1   **Can DNA help trace the local trade of pangolins? A genetic assessment of white-bellied**

2   **pangolins from the Dahomey Gap (West Africa)**

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23

24 **Abstract**

25 We conducted in the Dahomey Gap (DG) a pioneer study on the genetic tracing of the African

26 pangolin trade. We sequenced and genotyped 189 white-bellied pangolins from 18 forests and

27 12 wildlife markets using one mitochondrial fragment and 20 microsatellites loci. Tree-based

28 assignment procedure showed the 'endemicity' of the pangolin trade, as strictly fed by the

29 lineage endemic to the DG (DGL). DGL populations were characterized by low levels of

30 genetic diversity, an overall absence of equilibrium, inbreeding depression and lack of

31 geographic structure. We identified a 92-98% decline in DGL effective population size 200-

32 500 ya –concomitant with major political transformations along the 'Slave Coast'– leading to

33 contemporaneous estimates inferior to minimum viable population size. Genetic tracing

34 suggested that wildlife markets from the DG sourced through the entire DGL range. Our loci

35 provided the necessary power to distinguish among all the genotyped pangolins, tracing the

36 dispatch of same individuals on the markets and within local communities. We developed an

37 approach combining rarefaction analysis of private allele frequencies and cross-validation

38 with observed data that could trace five traded pangolins to their forest origin, c. 200-300 km

39 away from the markets. Although the genetic toolkit that we designed from traditional

40 markers can prove helpful to trace the pangolin trade, our tracing ability was limited by the

41 lack of population structure within DGL. Given the deleterious combination of genetic,

42 demographic and trade-related factors affecting DGL populations, the conservation status of

43 white-bellied pangolins in the DG should be urgently re-evaluated.

44

45   **Keywords:** Microsatellites, conservation genetics, demographic decline, trade tracing, white-

46   bellied pangolin, Dahomey Gap

Introduction

48   Pangolins (Order Pholidota) are considered the most trafficked mammals in the world, with c.

49   900,000 individuals seized over the last 20 years [1]. Although pangolins have been –

50   mistakingly– highlighted as potential intermediary hosts of the COVID-19 pandemia [2], the

51   volumes traded have remained unsustainably high [3]. As the exponential demand from the

52   Traditional Chinese Medecine (TCM) market has decimated the populations of Asian

53   pangolins, new routes of trafficking have emerged from Africa [4]. Between 2015 and 2019,

54   an estimated number of > 400,000 African pangolins were seized en route to Asian markets

55   [1, 5]. Consequently, African pangolins are currently experiencing unprecedented levels of

56   harvesting, for both local demands and the illegal international trade, with a possible influence

57   of the Chinese diaspora on the African trade networks and dynamics [4, 6, 7].

58   The white-bellied pangolin (WBP; *Phataginus tricuspis*) is the species with the largest range

59   in tropical Africa and is the pangolin most frequently sold on the African bushmeat market

60   stalls [5]. It is also the most represented African species in international seizures of pangolin

61   scales [4, 8]. Recent genetic investigations have shown that WBP consisted of six cryptic,

62   geographically traceable lineages [9], one of which occurs 'outside' the rainforest blocks, in a

63   West African savannah corridor interspersed with very fragmented forest cover, the Dahomey

64   Gap[10]. The Dahomey Gap lineage (DGL) is of peculiar patrimonial importance as it is

65   endemic to a unique biogeographical zone in western Africa (from Togo to Benin and

66   southwestern Nigeria; [9] and likely is the only pangolin species surviving in the Dahomey

67   Gap [11, 12].

68   DGL populations currently suffer from intense levels of deforestation and hunting.

69   Populations are fragmented into –generally small– patches of forest islands, and have

70   drastically decreased in abundance through the last decades [11, 12]. In Togo and Benin,

4

71   WBG is hunted for its meat and use in traditional medicine, both contributing to its

72   overexploitation [11, 12]. Because of the recent seizure of scales intended to the international

73   trade (Cotonou airport; [4]) the prominent proportion of DGL in Asian seizures [8] and the

74   established traffick hub in the neighbouring Nigeria [13], there is a serious risk that DGL

75   populations are exploited in unsustainable volumes.

76   The genetic toolkit has been successfully applied to trace the illegal wildlife trade, by

77   providing accurate information on the species traded and their geographic origins [14, 15].

78   Recently, the genetic distinctiveness among the different WBP lineages has been used to

79   assign the regional origins of international seizures of pangolin scales [8]. However, the

80   current lack of knowledge on the fine-scale population structure of WBP hampers any

81   attempts at tracing their local –e.g., country-scale– trade (see [16]). The stakes behind tracing

82   the local WBP trade are high, as they reside in, and, therefore, better Efficient conservation

83   actions to mitigate the local WBP trade will rely on identifying the source populations feeding

84   the market (and thus the market network) and accurate estimates of the number of individuals

85   traded (e.g., from scale seizures). We propose a pioneer investigation on the utility of the

86   genetic toolkit in tracing the local pangolin trade, based on microsatellite markers recently

87   developed for WBP [17]. Our general objective is to provide a detailed overview of the

88   genetic status of DGL populations and their traceability on the local pangolin trade. Our

89   specific objectives encompass the assessment of (i) population structure and diversity within

90   DGL, (ii) the demographic history of this endemic lineage, and (iii) the resolutive power of

91   our genetic markers to trace the pangolin trade in the Dahomey Gap.

92

93   **Material and Methods**

94

95   *Genetic sampling and wet laboratory procedures*

96   We collected a total of 189 WBP samples across Benin (6°10' - 11°00' N), Togo (8°10 - 9°0')

97   and southwestern Nigeria (6°10' - 11°00' N). Our sampling effort included all the occurrence

98   zones of the species in Benin [11] and one central forest in Togo (111 samples from 18

99   forests), nine major traditional medicine markets (TMMs; 71) from Benin and Togo, and three

100   bushmeat markets in Benin and Nigeria (7). Sample types varied from fresh tissue, skin and

101   tongue (59), to dried skin and tissue (60), and scale connective tissue (70) (see Table S1,

102   Supplementary information). Thirty-six samples were taken from carcasses having received

103   preservartive chemical treatments [7]. Free consent from hunters and market sellers was

104   obtained before collecting samples. We relied on an opportunistic sampling strategy [18],

105   without financial incentives. The samples collected from the forest were traced to their

106   original location after the information provided by hunters [11].

107   DNA extraction from fresh and scale connective tissues was performed using the

108   NucleoSpin® Tissue Kit (Macherey-Nagel, Hoerdt, France), following manufacturer's

109   recommendations. Final elution step was repeated twice in 50 µl Elution buffer to increase

110   DNA yield. The samples treated with chemicals were extracted following a modified CTAB

111   protocole including upstream TE washing baths and Dithiothreitol (DTT; [19]. Elution

112   volumes varied from 30 to 100 µl nuclease free water, depending on the size of the DNA

113   pellet. DNA concentrations were estimated on the NanoDrop 1000 Spectrophotometer

114   (ThermoFisher Scientific, Illkirch-Graffenstaden, France).

115   We amplified a mitochondrial fragment of 432 bp from Control region 1 (CR1) following

116   Gaubert   et   al.   [9].   PCR   products   were   sequenced   at   Genoscreen

117   (https://www.genoscreen.fr/en; Lille, France) and Macrogen Europe (https://dna.macrogen-

118   europe.com/en; Amsterdam, the Netherlands). Sequences were aligned by-eye with BioEdit

119   v7.0.5 [20] and the unique haplotypes were submitted to Genbank under accession numbers

120   OK275650- OK275662.

121   We amplified 20 microsatellite markers developed from the genome of WBP in four

122   multiplexes following Aguillon et al. [17]. PCR triplicates were conducted for dried and

123   chemically treated samples to mitigate the potential issue of allelic dropout and false alleles

124   [21]. Consensus was considered met when at least two out of the three replicates indicated the

125   presence of an allele. PCR products were separated on automated sequencer at Genoscreen

126   and GeT-PlaGe (https://get.genotoul.fr/; INRAE, Toulouse, France).

127

128   **Data analysis – Control region 1**

129

130   *Clustering*

131   We assessed the 'endemicity' of the pangolins from Togo, Benin and Nigeria (N = 168)

132   through a distance-tree analysis including all the CR1 sequences of WBP available in

133   Genbank (N = 100). All the sequences were aligned by-eye with BioEdit v7.0.5 [20].

134   Phylogenetic tree reconstruction was performed in MEGA-X v10.2.2 [22] using Neightbor

135   Joining, 1,000 bootstrap replicates, Kimura 2-parameter model [23] and Gamma distribution

136   (G). A pangolin was considered endemic to the DGL if its sequence clustered within the

137   Dahomey Gap lineage as defined by [9].

138   *Genetic diversity and structure*

139   Genetic diversity and structure in DGL were estimated from sequences without missing data

140   (N = 126). We used DnaSP v6.12 [24] to compute haplotype number (h), haplotype diversity

141   (Hd) and nucleotide diversity (π) (Table S2, Supplementary information) for the six WBP

142   lineages. We mapped the distribution of haplotypes in DGL using ArcGIS 10.1 (Esri France).

143   We used Network v10.2.0.0 to build a median-joining haplotype network with $\varepsilon = 0$ to

144   minimize alternative median networks.

145   *Demographic history*

146   Mismatch analysis was performed in Arlequin 3.5.2 [25] to test for signatures of demographic

147   and spatial expansion in DGL, by calculating the sum of squared deviations (SSD) between

148   observed and expected distributions using 1,000 boostrap replicates [26].

149   We also tested for deviation from neutrality by computing a series of statistics in DnaSP,

150   including Tajima's D [27], Fu's Fs [28], Harpending raggedness index r [29] and Ramos-

151   Onsins and Rozas' R2 [30]. We ran 1,000 replicates assuming a coalescent process with a

152   neutral, infinite-sites model and large constant population sizes [31], to calculate the P-value

153   of each observed statistics.

154

155   **Data analysis – Microsatellites**

156   *Genetic diversity*

157   Genious 9.0.5 [32] was used for allele scoring and genotype extraction through the

158   Microsatellites plugin (https://www.geneious.com/features/microsatellite-genotyping/). Only

159   the DGL individuals with at least 75% of genotyping success ($\geq$15 microsatellites markers)

160   were considered for the analyses (N = 169). Genetic diversity at each locus was characterized

161 through (i) number of alleles (Na), observed (*Ho*) and expected (*He*) heterozysity as

162 computed from GenAlEx 6.5 [33], (ii) allelic richness (A_R) and F_{IS} as estimated from FSTAT

163 2.9.4 [34]. Deviation from Hardy-Weinberg Equilibrum (HWE) was calculated for each locus

164 in GenAlEx. Linkage Deseliquilibrum (LD) for all the pairs of loci was tested in FSTAT

165 using 1000 randomisations with Bonferroni correction. Null allele detection, assuming

166 population at equilibrium, was done with Microcheker 2.2.3 [35] using Bonferroni correction.

167 *Genetic structure*

168 Global genetic variance within DGL was visualized through Principal Coordinates Analysis

169 (PCoA), using pairwise population matrix unbiased genetic distances in GenAlEx.

170 Pairwise differentiation (F_{ST}) among forest populations (N = 104) was computed in Arlequin

171 3.5 [36] using three partition schemes (Fig. S3, Supplementary information): (i) a 6-partition

172 scheme considering populations (i.e., from a same habitat patch) with ≥7 sampled individuals;

173 (ii) a 3-partition scheme including a southern forest block radiating from the Lama forest (c.

174 50 km maximal radius), a central forest block radiating from the Mont Kouffé forest (c. 45 km

175 maximal radius), and a central forest block in Togo radiating from the Assoukoko forest (c. 25

176 km maximal radius); and (iii) a 3-partition scheme using a latidunal-based grouping in Benin

177 (South, lower Centre, and upper Centre). Because the first partition scheme yielded the

178 highest levels of differentiation among populations (see Results), we calculated their

179 inbreeding coefficients (F_{IS}) in FSTAT.

180 We used STRUCTURE 2.3.4 [37] to conduct a clustering analysis on all the DGL individuals.

181 We performed 20 independent runs for K = 1–10 using 10^5 Markov chain Monte Carlo

182 (MCMC) iterations and burnin = 10^4, assuming admixture model and correlated allele

183 frequencies. STRUCTURE HARVESTER 0.6.94 [38] was used to detect the most likely

184   number of populations (K) using the ☐K method [39]. We also ran STRUCTURE using the

185   LOCPRIOR model (and same other parameters) to assess the amount of information carried

186   by the geographic distribution of populations (r). For r values > 1, the geographic information

187   is considered uninformative [37].

188   We also inferred the number of populations, spatial locations of genetic discontinuities and

189   population membership among georeferenced individuals using the *Geneland* package [40] in

190   R 4.0.5 (*R Team Development Core 2021*). Following Coulon et al. (2006), we first allowed K

191   to vary from 1 to 10 and launched five runs of 5.10^5 MCMC iterations (500 thinning and 500

192   burn-in) under a frequence-correlated model and 1 km of uncertainty for spatial coordinates.

193   Second, we fixed the number of estimated populations on the basis of the first analysis (K =

194   6-7), to perform 20 independant runs using the same parameters. We also performed 20

195   independent runs fixing K = 3 as obtained with STRUCTURE (see Results). For both

196   analyses, we assessed how stable was population assignment of individuals among the best

197   (i.e. with highest posterior probabilities) five runs. We used 500×500 pixels to map the

198   posterior probabilities of population membership.

199   Given the absence of any clear genetic structuring from the above analyses (see Results), we

200   performed spatial Principal Component Analysis (sPCA) using the Delaunay triangulation

201   connection network, which defines neighbouring entities based on pairwise geographic

202   distances. sPCA is a spatially explicit multivariate approach capable of investigating complex

203   and cryptic spatial patterns of genetic variability from allelic frequencies[41]. Such an

204   approach does not require data to meet Hardy–Weinberg or linkage equilibrium among loci.

205   sPCA was run with the DGL georeferenced individuals using the *adegenet* package [42] in R,

206   with 9,999 MCMC resampling to infer global *vs*. local structuration levels. Threshold distance

207   between any two neighbors was set to zero.

208   We tested isolation-by-distance (IBD) among (i) forest individuals and (ii) populations (6-

209   partition scheme; see above) by running a Mantel test in the R package *pegas* [43], where we

210   quantified the correlation (*r*) between genetic (Edward's) and geographic (Euclidean)

211   distances through 10,000 permutations. The geographic distances among populations were

212   calculated from the center of each forest.

213   *Ability of the microtellites data to trace the pangolin trade*

214   The discriminating power of our microsatellite markers among market and non-market

215   individuals was evaluated by (i) couting the number of identical genotypes among samples

216   with the Multilocus tagging option in GenAlEx (suboption Matches), (ii) computing the

217   probability of encoutering the same genotype more than once by chance using the R package

218   *poppr* (method=single; [44], and (iii) calculating values of unbiased probability of identity

219   and probability of identity among siblings (uPI and PIsibs) in Gimlet 1.3.3 [45].

220   We used the generalized rarefaction approach implemented in ADZE [46] to compute private

221   allele frequencies among various combinations of populations. We delineated six original

222   forest populations after partition scheme (i), and let population number vary from 2 to 6

223   (sample size rarefied from 2 to 7). Because the original scheme of six populations yielded the

224   greatest frequencies of inferred private alleles (see Results), we graphed the rarefaction curves

225   for each locus in these six populations to assess their respective contributions in the

226   identification of private alleles per population. Loci that reached a plateau or showed an

227   exponential trend of their estimated private allele frequencies were considered as potentially

228   useful for tracing the origin of pangolins found in the markets, whereas loci showing a

229   decreasing trend were discarded. We then crossed these results with the private alleles

230   actually observed for the six populations (GenAlEx), and only considered the loci that showed

231   both observed private alleles (GenAlEx output) and high potential for tracing (ADZE output).

232   Finally, we manually screened the genotypes of the market individuals to retrieve such private

233   alleles and attribute source populations.

234

235   *Demographic history*

236   We tested for the signature of bottleneck events in DGL using the Single Mutation Model

237   (SMM) and the Two Phase Model (TPM) in BOTTLENECK 1.2.02 [47]. We applied the

238   Wilcoxon sign-rank test to analyze the presence of heterozygote excess/deficit using 10,000

239   replications.

240   Demographic history was also assessed through the R package *varEff* [48], an approximate-

241   likelihood method that infers temporal changes in effective population size. Given the lack of

242   data on sexual maturity in WBP, we fixed a conservative generation time of 2 yrs based on

243   estimates from Asian species [49, 50]. Mutation rate was fixed to 5.10^{-4} based on published

244   average mutation rate [51]. The analysis was run with the single mutation, geometric mutation

245   and two phases mutation models, using 10,000 MCMC batches with a length of 1 thinned

246   every 100 batches and JMAX = 3. The first 10,000 batches were discarded as part of the burn-

247   in period. Confidence intervals for ancestral and current effective population size estimates

248   were calculated from the harmonic means for each mutation model.

249

250   **Results**

251   *Mitochondrial DNA*

252   Our ML tree based on 268 mitochondrial DNA (mtDNA) sequences recovered the six WBP

253   geographic lineages with robust node supports, including Western Africa, Ghana, Dahomey

254   Gap, western Central Africa, Gabon and Central Africa (Fig. 1). All the sequences produced

255   from Togo, Benin and southwestern Nigeria clustered into the Dahomey Gap lineage

256   (bootstrap support = 75%).

257   We identified fourteen CR1 haplotypes in DGL. The median-joining network did not show

258   any specific geographic structure in haplotype distribution (Fig S1, Supplementary

259   information). Two haplotypes were dominant in the DGL, with H5 (45%) being widely

260   distributed and H1 (25%) located in the central and northern part of the range (Fig. 2). Nine

261   haplotypes were found both in forests and wildlife markets, while four were only found in

262   wildlife markets. The proportion of H5 (40 %) and H1 (27%) in wildlife markets was

263   reflective of their frequencies observed in forest populations.

264   Mismatch analysis of CR1 haplotypes showed a bimodal distribution significantly deviating

265   from the sudden expansion demographic model (P(Sim. Rag. >= Obs. Rag.) = 0.005),

266   whereas the spatial expansion model could not be rejected (P(Sim. Rag. >= Obs. Rag.) =

267   0.15). The statistics D (-0.56496), r (0.2057) and R2 (0.0710) showed no significant deviation

268   from a scenario of large and constant population size through time ($p > 0.10$), whereas Fs (-

269   4.780, $p = 0.0431$) significantly rejected the model.

270

271   *Microsatellites*

272   Within DGL, the number of alleles (Na) varied from 2 to 11 (mean=5.3). Allelic richness (A_R)

273   ranged from 1.86 to 8.04 (mean = 4.27; sample size = 169), and observed heterozygosity (*Ho*)

274   and expected heterozygosity (*He*) from 0.072 to 0.775 (mean = 0.414) and 0.069 to 0.842

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275   (mean = 0.498), respectively. Eleven loci deviated significantly from *HWE* ($P<0.05$). Six of

276   them showed significant levels of heterozygosity deficiency ($P < 0.0025$) and three of them

277   were involved in LD (PT_839522, PT_1453906 and PT_353755). Null alleles were identified

278   in ten loci, including seven that deviated from *HWE* (Table 1).

279   Genetic variance within DGL individuals did not show any geographic structuring on the

280   main PCoA axes (PC1 to PC3 = 12.36 % of total variance), whether individuals from wildlife

281   markets were considered or not (Fig. 3).

282   Paiwise population differentiations using partition schemes (ii) and (iii) ranged from low (F_{ST}

283   = 0.0378) to moderate (F_{ST} = 0.116; between central Togo and southern Benin). In partition

284   scheme (i), differentiations among the six forest populations were the greatest and all

285   significant ($p<0.05$) and ranged from low to moderate (F_{ST} = 0.0528-0.1399; 67% of the F_{ST}

286   values) and high ($0.166 < F_{ST} < 0.244$; 27% of the F_{ST} values) (Table S3, Supplementary

287   information). The mean inbreeding coefficient (F_{IS}) in the six DGL populations was 0.172 and

288   varied from 0.098 to 0.317 (Table S4, Supplementary information).

289   Bayesian clustering analysis with STRUCTURE –without prior information on locations–

290   detected a most likely number of populations K = 3 (Fig S2, Supplementary information). The

291   three clusters did not correspond to exclusive geographic delineations, each being an

292   admixture of individuals from southern and central forest regions together with wildlife

293   markets. The assignment probabilities of the individuals to their respective populations were

294   generally low. Under the LOCPRIOR model, r was equal to 2.368.

295   Final inference of population number using *Geneland* was K = 7 (95% of the runs). The

296   geographic delineation of the populations did not show any spatially exclusive distribution.

297   Assignment probabilities to populations varied greatly among the best five runs (data not

298   shown). Similar results were observed when fixing K = 3 (Fig S4, Supplementary

299   information).

300   The eigenvalues observed from the spCA analysis (Fig. 4a) suggested a relatively strong

301   signal of "local structure", indicating negative autocorrelation between geographic and

302   genetic distances in pangolins from the Dahomey Gap. However, we could not detect any

303   significant global or local structure signal (p = 0.602 and 0.102, respectively) across the study

304   area.

305   There was significant IBD effect among forest individuals (r = 0.128; p = 0.001) and between

306   populations (forests with at least 7 individuals) (r = 0.779; p = 0.009) (Fig. 5).

307   A total of 164 samples (97 %) had a unique genotype. Five samples shared a genotype with

308   four other samples, including three pairs (A65-A66, B1-B12, B2-B15) and one triplet (D14-

309   D15-D16; see Table S1, Supplementary information). The null hypothesis of encoutering the

310   different genotypes more than once by chance was rejected in all cases (P < 0.0001). The

311   unbiased probability of identity (uPI) and the probability of identity among siblings (PIsibs)

312   were both low (uPI= 8.12 e^{-13}; PIsibs = 9.22 e^{-06}). A number of 7 microsatellite loci were

313   needed to reach the conservative value of PIsibs < 0.01 (Fig S5, Supplementary information).

314   Estimated mean frequencies of private alleles across the 20 loci (sample size = 7) using

315   ADZE ranged from 0.10 to 0.24. Within the eleven loci that presented appropriate private

316   allele signatures for one or several populations (see Fig S6 and S7, Supplementary

317   information), seven loci provided six observed private alleles in GenAlEx that could

318   potentially differentiate among four populations. On this basis, five individuals found on

319   wildlife markets could be traced back to their forests of origin: A2 (Gbèdagba market, Central

320   Benin) and A38 (Azovè market, southern Benin) to the Wari-Maro forest reserve, B50 (Azovè

321   market, southern Benin) to the forests of central Togo, and D13 (Avogbannan market) and

322   D26 (Dantokpa market) to the Ouémé supérieur forest reserve.

323   Bottleneck analysis on the DGL across 20 loci was not significant (Wilcoxon sign-rank test;

324     $p>0.05$) for both SMM and TPM models.

325   *VarEff* identified a pronounced and recent decline in the effective population size (*Ne*) of

326   DGL regardless of the models (Fig. 6). Our results suggested a 92-98% reduction of *Ne*, from

327   1682-3440 (ancestral *Ne*) to 78-135 (contemporaneous *Ne*) individuals as harmonic means

328   (95% CI: 263 to 487). The decrease in *Ne* was estimated to occur c. 200-500 years ago.

329

330   **Discussion**

331   *The endemic lineage of white-bellied pangolins from the Dahomey Gap feeds an endemic*

332   *trade*

333   Our mtDNA tree-based assignment procedure showed that all the white-bellied pangolins

334   (WBP) collected and traded in the Dahomey Gap –from Togo, Benin and southwestern

335   Nigeria– belong to the Dahomey Gap lineage (DGL; [9]). With 168 new samples sequenced

336   from different forests and TMM, we have circumscribed with more accuracy the geographic

337   delimitation of the DGL, from central Togo to northernmost and southernmost locations in

338   Benin (Ouémé supérieur and Gnanhouizounmè, respectively), and Asejire in southwestern

339   Nigeria. We have also confirmed the absence of range overlap with other WBP lineages,

340   notably from the neighboring Ghana. This last result is to be tempered by the fact that

341   introgression between WBP lineages cannot be excluded –but has not been reported to date–

342   and could have passed undetected for the few samples sequenced at a single locus (mtDNA)

16

343   in our study. The endemic pattern of DGL superimposes with DNA-based delineation recently

344   found in mammals and plants from the Dahomey Gap [52, 53], further emphasizing the

345   patrimonial importance of the area for West African forest taxa.

346   Contrary to the bushmeat markets in Têgon and Hounkpogon (Benin) and Asejire

347   (southwestern Nigeria) that are known to source the game from nearby forests [18], this

348   study), the endemicity of the pangolin trade was not expected from the traditional medicine

349   markets (TMMs). Indeed, the geographic amplitude of the TTM network was shown in

350   previous investigations from Benin and Nigeria, revealing the long-distance trade of non-

351   native species [54, 55]. Moreover, there is a great demand for pangolins in the Dahomey Gap

352   [56], notably from the Chinese diaspora [7], and the trade of pangolins across borders have

353   been reported elsewhere in tropical Africa [57]. The endemicity of the pangolin trade in the

354   Dahomey Gap might translate into a huge hunting pressure on such a geographically restricted

355   lineage. This is especially true as what we observed on the markets might not apprehend the

356   full dimension of the pangolin trade, which may also feed the international market through

357   alternative networks [7, 57, 58].

358

359   *White-bellied pangolins in the Dahomey Gap show genetic diversity erosion and recent, sharp*

360   *demographic decline*

361   Overall, DGL populations were characterized by low levels of genetic diversity.

362   Mitochondrial (CR1) haplotype and nucleotide diversity was lower compared to all the other

363   WBP lineages. Mean allelic richness based on microsatellites was also lower than what was

364   found in WBP from Cameroon using the same markers (A_R = 4.63 *vs.* 6.74, respectively;

365   minimum sample size = 37; see [17]). Compared to genetic diversity estimates based on ten

17

366   WBP samples from Ghana, mean observed heterozygosity was again lower in DGL ($Ho =$

367   0.541 vs. 0.414, respectively; see [59]).

368   Low levels of genetic diversity are assumed to be negatively correlated with fitness and

369   adaptability [60]. The overall absence of equilibrium detected from our microsatellite dataset

370   suggests that inbreeding depression is one of the driving factors of the low genetic diversity

371   observed in DGL. Although deviations from Hardy-Weinberg equilibrium (55% of the loci in

372   this study) can be due to a number of factors including inbreeding, population structure and

373   genotyping errors [61], we can reasonably discard the two latter given (i) the apparent lack of

374   population structure in DGL and (ii) the optimized loci and genotyping approach that we

375   used. Besides, it is well known that populations going through inbreeding will produce

376   upwardly biased estimates of null allele frequencies [62], as observed in DGL (50% of the

377   loci). The deficit of heterozygotes observed in 75% of the loci, 30% of which having

378   significant levels of deficiency, also supports the view that DGL populations are subject to

379   inbreeding depression, possibly indicative of non-random mating [63].

380   Our demographic analyses based on microsatellites identified a sharp and recent decline in the

381   effective population size (Ne) of DGL c. 200-500 years ago (100-250 generations), leading to

382   a 92-98 % reduction to contemporaneous Ne below the conservative thresholds of minimum

383   viable population size (500-5000; [64, 65]). As variation in N_e is crucial in determining levels

384   of genetic diversity [66], the state of genetic depauperation observed in DGL may be directly

385   linked to the recent demographic decline affecting the lineage. The time of decline

386   corresponds to a period of major transformations along the 'Slave Coast', where from the 17th

387   century the Dahomey kingdom expanded as a state bureaucracy benefiting from the growing

388   trade of slaves and agricultural goods with Europeans [67, 68]. Whether such political growth

389   was followed by agricultural expansion and deforestation causing the decline of pangolins in

390   the region is uncertain, but similar declines have been observed in commercially exploited

391   species of vertebrates through the last centuries [68–70]. Our results are important for the

392   conservation of DGL, because inbreeding depression together with high levels of genetic drift

393   will potentially lead to the fixation of mildly deleterious alleles that could drive an extinction

394   vortex in this lineage [71].

395   Earlier events such as the expansion of agriculture in West Africa c. 4,200 BP [72] and

396   natural forest fragmentation caused by cyclical drier climatic conditions in the Dahomey Gap

397   from 4,500 BP [10] do not seem to have affected the demographic history of DGL

398   populations. Indeed, mtDNA-based demographic analyses showed no deviation from a model

399   of large, constant Ne through time, indicating long-term matrilineal stability. The only

400   exception was the Fu's statistics, which has maximum power to detect sudden demographic

401   decline events [73] and thus can be related to the recent decline discussed above. However,

402   further analyses based on nuclear genomic markers (SNPs) will have to be conducted to

403   assess the ancient demographic history of DGL, the origin of which dates back to 120-240

404   kya [9]. The Dahomey Gap is a broad savannah corridor intermixed with forest patches that

405   seperates the two African rainforest blocks, and as such can be considered a sub-optimal

406   habitat for WBP that heavily relies on rainforest cover [9]. Because the area underwent drastic

407   alternations of dry and humid periods since at least the last 150,000 yr [74], it is possible that

408   DGL populations were affected by early, successive founder effects and bottlenecks due to

409   Late Pleistocene climatic pejoration [53]. Such demographic events could also have shaped

410   the genetic diversity and absence of population structure (see below) observed today in DGL.

411   *The fragmented populations of white-bellied pangolins in the Dahomey Gap show no genetic*

412   *structure*

413   Our analyses based on mtDNA and microsatellites generally suggested that there was no

414   geographic structuring across the Dahomey Gap, against the expectation that habitat

415   fragmentation leads to genetic isolation [75]. One potential explanation to the lack of

416   population structure would involve a fair level of long-range dispersals. Pangolins have been

417   reported to disperse up to 300 km in four months, with a marked period of mobility for

418   unestablished young individuals through –notably– anthropized areas [76, 77] However, the

419   dispersal ecology of pangolins remains poorly known, especially in WBP. The species seems

420   to heavily rely on forest cover and old trees for its nocturnal activities [78], exploring its range

421   up to 1.8 km per night [79]. In the Dahomey Gap, WBP may occur in disturbed habitats

422   including commercial plantations of teaks and palm trees, fallows and farmlands [80].

423   However, evidence of long-range dispersal is lacking and the general absence of structural

424   connectivity among the remnant forest islands of the Dahomey Gap, especially in Benin (see

425   [81, 82]) does not support such scenario.

426   Although we could not find any clear population structure in DGL, we detected significant

427   levels of differentiation and isolation-by-distance among both individuals and populations.

428   We observed the strongest differentiations between the most distant Togolese and Beninese

429   populations, and some cases of moderate differentiation between geographically close

430   populations (e.g., the contiguous Ouémé supérieur and Wari-Maro protected areas). Genetic

431   differentiation among populations is determined by the interplay between homogenizing

432   processes such as gene flow and differentiating processes including local adaptation, different

433   adaptive responses to shared environmental conditions, and genetic drift [83]. If we posit that,

434   in the case of DGL, (i) gene flow between populations is not an option (see above), (ii)

435   genetic drift in isolated, inbred populations should have resulted in detectable geographic

436   structure [84], and (iii) environmental conditions are similar across the DGL range (Guineo-

437   Congolian and Sudano-Guinean zones; [85]), then different adaptive responses of populations

20

438   to a similar environment could be a candidate scenario to explain the level of differentiation

439   observed among populations from the Dahomey Gap. This could notably be expectable in the

440   case of populations trapped into sub-optimal environmental conditions and impacted by

441   frequent climatic oscillations (see [9]). However, microsatellites markers generally reflect

442   neutral genetic variation, and should not be affected by signatures of local adaptation [86].

443   A more plausible scenario would relate to a former, possibly recent, spatial expansion in

444   subdivided populations across the DGL range, as mtDNA did not reject this model. It is

445   possible that forest-restricted DGL populations underwent a spatial expansion following the

446   last recent increase in rainforest cover during the last Interglacial or early Holocene periods

447   [10]. Such event would explain the absence of population structure together with some level

448   of population differentiation as observed in this study, provided that dispersal among

449   populations would have been maintained long enough to counter-balance the effect of genetic

450   drift in isolated populations later induced by drier climatic conditions,[10]) and anthropogenic

451   pressure in the Dahomey Gap. Overall, our study has the merit to posit a number of

452   hypotheses that could explain the puzzling pattern observed in the population structure of

453   DGL. Such hypotheses will have to be tested through a demographic scenario-based strategy,

454   preferentially using versatile and powerful genomic resources.

455

456   *Tracing the pangolin trade in the Dahomey Gap: specimen dispatch and evidence for long-*

457   *distance trade*

458   The genetic diversity of WBP sold on the markets was reflective of the overall genetic

459   diversity (haplotype and allelic frequencies) observed in DGL forest populations, suggesting a

460   widespread sourcing of pangolins through the entire Dahomey Gap. Our 20 microsatellites

461   loci provided the necessary power to confidently distinguish among all the DGL indivuals,

462   and only seven best microsatellite loci were needed to reach the conservative value of

463   probability of identity < 0.01 [87]. The probability that two individuals drawn at random from

464   a population, including or not including siblings, will have the same genotype was low (but

465   higher than in previous studies,[17, 77]). This has important implications for the genetic

466   tracing of the pangolin trade in the Dahomey Gap, as one of the main inputs of the genetic

467   toolkit is its potential for tracing the trade at the individual level [88]. For instance, our

468   markers would be capable of estimating the exact number of individuals from scale seizures, a

469   major challenge that conservationists are currently facing [89]. We also demonstrated that our

470   genotyping approach was useful in tracing the dispatch of a same pangolin sold on the market

471   (one individual detected on two different stalls in Dantokpa) or kept by local communities

472   (scales of two different individuals shared between villagers in Mont Kouffé and Lama

473   forests). Such application is especially relevant for tracing the pangolin trade, which often

474   translates into separate networks specialized in the selling of specific parts (scales, organs,

475   meat), notably in Benin [7].

476   Given the lack of population structure in DGL and the negative autocorrelation between

477   geographic and genetic distances (for individuals and populations), classical assignment

478   procedures [90, 91] are hardly usable to trace the pangolin trade in the Dahomey Gap. We

479   have developed a conservative approach combining rarefaction analysis of private allele

480   frequencies in each population and cross-validation with observed data that could partly

481   circumvent that issue. Such method may be applicable to any taxon or lineage without

482   observed genetic structure across its range, notably at the local scale. We identified seven

483   private alleles (from seven loci) that could potentially differentiate among five DGL

484   populations. Five pangolins were traced to their forest of origin using three private alleles,

485   illustrating the long-distance trading routes that feed TMMs in the Dahomey Gap (see [7]).

486   Indeed, pangolins from the forests of central Togo, Wari-Maro and Ouémé Supérieur were

487   found on the markets of Dantokpa, Gbèdagba, Azovè and Avogbannan, c. 200-300 km away

488   from the source forests.

489   Despite a relatively fair number of loci (20) and an exhaustive sample set across Benin, we

490   could only trace c. 8% of the WBP genotyped from TMMs. Such performance could be

491   improved with additional geographic sampling, notably from Togo and southwestern Nigeria,

492   and denser sampling of populations, although the ideal standards for reaching confident

493   estimates of allele frequencies when applied to forensic use might be unreachable (100-150

494   individuals per population; [92]). Future analyses based on bi-allelic markers such as SNPs

495   will have to be considered as they significantly reduce the sample size required for reliable

496   estimates of allele frequency distribution [93].

497

498   **Conclusion**

499   Our study is the first to provide a comprehensive population genetic assessment of an African

500   pangolin species/lineage, filling an important knowledge gap for the future conservation of

501   pangolins in Africa [16]. Overall, we showed that the DGL populations suffered from

502   inbreeding, genetic diversity erosion and a drastic decline in effective population size. Given

503   the multi-purpose trade that DGL populations are the target of (this study, [7]), and the

504   observed reduction of the DGL range during the last two decades (in Benin, [11]), the

505   conservation status of WBP in the Dahomey Gap should be urgently re-assessed.

506   Conservation measures are to be implemented before the species becomes locally extinct, as it

507   may already be the case for the giant pangolin [11]. Measures should include the

508   reinforcement of national protection status, the creation of dedicated protected areas and

509   forest corridors, campaigns of public awareness, and breeding programs.

510

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517

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523

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755

756 Table 1. Genetic diversity estimates at the 20 microsatellite loci used in this study.

757 N = number of genotyped individuals; Na = number of alleles; A_R = allelic

758 richness; H_O = observed heterozygosity; H_E = expected heterozygosity; $P(HWE)$ = P-value for

759 deviation from the null hypothesis of Hardy–Weinberg equilibrum; F_{IS} = inbreeding

760 coefficient. *P*-values * <0.05, *** <0.001.

761

Locus	N	Na	A_R	Ho	He	$p(HWE)$	F_{IS}
PT_1162028	169	4	3.123	0.426	0.6	0.000***	0.292*
PT_1753627	162	11	7.993	0.642	0.689	0.019*	0.071
PT_796077	168	3	2.117	0.16	0.186	0.281	0.145
PT_1973508	169	5	4.339	0.562	0.48	0.028*	-0.167
PT_839522	168	7	6.168	0.524	0.788	0.000***	0.338*
PT_464918	168	8	7.025	0.56	0.766	0.000***	0.272*
PT_1453906	168	4	3.851	0.321	0.646	0.000***	0.505*
PT_34432	151	2	1.863	0.086	0.082	0.580	-0.042
PT_1594892	151	3	2.988	0.192	0.501	0.000***	0.619*
PT_308752	169	11	7.203	0.657	0.678	0.000***	0.034
PT_1669238	169	3	2.233	0.314	0.361	0.276	0.134
PT_1225378	167	3	1.903	0.072	0.069	0.972	-0.031
PT_739516	167	4	3.363	0.461	0.498	0.612	0.077
PT_619913	168	2	1.959	0.113	0.128	0.142	0.116
PT_338821	165	7	5.930	0.503	0.721	0.000***	0.305*
PT_18497228	168	4	3.848	0.595	0.6	0.611	0.01
PT_378852	168	2	1.969	0.113	0.138	0.020*	0.182

|							
PT_353755	160	9	8.044	0.775	0.842	0.000*	0.083
PT_276641	166	5	4.168	0.699	0.696	0.535	-0.002
PT_2019332	169	8	5.319	0.503	0.498	0.309	-0.007

762

763 **Figures**

764

765   Figure 1. Neighbor-joining tree of white-bellied pangolins based on 268 control region

766   sequences, showing the six main geographic lineages (following Gaubert et al. 2016)

767   collapsed. Bootstrap supports are given at nodes. All the individuals from the Dahomey Gap

768   belong to the Dahomey Gap lineage (see Fig S8, Supplementary information, for the

769   expanded tree).

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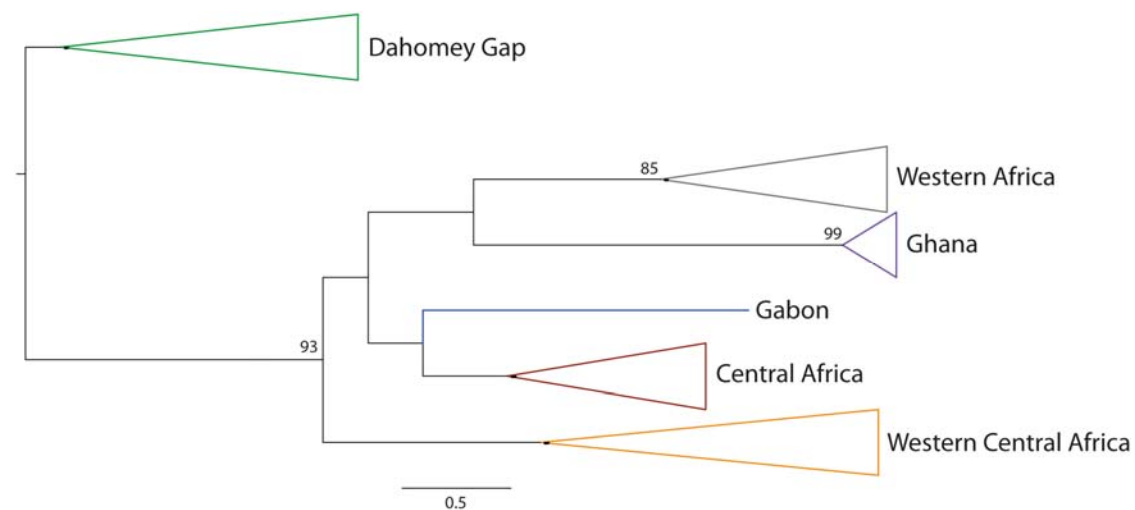
773   Figure 2. Distribution of control region haplotypes in white-bellied pangolins from the forests

774   and wildlife markets of the Dahomey Gap. Haplotype numbers refer to Table S2

775   (Supplementary information). Top left shows the location of the study zone in Africa (in

776   black).

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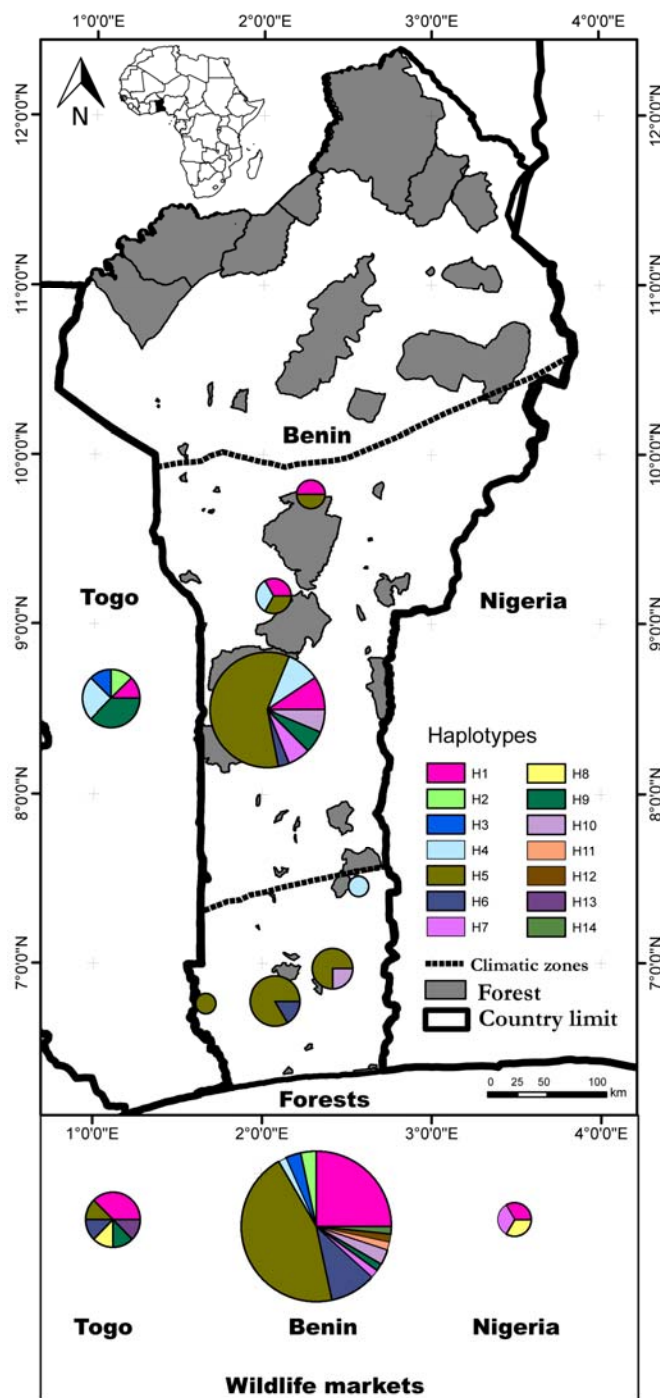
778   Figure 3. Distribution of genetic variance (PCoA) within white-bellied pangolins from the Dahomey Gap lineage. A- including forests and

779   wildlife markets; B- excluding wildlife markets.
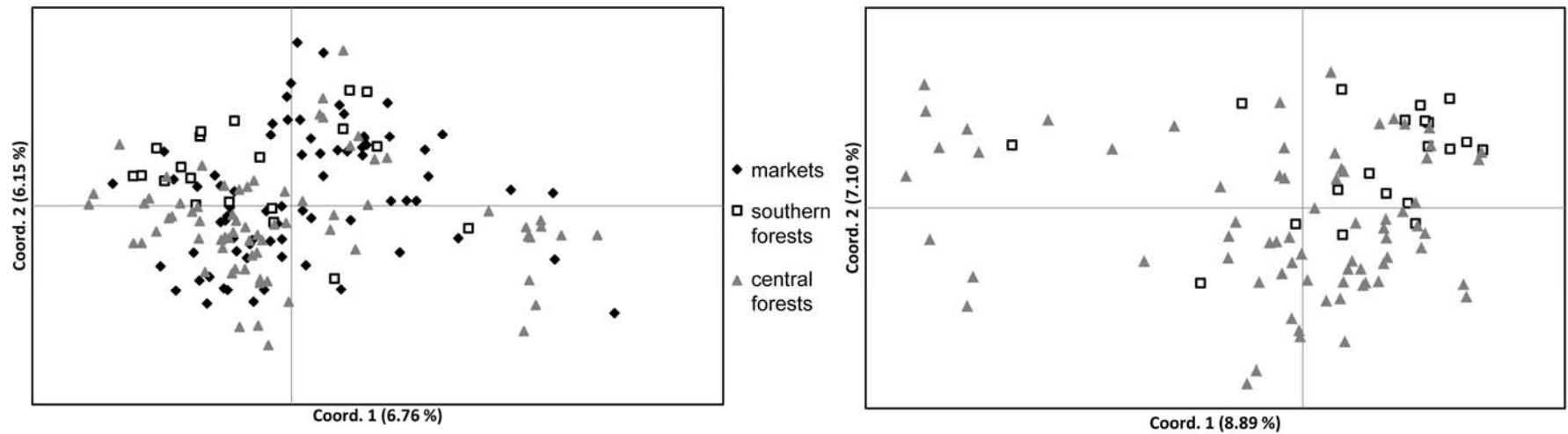
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781 Figure 4. Spatial clustering in white-bellied pangolins from the Dahomey Gap obtained from spatial Principal Component Analysis (sPCA). Left:

782 Positive and negative sPCA eigenvalues indicating global and local structures, respectively. Right: Map of the first global sPCA score among

783 sampling localities. Large white and black squares stand for highly negative and positive scores respectively. Large white squares are genetically

784 well differentiated from large black squares, while small squares are less differentiated.
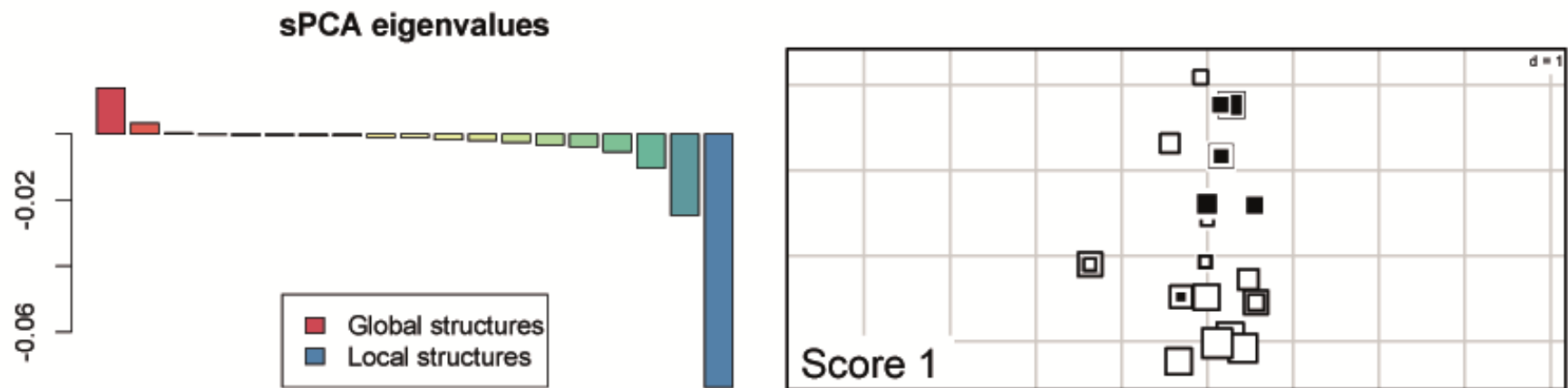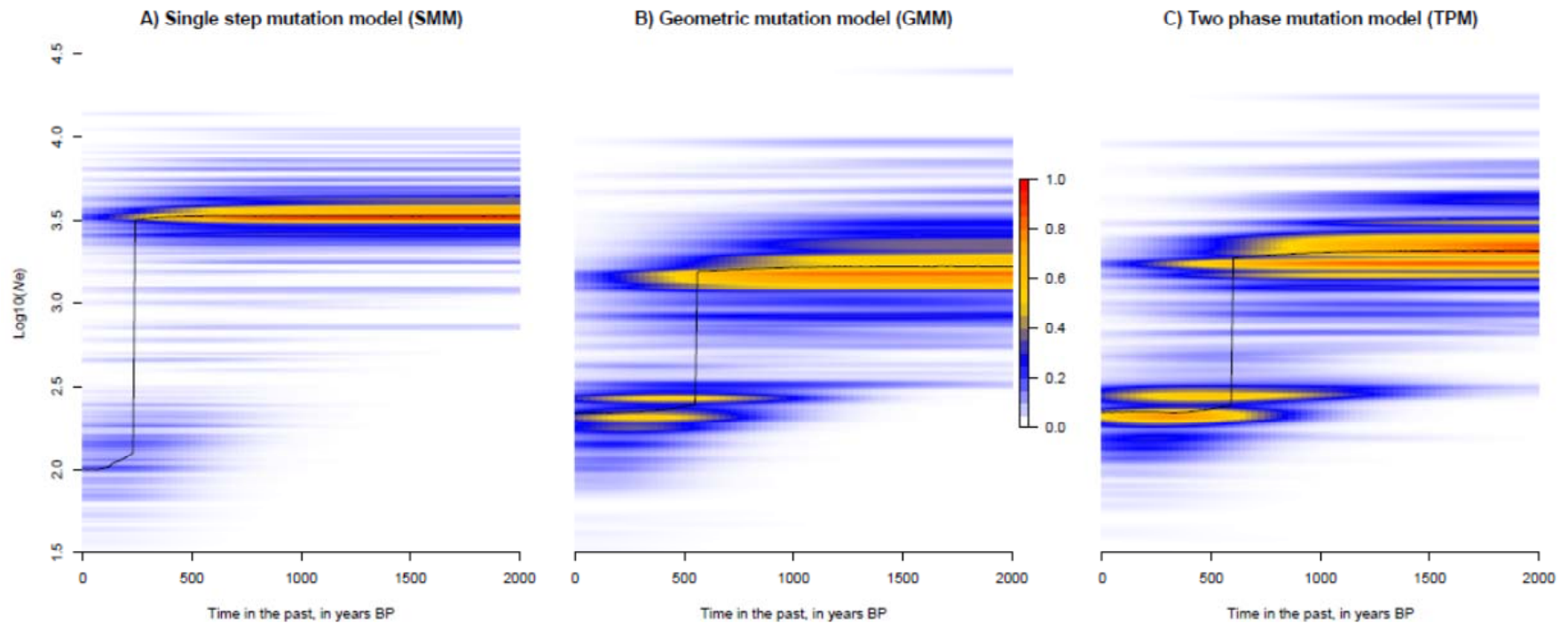

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786   Figure 5. Isolation by distance among (left) individuals and (right) populations of white-bellied pangolins from the Dahomey Gap as inferred

787   from 20 microsatellite loci. Dashed curve indicates linear regression.

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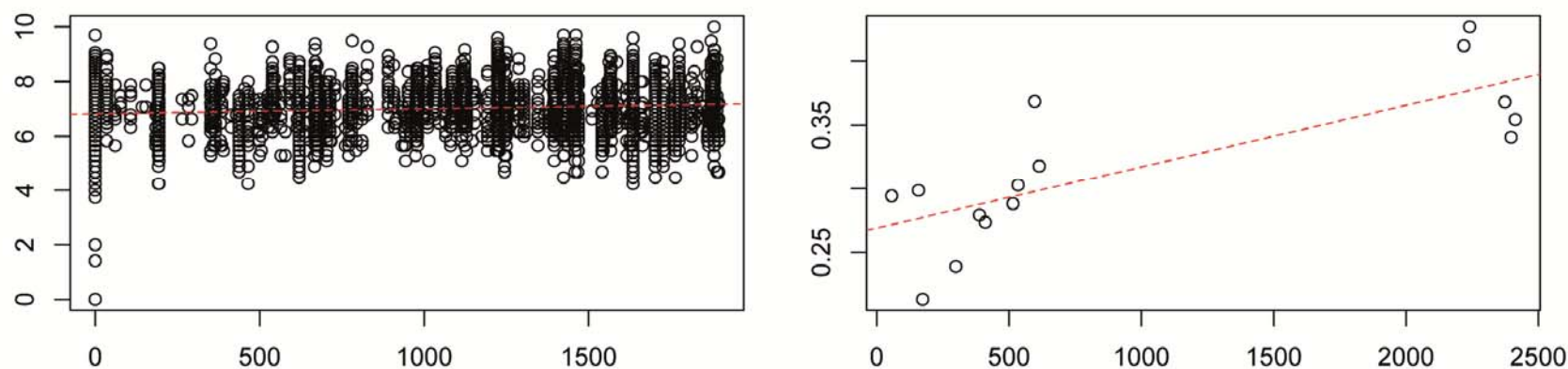
Figure 6. Temporal change in the effective population size of white-bellied pangolins in the Dahomey Gap, as estimated from *VarEff* under three different mutation models. Mode (black line) and kernel density (color scale) of effective population size (*Ne*) posterior distributions are given in years BP.