

1 **Title**

2 **Limited Introgression Supports Division of Giraffe into Four Species**

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17 **Abstract**

18 All giraffe (*Giraffa*) were previously assigned to a single species (*G. camelopardalis*) and nine
19 subspecies. However, multi-locus analyses of all subspecies have shown that there are four
20 genetically distinct clades and suggest four giraffe species. This conclusion might not be fully
21 accepted due to limited data and lack of explicit gene flow analyses. Here we present an
22 extended study based on 21 independent nuclear loci from 137 individuals. Explicit gene flow
23 analyses identify less than one migrant per generation, including between the closely related
24 northern and reticulated giraffe. Thus, gene flow analyses and population genetics of the
25 extended dataset confirm four genetically distinct giraffe clades and support four
26 independent giraffe species. The new findings call for a revision of the IUCN classification of
27 giraffe taxonomy. Three of the four species are threatened with extinction, mostly occurring
28 in politically unstable regions, and as such, require the highest conservation support possible.

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32 **Keywords**

33 Conservation, gene flow, hybridization, speciation

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40 Introduction

41 Traditionally, giraffe were classified as a single species (*Giraffa camelopardalis*) with up to
42 eleven subspecies proposed (Lydekker, 1904). However until recently, the classification into
43 nine subspecies was most widely accepted (Dagg & Foster, 1976). It has been shown that in
44 captivity some giraffe subspecies hybridize (Gray, 1972; Lackey, 2011; Lönnig, 2011), which
45 seemed to supported the traditional single species concept for giraffe. However, multi-locus
46 analyses of wild giraffe nuclear loci identified four monophyletic, distinct and evolutionary old
47 groups of giraffe that should be recognized as four distinct species (Fennessy et al., 2016). This
48 finding conflicts with former classifications and has been questioned based on the limited
49 interpretation of traditional data e.g. pelage pattern, number of ossicones and geographic
50 distribution (Bercovitch et al., 2017). The initial findings of four giraffe species (Fennessy et
51 al., 2016) could better be criticized, because it did not involve explicit gene flow analyses.

52 Imperative to understanding speciation in general from a genetic perspective is gene flow
53 analyses, especially as the keystone of the biological species concept (BSC) is reproductive
54 isolation (Coyne & Orr, 2004). The BSC implies that there is no or only very limited gene flow
55 between species. It has been proposed that one or a limited number of effective migrants (up
56 to 10) per generation (N_{em}) avoids genetic differentiation of populations and escapes a
57 substantial loss of genetic diversity for neutral traits (Lacy, 1987; Mills & Allendorf, 1996;
58 Vucetich & Waite, 2000; Wright, 1969). Thus, it is a conservative estimate that limited gene
59 flow of less than one migrant per generation ($N_{em} < 1$) can lead to speciation, despite the
60 occurrence of hybridization between mammal species. As shown, the BSC might need to be
61 revised as some species naturally hybridize in the wild and produce fertile offspring e.g. bears
62 (Arnold, 2016; Kelly, Whiteley, & Tallmon, 2010; Kumar et al., 2017) and whales (Bérubé &

63 Aguilar, 1998; Spilliaert et al., 1991), and divergence can occur under genetic exchange
64 (Arnold, 2016). Whilst the distinction of four giraffe species is consistent with population
65 genetic analyses (Fennessy et al., 2016), gene flow among giraffe species has not yet been
66 sufficiently analyzed. Here, we revisit the hypotheses of four giraffe species using population
67 genetic methods that explicitly involve gene flow analyses with an increased dataset of 21
68 nuclear loci and 137 giraffe individuals from 21 locations across Africa (Fig. 1, Supplementary
69 Table 1).

70

71 **Materials and Methods**

72 **Sampling and DNA Extraction**

73 Tissue samples from all giraffe species and five subspecies were collected by the Giraffe
74 Conservation Foundation (GCF) and partners using remote biopsy darts with country-specific
75 research permits between 2009 and 2016 in accordance with ethical guidelines and
76 regulations of the respective governments and institutions. All samples were stored either in
77 RNAlater (Invitrogen) or > 95% ethanol. Additional giraffe samples were added to the dataset
78 of Fennessy et al. (2016) resulting in a total number of 217 giraffe individuals, mostly southern
79 giraffe. The geographical origins and individual IDs are shown in Supplementary Table 1.
80 Sample locations and geographical distributions are shown in Fig. 1. Additional southern
81 giraffe individuals were only included if they are from a hitherto unrepresented region. DNA
82 was extracted using either a Macherey-Nagel NucleoSpin Tissue Kit or a standard phenol-
83 chloroform extraction method. All experimental protocols are in compliance with the
84 guidelines for the best ethical and experimental practices of the Senckenberg Society, as well
85 as with national guidelines of the respective countries.

86

87 **Amplification and sequencing**

88 We PCR amplified and sequenced the seven intron markers previously published (Fennessy et
89 al., 2016) for 32 new individuals and developed 14 additional intron markers as described
90 (Fennessy et al., 2016). The 14 new intron markers were amplified and sequenced for a total
91 number of 137 individual giraffe and the okapi (*Okapia johnstoni*). PCRs were performed with
92 10 ng genomic DNA giraffe and okapi specific primers (see Supplementary Table 2 for primer
93 sequences and PCR conditions). We also amplified and sequenced the mitochondrial
94 cytochrome b and control region for all new individuals as described previously (Bock et al.,
95 2014). Each PCR was examined using agarose gel electrophoresis on a 1% agarose gel with
96 ethidium bromide.

97 Sanger sequencing was performed for the forward and reverse strand using the BigDye
98 terminator sequencing kit 3.1 (Applied Biosystems) with 5 ng of PCR product for each reaction
99 and analyzed on an ABI 3730 DNA Analyzer.

100 The sequences were manually edited and aligned in Geneious v.6.1.8 (Kearse et al., 2012).
101 Heterozygous insertions/deletions of nuclear sequences were resolved by hand or using
102 Indelligent v.1.2 (Dmitriev & Rakitov, 2008) and verified by allele-specific primers if necessary.
103 PHASE implemented in DnaSP v.5.10.01 (Librado & Rozas, 2009) was used to derive the allele
104 haplotypes of the nuclear sequences using a threshold of 0.6 and allowing for recombination.
105 All analyses, except of a mtDNA tree analysis, were performed using only nuclear allele
106 haplotype data.

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108

109 **Tree Analyses**

110 The mitochondrial cytochrome b and control region sequences of 217 giraffe including newly
111 generated as well as already published sequences (Bock et al., 2014; Brown et al., 2007;
112 Fennessy et al., 2016, 2013; Hassanin et al., 2012, 2007; Winter, Fennessy, Fennessy, & Janke,
113 2018) (Supplementary Table 1) were aligned, as well as concatenated, and a Neighbor-Joining
114 analysis was reconstructed in Geneious v.6.1.8 (Kearse et al., 2012). We used the HKY model
115 of sequence evolution (Hasegawa, Kishino, & Yano, 1985), as suggested by jModelTest v.2.1.1
116 (Darriba, Taboada, Doallo, & Posada, 2012) with 1,000 Bootstrap replicates and sequences of
117 two okapis were used as an outgroup.

118 A multi-locus Bayesian phylogenetic tree of the 21 intron markers for 137 individuals and the
119 okapi as outgroup was generated with the StarBEAST2 (Ogilvie, Bouckaert, & Drummond,
120 2017) package in BEAST v.2.4.5. (Bouckaert et al., 2014, p. 2) under the JC model of nucleotide
121 evolution as suggested as best fitting model by jModelTest v.2.1.1 (Darriba et al., 2012). A
122 lognormal relaxed clock was used with 10^9 generations and sampling every 20,000th iteration.
123 Convergence of the MCMC runs was analyzed with Tracer v.1.6.0 (Rambaut, Suchard, Xie, &
124 Drummond, 2014), and TreeAnnotator v.2.4.5 (Rambaut & Drummond, 2016) was used to
125 construct a maximum clade credibility tree with 30% burn-in for the nuclear markers.

126

127 **Population Genetic Analyses**

128 Haplotype information for each locus deduced by DnaSP (Librado & Rozas, 2009) was used to
129 code each individual. The haplotype matrix was then used to infer admixture with the
130 Bayesian clustering algorithm implemented in STRUCTURE v.2.3.4 . For the maximum number
131 of populations (K) between 1-10, we sampled 250,000 steps following a 100,000-step burn-in,

132 with 40 replicates each. The CLUMPAK webserver (Kopelman, Mayzel, Jakobsson, Rosenberg,
133 & Mayrose, 2015) was used to average the results, and to infer the most likely K based on the
134 posterior probability of K (Pritchard et al., 2000) and ΔK (Evanno et al., 2005). Additionally,
135 the most likely K was deduced based on the estimated Ln probability of data ($\text{Ln Pr}(X|K)$)
136 (Pritchard et al., 2010) using Structure Harvester (Earl & vonHoldt, 2012). Principal Component
137 Analyses were performed with the R package adegenet (Jombart, 2008) in R v.3.2.3 (R Core
138 Team, 2015) to assess the degree of similarity between defined population scenarios. Pairwise
139 Fixation index (F_{st}) values were calculated in Arlequin v.3.5.2.1 (Excoffier & Lischer, 2010)
140 based on the nuclear haplotypes.

141

142 **Gene flow analyses**

143 Long-term average gene flow among and within the giraffe species were calculated in the
144 coalescent genealogy sampler MIGRATE-N v.3.6.11 (Beerli, 2006; Beerli & Felsenstein, 2001)
145 by estimating the mutation-scaled population sizes (Θ) for each population and migration
146 rates (M) for each direction between a pair of populations. We used the Brownian motion
147 mutation model and the Bayesian inference analysis strategy, as some parameter
148 combinations are better estimated using the Bayesian approach compared to the Maximum-
149 likelihood approach (Beerli, 2012). The transition/transversion ratio was set to 2.31 as
150 estimated in MEGA v.7.0.16 (Kumar, Stecher, & Tamura, 2016) based on a concatenated
151 alignment of all 21 loci. Variable mutation rates were considered amongst loci. We used the
152 default settings for the Θ uniformpriors, and adjusted the M uniformpriors (0; 5,000; 10,000;
153 1,000), because the upper prior boundary appeared to be too small in initial first analyses.
154 Several short-runs were performed to check for convergence of the runs. A long-chain run was

155 performed for 6 million Markov Chain Monte Carlo (MCMC) iterations (60,000 recorded steps)
156 and a burn-in of 600,000 iterations. An adaptive heating scheme was used with four chains
157 and temperatures set by default with a swapping interval of 1. Convergence of the runs was
158 checked by the posterior distributions, Effective Sample Size (ESS) and consistency of results
159 between runs. In addition, we estimated short-term gene flow, as well as the probability of
160 recent hybridization for each individual in BayesAss v.3.0.4 (Wilson & Rannala, 2003) using
161 100 million MCMC iterations, a burn-in of 10 million and a sampling interval of 1000 iterations.
162 Mixing of the chain was improved by adjusting the acceptance rates for proposed changes to
163 the parameters (allele frequencies and inbreeding coefficient) by adapting the mixing
164 parameters for allele frequencies (ΔA) and inbreeding coefficients (ΔF) to 0.30. Convergence
165 was checked in Tracer v.1.6.0 (Rambaut et al., 2014) and by consistency of results of several
166 runs with different initial seeds. Results for short-term gene flow were visualized in circos plots
167 using the Circos Table Viewer v.0.63-9 (Krzywinski et al., 2009).

168

169 **Calculation of gene flow rate**

170 We calculated the effective number of migrants per generation ($N_e m$) or rate of gene flow
171 using two different methods. The first method to calculate $N_e m$ was based on the pairwise F_{st}
172 values using equation (1) by Wright (1951):

173

$$174 \quad (1) \quad N_e m = \frac{(1/F_{st} - 1)}{4}.$$

175

176 In addition, we calculated the $N_e m$ using the coalescent-based estimates for the mutation-
177 scaled population size Θ and the mutation-scaled immigration rate M derived from MIGRATE-

178 N. For autosomal markers equation (2) expresses the relationship between Θ_j (population size
179 of the population receiving migrants) and M_{ij} (corresponding migration rate) (Marko & Hart,
180 2011):

$$181 \quad (2) N_e m_{i>j} = \frac{M_{i>j} \times \Theta_j}{4}.$$

182

183 Results

184 The putatively independent 21 nuclear gene loci are on different chromosomes or are clearly
185 separated from each other in the bovine genome, a close relative with available chromosome
186 level genome data (Supplementary Table 2). A Bayesian multi-locus tree analysis of the 21
187 nuclear loci (total of 16,969 nucleotides) for 137 giraffe, including all traditionally recognized
188 giraffe subspecies (Fig. 2a), implies a clear separation into four giraffe clades: (1) a northern
189 giraffe cluster including West African (*G. c. peralta*), Kordofan (*G. c. antiquorum*), and Nubian
190 giraffe (*G. c. camelopardalis*) which includes the former Rothschild's giraffe (*G. c. rothschildi*),
191 (2) the reticulated giraffe (*G. reticulata*), (3) the Masai giraffe (*G. tippelskirchi*) including
192 former Thornicroft's giraffe (*G. c. thornicrofti*), and (4) a southern giraffe cluster (*G. giraffa*)
193 including South African (*G. g. giraffa*) and Angolan giraffe (*G. g. angolensis*). The monophyly
194 of each of these four clades is supported by a posterior probability of $p \geq 0.95$. However, in
195 the analyses the exact relationships of southern and Masai giraffe relative to northern and
196 reticulated giraffe could not be determined with significant probability ($p \approx 0.81$) (not shown).
197 A mtDNA Neighbor-Joining tree (Supplementary Fig. 1) confirms the reciprocal monophyly of
198 seven distinct subspecies clusters with Bootstrap support of $\geq 80\%$ (Bock et al., 2014; Brown
199 et al., 2007; Fennessy et al., 2016). MtDNA do not support two subspecies of Masai giraffe
200 because individuals which are designated Masai giraffe individuals, disrupt a possible

201 reciprocal monophyly. For reticulated giraffe, three individuals do not group as expected but
202 rather fall within the northern giraffe, indicating possible hybridization. However, two of these
203 individuals are from zoos, where hybridization is common, and have an unknown history. The
204 third individual is a wild giraffe from a geographic range adjacent to the northern giraffe and
205 is a possible natural hybrid, but during sampling was identified as a phenotypically reticulated
206 giraffe.

207 Multi-locus population STRUCTURE analyses (Pritchard, Stephens, & Donnelly, 2000) of 21
208 nuclear loci (Fig. 2b) proposes the best clustering into four distinct populations (optimal $K = 4$)
209 based on the graphical display. At $K = 3$ the analyses merge the reticulated and the northern
210 giraffe and at $K \geq 5$ the analyses do not produce further clustering. Three different statistical
211 methods to interpret the STRUCTURE results (Evanno, Regnaut, & Goudet, 2005; Pritchard et
212 al., 2000; Pritchard, Wen, & Falush, 2010) confirm $K = 4$ being significantly the best fitting
213 number of populations (Supplementary Fig. 2). These four clusters conform with the four
214 giraffe clades identified by tree analyses. Intriguingly, STRUCTURE also identifies three
215 potential hybrids between the northern and reticulated giraffe within the reticulated giraffe
216 clade (Fig 2a, Fig 2b). The distinctness of four unique giraffe clades is in addition supported by
217 Principal Component Analyses (PCAs) (Fig. 2c) with significant non-overlapping 95%
218 confidential intervals. PCAs using groups of the seven mtDNA clades do not find more than
219 four distinct clusters (Supplementary Fig. 3). Finally, pairwise fixation indices (F_{st}) of ≥ 0.237
220 (statistically significant at $p < 0.001$) are consistent with the four distinct clusters of giraffe in
221 the tree analyses (Supplementary Table 3).

222 Separate PCAs and STRUCTURE analyses for each species (Supplementary Fig. 4-7) indicate
223 population substructure within northern giraffe, and potentially in the Masai giraffe, but no

224 further population substructure in southern and reticulated giraffe. Within northern giraffe
225 STRUCTURE and PCAs up to four clusters can be identified. However, the sample sizes of some
226 populations (three for Ethiopia) are arguably insufficient to draw definitive conclusions.
227 Within the Masai giraffe STRUCTURE and PCAs identify potentially two separate clusters,
228 indicating a possible separation of the two geographically most distant populations that have
229 been analyzed for nuclear SNPs (single nucleotide polymorphisms) to date. Consistent with
230 the STRUCTURE and PCA analyses, pairwise F_{st} analyses within each giraffe species finds a high
231 level of population differentiation within northern and possibly Masai giraffe, and little
232 differentiation within southern and reticulated giraffe (Supplementary Table 4).

233

234 We estimated long-term gene flow within all four giraffe clades, as well as among subspecies
235 within each of the giraffe species which show population substructure in STRUCTURE and
236 PCAs using MIGRATE-N (Beerli, 2006; Beerli & Felsenstein, 2001). Assuming similar mutation
237 rates among all giraffe species, the mutation-scaled population size θ estimates for
238 the four species suggest that the effective population size (N_e) is smaller in southern giraffe
239 and Masai giraffe than in northern and reticulated giraffe (Supplementary Table 5a). Thus, the
240 population size in the northern and reticulated giraffe had been larger in the past. The
241 calculated effective numbers of migrants per generation or gene flow rate (N_{em}) based on θ
242 and the mutation-scaled migration rate (M) (Supplementary Table 5b) indicate generally very
243 low level of gene flow among most of the four giraffe clades with a maximum of one migrant
244 per five generations ($N_{em} \leq 0.179$), with one exception. A higher N_{em} occurs between the
245 northern and reticulated giraffe, with nearly one migrant per generation in the direction of
246 the reticulated giraffe ($N_{em} = 0.945$), but much less migration is observed in the opposite

247 direction to northern giraffe ($N_{em} = 0.179$). There is little (ca. one in ten) directional gene flow
248 from Masai to reticulated giraffe ($N_{em} = 0.107$) and from southern to reticulated giraffe (N_{em}
249 $= 0.104$) with nearly zero gene flow in the opposite direction. The gene flow rates for all other
250 species pairs are extremely low ($N_{em} < 0.065$). Within species long-term gene flow rates are
251 on average higher ($N_{em} > 1$) (Supplementary Table 6b). However, between some subspecies
252 gene flow is also limited, in particular the geographically extremely isolated West African
253 giraffe (WA).

254

255 Gene flow rates that were calculated on pairwise F_{st} values between species corroborate the
256 MIGRATE-N analyses, but provide no information about the direction of gene flow and the
257 rates are somewhat higher (Supplementary Table 3). In agreement with the MIGRATE-N
258 analyses, the F_{st} based analyses find the highest rate of gene flow between northern and
259 reticulated giraffe ($N_{em} = 0.804$), and a much lower gene flow rate (N_{em} ranges from 0.113 to
260 0.186) is observed among all other population pairs.

261

262 Finally, short-term migration rates (m) estimated with BayesAss (Wilson & Rannala, 2003)
263 (Figure 3, Supplementary Table 5b) confirm low levels of gene flow among the four giraffe
264 species for the past three generations. The highest migration rates occur from northern, Masai
265 and southern giraffe in the direction of reticulated giraffe. The data suggest that
266 approximately 2% ($m = 0.021$) of the reticulated giraffe population are derived from each of
267 these neighboring species, which is expected. In comparison, to the other gene flow analyses,
268 BayesAss identifies somewhat higher recent migration rates (m) among subspecies within
269 species (Supplementary Table 6b). This is consistent with the lack of genetic differentiation

270 identified by PCA and F_{st} analyses. The recent migration rates estimated by BayesAss analyses
271 suggest directional gene flow between West African and Kordofan giraffe ($m = 0.064$), and
272 find gene flow between South African and Angolan giraffe ($m = 0.052$). Most importantly,
273 however is that BayesAss does not find any first or second generation hybrids.

274

275 **Discussion**

276 Morphology and genetic analyses suggest that there is more than one giraffe species (Brown
277 et al., 2007; Fennessy et al., 2016; Groves & Grubb, 2011). Here we expand our previous
278 dataset three-fold and improve the sampling of northern and reticulated giraffe, to further
279 study if there are indeed more than one species. The new data set allows for the first-time
280 detailed gene flow and migration analyses. Among the four giraffe species⁶, gene flow and
281 migration is very limited. As such, the new analyses of the extended nuclear data corroborate
282 the identification of four genetically distinct giraffe species (Fennessy et al., 2016).

283 Several attempts have been made to define a species, but a unequivocal consensus has not
284 yet been reached (Coyne & Orr, 2004; De Queiroz, 2007). The most commonly applied model
285 is the BSC, which suggests that reproductive isolation is essential to delineate species
286 (Dobzhansky, 1970; Mayr, 1942). By contrast, subspecies or evolutionary significant units
287 (ESU) are sometimes arbitrary distinctions within a species. Reproductive isolation is also a
288 cornerstone of other species concepts that define species as distinct ESUs with limited gene
289 flow to other such units (Avice & Ball, 1990). Therefore, analyzing gene flow among species is
290 a central analysis to delineate species, especially if, like in giraffe, they possibly hybridize in
291 nature. It has been suggested that gene flow among species must be limited to allow genetic
292 differentiation, and a value below one migrant per generation ($< 1 N_e m$) is a conservative

293 estimate (Wright, 1969), even if other studies are more liberal and suggest that gene flow
294 rates of $< 5 N_e m$ (Lacy, 1987) or even $< 10 N_e m$ (Mills & Allendorf, 1996; Vucetich & Waite,
295 2000) can allow genetic differentiation and consequently speciation.

296 The initial finding of four giraffe species was unexpected (Fennessy et al., 2016), because
297 giraffe seem to be a morphologically homogenous group, can interbreed in captivity (Gray,
298 1972; Lackey, 2011; Lönnig, 2011), and are highly mobile (Flanagan, Brown, Fennessy, &
299 Bolger, 2016). To avoid differentiation between populations, and if giraffe was in fact one
300 species, gene flow rates in excess of 1-10 migrants per generation are expected based on
301 mathematical models (Lacy, 1987; Mills & Allendorf, 1996; Vucetich & Waite, 2000; Wright,
302 1969).

303 Our study show that the long-term average estimates of gene flow rate among giraffe are
304 below one migrant per generation ($N_e m < 1$). The introgression and population genetic
305 analyses of the expanded data set are thus consistent with the previously proposed
306 classification of four giraffe species by Fennessy et al. (2016). The highest gene flow rates are
307 observed between the northern and reticulated giraffe, but the rate is below Wright's (1969)
308 conservative estimate of $< 1 N_e m$. Compared to other giraffe species, higher gene flow rates
309 among northern and reticulated giraffe is not unexpected, because they are the closest
310 related (Fennessy et al., 2016) and their current neighboring geographic ranges might have
311 overlapped historically (Fig. 1).

312 Yet, even among the closely related and neighboring northern and reticulated giraffe lower
313 gene flow rates than $< 1 N_e m$ are observed, which is consistent with being genetically
314 differentiated species. In addition, among all 137 individuals from a wide geographic

315 distribution, only one natural hybrid has been genetically identified yet. The rare occurrence
316 of hybrids further supports the existence of four giraffe species.

317 Population genetic analyses, such as STRUCTURE and PCA of the data set support the results
318 from the gene flow analyses. The new results are inconsistent with past suggestions of
319 possibly six or seven distinct giraffe species (Brown et al., 2007). These results were based on
320 non-stringent conclusions from STRUCTURE analyses with 11 separate genetic clusters at $K =$
321 13 based on 14 microsatellites, and results of six to seven giraffe clusters based on mtDNA
322 phylogeny (Brown et al., 2007): “11 of the 18 sampling localities resolved as distinct genetic
323 clusters at $K=13$ ”, however the authors concluded that only “the seven lineages that are
324 reciprocally monophyletic in the mtDNA tree need to be considered evolutionary significant
325 units if not species”. Other findings of up to eight giraffe species were proposed based on a
326 combination of limited genetic analyses (Brown et al., 2007; Hassanin, Ropiquet, Gourmand,
327 Chardonnet, & Rigoulet, 2007) and morphological characteristics (Groves & Grubb, 2011),
328 however the location of some samples were inaccurate.

329 Both Fennessy et al. (2016) and Bock et al. (2014) suggested to subsume Rothschild’s giraffe
330 (MF) into the Nubian giraffe, as well as Thornicroft’s giraffe (LVNP) into the Masai giraffe,
331 because they lack differentiation at mtDNA sequences. Evolutionary differentiation of
332 populations is often first evident in mtDNA, because theory suggests that this locus, due to its
333 maternal inheritance and non-recombining nature, reaches fixation 4-times more rapidly than
334 nuclear loci (Zink & Barrowclough, 2008). Thus, differentiation into subspecies is often first
335 evident on mtDNA, rather than nuclear sequences. Such population differentiation processes
336 have been reported in natural population of bears (Hailer et al., 2012), humpback whales
337 (Palumbi & Baker, 1994) and macaques (Melnick & Hoelzer, 1992).

338 While the current mtDNA analyses support previous findings (Fennessy et al., 2016) of
339 Thornicroft's giraffe being subsumed into the Masai giraffe, new and extended nuclear gene
340 datasets identify some substructure among them. We emphasize however, that the nuclear
341 loci have only been sampled from across a limited distribution of the Masai giraffe⁶. Additional
342 sampling of intermediate Masai giraffe populations and additional nuclear gene loci will be
343 necessary to yield more definite results. The first detailed mtDNA analyses on Thornicroft's
344 giraffe (Fennessy, Bock, Tutchings, Brenneman, & Janke, 2013) proposed that while they are
345 not reciprocal monophyletic, the geographic location in Zambia's Luangwa Valley is unique
346 and should, for conservation efforts, tentatively maintain its subspecies status as Thornicroft's
347 giraffe within Masai giraffe (*Giraffa tippelskirchi thornicrofti*).

348 Within the northern giraffe some substructure is evident in PCAs and STRUCUTURE analyses
349 for nuclear sequences (Supplementary Fig. 6). However, the West African giraffe is a
350 geographically very isolated and small population of ~600 individuals. As described (Fennessy
351 et al., 2016), the geographic distinction between the former Nubian and Kordofan giraffe is
352 unclear and current data suggest that they are not genetically isolated.

353 Multi locus phylogenies, population genetic and gene flow analyses support the hypothesis of
354 four genetically distinct giraffe species (Fennessy et al., 2016). The molecular data show that
355 there is only very limited gene flow between the four species, which is in agreement with the
356 BSC.

357 With little more than 5,000 northern giraffe, <8,700 reticulated giraffe and ~34,000 Masai
358 giraffe remaining in the wild (Giraffe Conservation Foundation, 2017), recognizing these – and
359 the southern giraffe – as separate species has an impact on giraffe conservation. Their decline
360 in numbers over the last thirty years (three generations) – northern giraffe (~95 %), reticulated

361 giraffe (~80%) and Masai giraffe (~52 %), highlight that these species are threatened with
362 extinction(IUCN, 2017). Giraffe, as a single species, and not four, were recently listed as
363 “Vulnerable” on the IUCN Red List (Muller et al., 2016). The mounting evidence of four giraffe
364 species proposes a re-evaluation of the current IUCN giraffe taxonomy to raise the
365 conservation classification to a higher level of threat, and in turn increased conservation
366 management actions.

367

368 **Acknowledgements**

369 We thank Susanne Gallus, for support in the laboratory, and Fritjof Lammers as well as Markus
370 Pfenninger for support with analyses. Stephanie Fennessy and Maria Nilsson gave valuable
371 comments on the manuscript. We thank Jon B. Hlidberg for the artwork of a Nubian giraffe.
372 This study was supported by the Leibniz Association, the Giraffe Conservation Foundation-
373 USA, the Leiden Conservation Foundation, and various African government partners and
374 international zoo and private supporters.

375

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542 **Data Accessibility Statement**

543 The authors declare that all the data supporting the findings of this study are available within
544 the article and its Supplementary Information files. Sequences generated during the study are
545 available at GenBank (<https://www.ncbi.nlm.nih.gov/nucleotide/>) under the Accession
546 Numbers MG257948 - MG262301.

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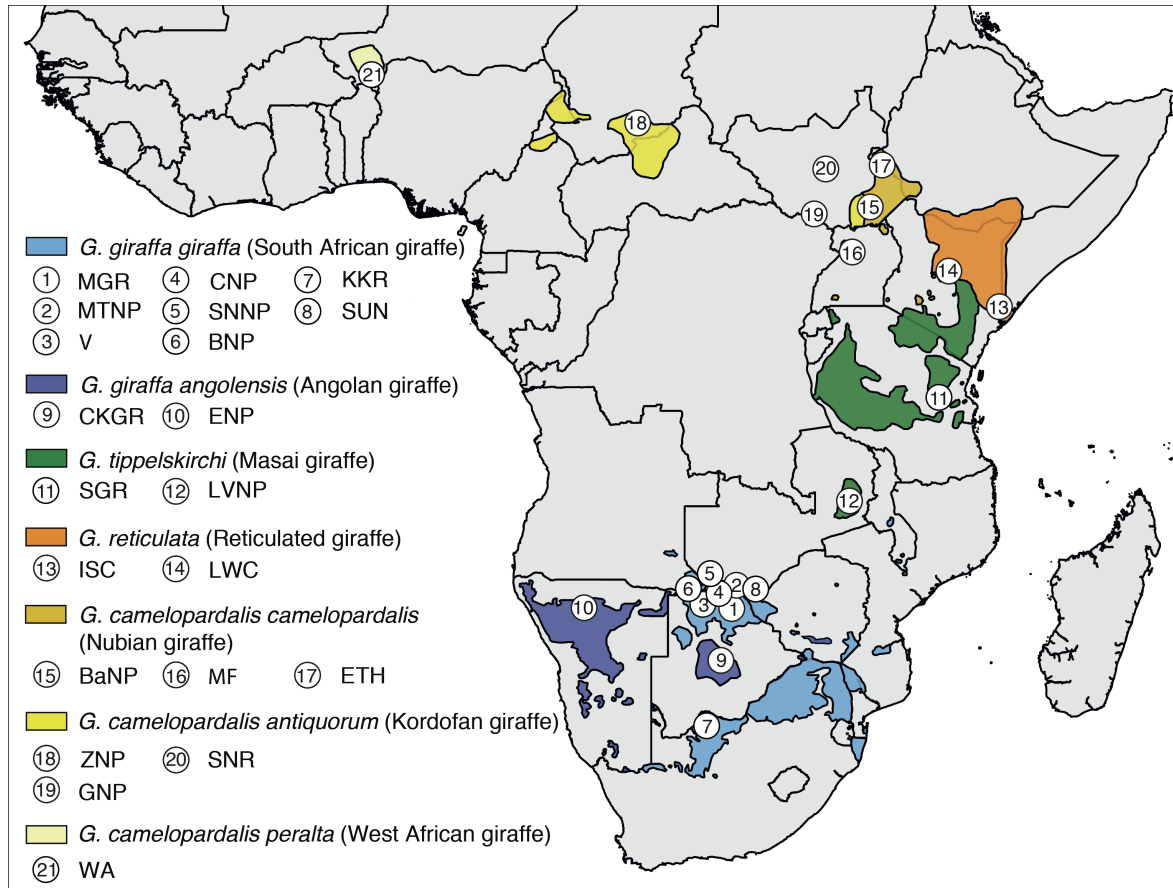
548 **Author Contributions**

549 AJ, JF and SW designed and conceived the study. AJ and JF funded the project, JF collected
550 the samples and provided biological data. SW developed markers, generated and analyzed the
551 data. SW and AJ wrote the manuscript with input from JF.

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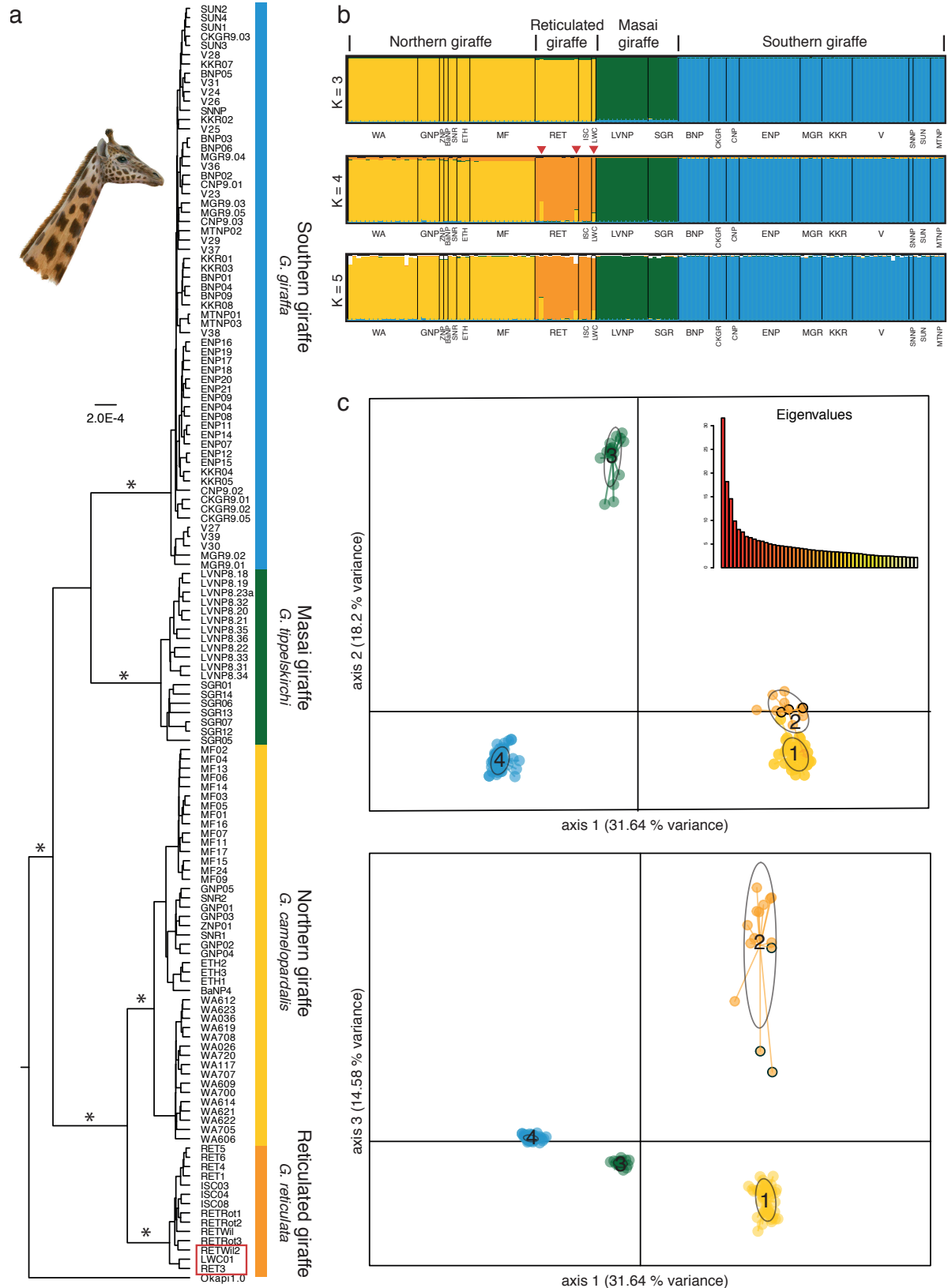
554 **Figures**



555

556 **Fig. 1 Map of Sub-Saharan Africa with giraffe (sub)species distributions and sampling**
 557 **locations.**

558 Geographic ranges (colored shadings) of giraffe as identified by the Giraffe Conservation
 559 Foundation (2017) were plotted on a map of Sub-Saharan Africa. Numbered circles represent
 560 sampling locations (for details see Supplementary Table 1). Species and common names as
 561 per Fennessy et al. (2016).



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564 **Fig. 2 Nuclear phylogeny and population structuring of giraffe**

565 (a) Bayesian multi-locus tree from 21 nuclear loci and 137 giraffe individuals reconstruct four
566 significant supported ($p \geq 0.95$) giraffe clades, corresponding to the four giraffe species
567 (Fennessy et al., 2016). The okapi is used as the outgroup. The asterisks indicate branches
568 with statistical significant support ($p \geq 0.95$). The red frame indicates the potential hybrids.

569 (b) STRUCTURE analysis of the dataset, excluding the okapi. The colors indicate the
570 membership in a cluster for each sampling location and individual. $K = 4$ shows four well-
571 resolved groups and is supported as best fitting number of clusters by several statistical
572 methods (see Supplementary Fig. 2). The grouping into four clusters is consistent with the
573 Bayesian multi-locus analysis: yellow: northern giraffe, orange: reticulated giraffe, green:
574 Masai giraffe, and blue: southern giraffe. Three individuals within the reticulated giraffe
575 cluster (red arrowheads) indicate potential hybridization with admixture from the northern
576 giraffe. $K = 3$ merges northern and reticulated giraffe, and at $K \geq 5$ no further clustering is
577 evident.

578 (c) PCA axes 1-2 and axes 1-3 for four distinct giraffe clusters (1: northern; 2: reticulated; 3:
579 Masai; 4: southern). Colors as in Fig 2b. The 95% confidence intervals are shown as oval
580 outlines. Note that the non-overlapping confidential intervals in the PCA axes 1-2, as well as,
581 axes 1-3 indicate significantly different clusters. Potential hybrids are indicated by black
582 circles.

583 Note – The drawing by Jon B. Hlidberg shows a Nubian giraffe.

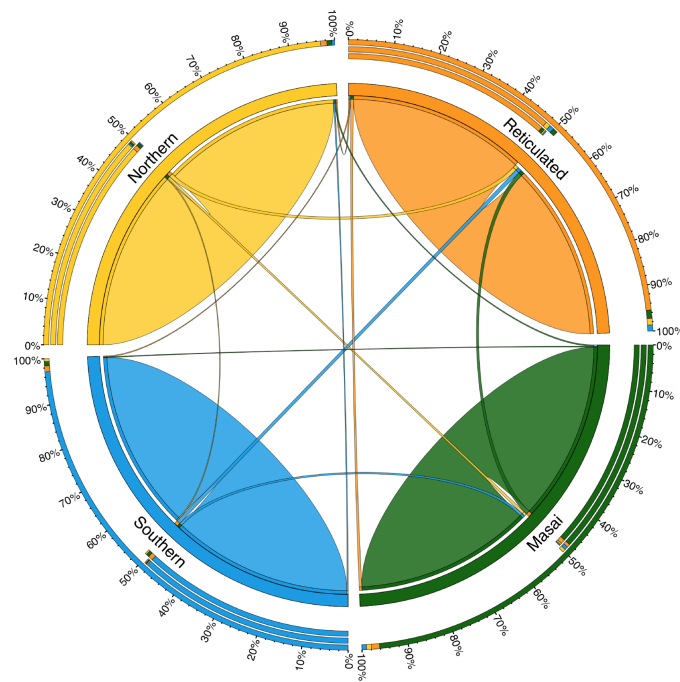
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590 **Fig. 3 Circular migration plot of recent migration rates among four giraffe clades.**

591 Recent directional migration rates (m) as estimated by BayesAss and indicated by ribbons
592 connecting one species to another. The color coding of the four species is according to the
593 STRUCTURE clusters (Fig 2b). Peripheral concentric stack bars show relative migration rates in
594 percent. Whereas the inner stack bar shows the outgoing ribbon sizes, the middle stack bar
595 the incoming ribbon sizes and the outer stack bar the combination of both.