time/2 x divergence time. We
556 assumed a divergence time coinciding with that of the Miocene/Pliocene boundary (5.3 Ma) as
557 the stem lineage (*C. neumayri*) was common during the late Miocene (7-9 Ma) but had already
558 split into *D. praecox* and *C. mauritanicum* lineages by the Pliocene (Geraads 2005, 2017). A
559 generation time of 24 years was assumed for the black rhinoceros (Moodley et al, 2017) and 27
560 years for the white rhinoceros (Moodley et al. 2018). Moreover, we used the per year mutation
561 rate calculated by comparing the black and white rhinoceros to estimate the within species
562 divergence dates based on the within species average pairwise distances when mapping to the
563 conspecific reference genome.

564

565 *Demographic analyses*

566 We ran demographic analyses on the diploid genomes of all four African rhinoceros
567 individuals using Pairwise Sequentially Markovian Coalescent model (PSMC) (Li and Durbin
568 2011). Using this method, it is possible to infer changes in effective population size through time
569 for diploid (high coverage) genomes from the distribution of its heterozygous sites across the
570 genome. We called diploid genome sequences using SAMtools and bcftools (Narasimhan et al.
571 2016) specifying a minimum quality score of 20 and minimum coverage of 10. We removed
572 scaffolds found to align to sex chromosomes in the previous step and scaffolds shorter than
573 100KB. We ran PSMC specifying atomic intervals previously shown to be suitable for human
574 datasets (4+25*2+4+6) and performed 100 bootstrap replicates to investigate support for the
575 resultant demography. We overlaid the resultant PSMC plots as the point in time in which the
576 demographic trajectories of two individuals diverges can be interpreted as a rough measure of
577 the end of panmixia in that species. Moreover, as one of our individuals (northern white rhino)
578 was only ~16-fold coverage, we downsampled our (~35-fold coverage) southern black rhinoceros
579 genome to 16-fold coverage to investigate the effect this may have on our inferences. We ran a
580 PSMC analysis on the downsampled genome and compared it to the results recovered for the
581 same genome using the much higher coverage data.

582

583 *Inter and intra-specific post-divergence gene flow*

584 For the gene flow analyses, we mapped the raw reads from the four African species to the
585 Sumatran rhinoceros following the same methods mentioned above to avoid any ascertainment
586 bias that may occur when mapping to an ingroup African rhinoceros species (Westbury *et al.*
587 2019). We performed multiple different analyses to test for post divergence gene flow between
588 African rhinoceros. First, we implemented the four-taxon ABBA/BABA or D-statistics approach
589 (Durand *et al.* 2011) with ANGSD. We called bases using a random base call (-doAbbababa 1),
590 only considered scaffolds over 100KB in length, specified the Sumatran rhinoceros as outgroup,
591 and applied the following filters; -minMapQ 25, -minQ 25, -uniqueOnly 1, -remove_bads 1. We
592 also adjusted quality scores around indels (-baq 1) (Li 2011). ANGSD performs all possible
593 combinations but we only investigated the output with conspecifics in the H1 and H2 positions
594 and an individual from the other species in the H3. Any other combination would go against the
595 species tree and therefore produce invalid signs of admixture driven by more recent common
596 ancestry as opposed to true admixture. To investigate the significance of our result, we performed
597 a weighted block jackknife test using 5MB non-overlapping blocks. D-values more than three
598 standard errors different from zero ($-3 < Z > 3$) were considered as statistically significant.

599 Following the D-statistics, we implemented Dfoil (Pease and Hahn 2015), a more detailed,
600 expanded version of D-statistics using five-taxa to test for gene flow, with the Sumatran rhinoceros
601 as an outgroup. Dfoil implements four independent D-statistics in a sliding window fashion which
602 are then combined before inferences are made. This has the advantage over the four-taxon test
603 in that it can infer the direction of gene flow and uncover whether gene flow occurred between
604 ancestral lineages. For this analysis, we also mapped all rhinoceros to the Sumatran rhinoceros.
605 We then constructed fasta files for each individual using ANGSD and specifying maximum
606 effective base depth (-doFasta 3) and the following parameters: -minMapQ 25, -minQ 25, -
607 uniqueOnly 1, -remove_bads 1. Additionally, we removed all scaffolds shorter than 1MB and
608 trimmed the ends of the remaining scaffolds down to the nearest 100KB, leaving us with 937.2MB.
609 The resultant fasta files were converted into an mvf file (ConvertFasta2MVF) which was then
610 converted into three independent Dfoil input files (CalcPatternCount) of window sizes 100KB,
611 50KB, and 20KB with mvftools (Pease and Rosenzweig 2018). Regions showing signs of
612 admixture between the ancestral white rhinoceros and either the southern or eastern black
613 rhinoceros were extracted and compared between window sizes. We cross referenced the
614 introgressed genomic segments consistently showing signs of admixture despite window size
615 against the white rhinoceros annotation to uncover putative protein coding genes in these regions.
616 We then tested for GO enrichment terms with GOrilla (Eden *et al.* 2009). We further investigated
617 the contiguity of introgressed regions by extracting all regions showing any signs of introgression,
618 regardless of direction, and investigating how long the stretches of these introgression windows
619 were for all window sizes independently.

620 To further test for admixture, we implemented D3 (Hahn and Hibbins 2019), a three taxon
621 test for introgression that makes use of pairwise distances and does not require an outgroup
622 genome using the topology ((A, B), C) and the equation $(BC-AC)/(BC+AC)$. We computed
623 pairwise distances between the four African rhinoceros based on a consensus base using
624 ANGSD -doIBS 2 and the following parameters; -makeMatrix 1 -uniqueOnly 1 -remove_bads 1 -
625 doMajorMinor 1 -minInd 4 -GL 1 -setMinDepthInd 5 -minmapq 25 -minq 25. We did this twice

626 independently specifying two different non-overlapping window sizes (100KB, and 1MB) to test
627 the significance of our results. We calculated a p-value for each comparison to evaluate the
628 difference from 0 by calculating the mean, standard deviation, and assuming a normal distribution
629 in R v3.6.0 (R Core Team, 2019) using the pnorm function. We also ran Treemix v 1.13 (Pickrell
630 and Pritchard 2012) with various migration edges as well as the threepop and fourpop tests,
631 otherwise known as the F3- and F4-statistics (Reich *et al.* 2009, Keinan *et al.* 2007), to determine
632 the presence or absence of gene flow among African rhinoceros using the software available in
633 the Treemix toolsuite (see Supplementary methods).

634

635 *Timing of reproductive isolation and the cessation of gene flow*

636 To add a temporal element to the onset of reproductive isolation and the cessation of gene
637 flow between African rhinoceros, we used the F1 hybrid pairwise sequentially Markovian
638 coalescent model, hPSMC (Cahill *et al.* 2016). To address whether ascertainment bias may have
639 played a role in our results, we performed this analysis twice independently for the between
640 species comparisons, white vs black rhinoceros, once using the black rhinoceros as reference
641 genome, and once using the white rhinoceros as reference genome. Within species comparisons
642 were only computed once using the conspecific genome as reference. We constructed haploid
643 consensus sequences for the four individuals using ANGSD by considering the base with the
644 highest effective depth, the following quality filters; -minQ 25, -minmapq 25, -uniqueonly 1, -
645 remove_bads 1, and only considering autosomes and scaffolds over 100KB. We merged these
646 resultant haploid consensus sequences together into a pseudo diploid sequence using the
647 hPSMC tool suite. These were then run through PSMC and plotted using an intermediate mutation
648 rate per generation and generation time. When comparing the black and white rhinoceros we
649 used a generation time of 25.5 years and a mutation rate of 2.2×10^{-8} mutations per generation.
650 When comparing within species, we used intraspecific mutation rates and generation times. From
651 this, we manually estimated the pre-divergence N_e by outputting the text file (-R) using the plot
652 script from the PSMC tool suite and looking into the output text file. Using the pre-divergence N_e
653 estimated from this output, we then ran simulations to infer the confidence intervals using Ms
654 (Hudson 2002) with the hPSMC_quantify_split_time.py python script from the hPSMC tool suite,
655 while specifying the time windows we wanted to simulate, and the remaining parameters as
656 default. When comparing black and white rhinoceros, we estimated a pre-divergence N_e of
657 60,000 and ran simulations using divergence times between 3,000,000-7,000,000 years in
658 100,000 year intervals. When comparing northern and southern white rhinoceros, we estimated
659 a pre-divergence N_e of 7,000 and ran simulations using divergence times between 50,000-
660 450,000 years in 10,000 year intervals. When comparing eastern and southern black rhinoceros,
661 we estimated a pre-divergence N_e of 13,000 and ran simulations using divergence times between
662 0-400,000 years in 10,000 year intervals. Results were plotted and the simulations with an
663 exponential increase in N_e closest to the real data, within 1.5x and 10x of the pre-divergence N_e ,
664 were taken as the time interval in which gene flow stopped. We considered the portion between
665 1.5x and 10x of the pre-divergence N_e as suggested by the original manuscript. This was
666 suggested in order to capture the portion of the hPSMC plot most influenced by the divergence
667 event. The lower bound is set to control for pre-divergence increases in population size and the

668 upper bound is to avoid exploring parameter space in which little information is present (Cahill *et*
669 *al.* 2016).

670

671 *Evaluating the role of ancestral gene flow on D-statistics results*

672 In order to evaluate the influence of ancestral gene flow on D-statistics results, we ran a simple
673 model simulation in MSMS (Ewing and Hermisson, 2010) specifying gene flow between the
674 ancestral lineages as shown in Figure 4. This was done using the following command: msms 82
675 500 -l 5 2 20 20 20 20 0 -t 1760 -r 352 -ej 0.2375 5 4 -ej 0.2375 125 3 2 -em 1.875 4 2 {migration
676 rate} -em 1.875 2 4 {migration rate} -ej 2.5 4 2 -ej 12.5 2 1. In brief we specified window sizes of
677 1MB, an effective population size of 20,000 for all five populations, with constant population sizes,
678 a generational mutation rate of 2.2×10^{-8} and a recombination rate one fifth of the mutation rate
679 (4.4×10^{-9}), three independent runs of 20,000 windows, each with different migration rates ($m =$
680 0.5, 1, and 2), a divergence time of 200,000 generations between the black and white rhinos, the
681 end of gene flow between the black and white rhinoceros as 150,000 generations, the within
682 species divergence as 19,000 generations and assuming a generation time of 25.5 years. The
683 output of the simulations were then run through a custom python script which calculated the D-
684 score for each 1MB window independently. Finally, we performed a block jackknifing approach
685 with the resample library in R v3.6.0 to test for significance of the results.

686

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688

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701

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894

895 **Figure legends**

896

897 **Figure 1.** The distribution of African rhinoceros taxa in time and space. The distribution of co-
898 occurring Pliocene fossils of *Diceros praecox* (precursor to the black rhinoceros) and
899 *Ceratotherium mauritanicum* (precursor to the white rhinoceros) are given in red-grey squares.
900 Pleistocene fossil distributions of modern black and white rhinoceros are given in black, white or
901 black-white squares and Holocene distributions (after Rookmaaker and Antoine, 2012) of eastern
902 (green) and southern (yellow) black rhinoceros and northern (blue) and southern (red) white
903 rhinoceros are depicted.

904

905 **Figure 2.** Evolutionary and demographic histories of the black and white rhinoceros. **A.** Species
906 tree and intraspecific divergence times assuming an ancestral split at the Miocene-Pliocene
907 boundary. **B.** Demographic reconstructions for each African rhinoceros species showing windows
908 for the end of panmixia within both species. **C.** Paleoclimatic reconstruction for the last 1.4 million
909 years, modified from Zachos *et al.* (2001).

910

911 **Figure 3.** Inferring the cessation of gene flow between and within black and white rhinoceros
912 using hPSMC and simulations. **A.** hPSMC plot between black and white rhinoceros and
913 simulations of different divergence times spanning 3-7 Ma in 100,000-year intervals. **B.** hPSMC
914 between northern and southern white rhinoceros and simulations of divergence times spanning
915 50,000-450,000 years in 10,000-year intervals. **C.** hPSMC between eastern and southern black
916 rhinoceros and simulations of divergence times spanning 0-400,000 years ago in 10,000-year
917 intervals. Greyed out regions represent 1.5x and 10x the pre- divergence effective population
918 size. Bold red lines represent the hPSMC results based on the real data. Thin grey lines represent
919 the simulated data while thin black lines represent the simulations closest to the real data without
920 overlapping it, which was used to infer the time interval when gene flow ceased.

921

922 **Figure 4.** Model of gene flow and the evolution of specialization in white and black rhinoceros.
923 Our analyses indicate that ongoing gene flow between speciating *Ceratotherium* and *Diceros*
924 lineages continued for up to two million years after initial divergence. Black asterisks indicate the
925 first appearances of both lineages in the fossil record. The grey dashed line marks the time at
926 which fossils of both lineages were present at Kanapoi in East Africa.