1   **Title:**

2   **Ancient genomes redate the extinction of *Sussemionus*, a subgenus of**

3   ***Equus*, to late Holocene**

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Abstract

29   The exceptionally-rich fossil record available for the equid family has provided

30   textbook examples of macroevolutionary changes. Horses, asses and zebras represent

31   three extant subgenus of *Equus* lineage, while the *Sussemionus* subgenus is another

32   remarkable *Equus* lineage ranging from North America to Ethiopia in Pleistocene. We

33   sequenced 26 archaeological specimens from northern China in Holocene showing

34   morphological features reminiscent of *Equus ovodovi*, a species representative of

35   *Sussemionus*, and further confirmed them as this species by genetic analyses. Thus,

36   we present the first high-quality complete genome of the *Sussemionus* that we

37   sequenced to 12.0× depth-of-coverage and demonstrate that it survived until ~3,500

38   years ago, despite the continued demographic collapse during the Last Glacial

39   Maximum and the great human expansion in East Asia. We also confirmed the *Equus*

40   phylogenetic tree, and found *Sussemionus* diverged from the ancestor of non-caballine

41   equids ~2.3-2.7 Million years ago and admixture events could have taken place

42   between them. Our works suggest the small genetic diversity but not the enhanced

43   inbreeding mainly limited the chances of survival of the species, and illustrates how

44   ancient DNA can inform on extinction dynamics and the long-term resilience of

45   species surviving in cryptic population pockets.

46

Introduction

48   Today, all of the seven extant species forming the horse family belong to one single

49   genus, *Equus*. It emerged in North America some 4.0-4.5 million years ago (L.

50   Orlando et al., 2013), and first spread to Eurasia ~2.6 million years ago, via the

51   Beringia land bridge (Lindsay, Opdyke, & Johnson, 1980). This first vicariance and

52   expansion out-of-America led to the emergence of the ancestors of zebras, hemiones

53   and donkeys, a group collectively known as non-caballine equids. Another expansion

54   through Beringia occurred later, bringing caballine equids (i.e. those most closely

55   related to the horse) into the Old-World, where they survived until their domestication

56   some ~5,500 years ago (Gaunitz et al., 2018; Outram et al., 2009).

57   In the recent years, ancient DNA (aDNA) data have revealed that the genetic

58   diversity of non-caballine *Equus* was considerably larger diversity in the past than it is

59   today (Librado & Orlando, 2020; Ludovic Orlando, 2020), especially as the first

60   mitochondrial DNA (mtDNA) data of *Equus* (*Sussemionus*) specimens were collected

61   (hereafter referred to as Sussemiones) (Eisenmann, 2010). This lineage radiated

62   across North America, Africa, and Siberia, and developed multiple adaptations to a

63   whole range of arid and humid environments (Eisenmann, 2010). Sussemiones was

64   first believed to have become extinct during the Middle Pleistocene as the last known

65   specimen showing typical morpho-anatomy dated back to approximately 500,000

66   years ago (Vasiliev, 2013). However, DNA results obtained on multiple osseous

67   remains within the radiocarbon range and showing morphological traits reminiscent of

68   the Eurasian Sussemiones species indicated that the lineage in fact survived until the

69   Late Pleistocene (Druzhkova et al., 2017; L. Orlando et al., 2009; Vilstrup et al., 2013;

70   Yuan et al., 2019). Pioneering publications indicated survival dates 40-50 kya in

71   southeastern Siberia, Russia (Proskuryakova cave) (L. Orlando et al., 2009; Vilstrup

72   et al., 2013), ~32 kya at the Denisova cave (Druzhkova et al., 2017), and ~12.6 kya at

73   northeastern China (Yuan et al., 2019).

74   Despite an abundant fossil material, only a limited number of Sussemiones

75   specimens have been investigated for Ancient mitochondrial DNA (aDNA), which

76   showed that Sussemiones formed a non-caballine equine lineage. However, the exact

77   placement of Sussemiones could not be resolved (Heintzman et al., 2017; L. Orlando

78   et al., 2009; Vilstrup et al., 2013). In this study, we have carried out archaeological

79   excavations in three Holocene sites in China, and uncovered equine samples showing

80   distinct morphological features when compared to horses and donkeys (Figure

81   1—figure supplement 1). The whole mitochondrial and nuclear genome data allowed

82   us to unveil the phylogenetic placement of Sussemiones within the *Equus*

83   evolutionary tree, the timing of its divergence to other non-caballine equids,

84   signatures of demographic collapse and adaptations specific to this lineage, and the

85   extinction dynamics of this lineage.

86

4

Results

Archaeological samples and sequencing data

All the equine specimens investigated in this study were excavated from three archaeological sites in China (Figure 1 and Table S1) (Honghe, Heilongjiang Province (Figure 1—figure supplement 2); Muzhuzhuliang, Shaanxi Province; Shatangbeiyuan, Ningxia Province). They showed morphological and genetic signatures distinct from those of extant horses and donkeys. The morphological differences were especially marked in the second and third molars, which appeared to be smaller than in modern horses, and were reminiscent of the third molars paracones and metacones observed in Sussemiones specimens (Figure 1B). Combined, these samples were radiocarbon dated to 3,477-4,481 calibrated years before the present (cal BP), including the latest sample HH13H with 3,477-3,637 cal BP (Table S2). They could, thus, represent some of the latest surviving Sussemiones individuals prior to their extinction.

We next aimed at genetically characterizing and identifying the taxonomic status of these specimens using high-throughput DNA sequencing technologies. We extracted ancient DNA from a total of 26 specimens and sequenced the whole nuclear genome at ~0.002 to 12.0 times coverage, including three samples from Honghe provided 12.0×, 3.5× and 1.0× nuclear genome (Table S1). Comparison of the X chromosome and autosomal coverage revealed the presence of 15 males and 11 females.
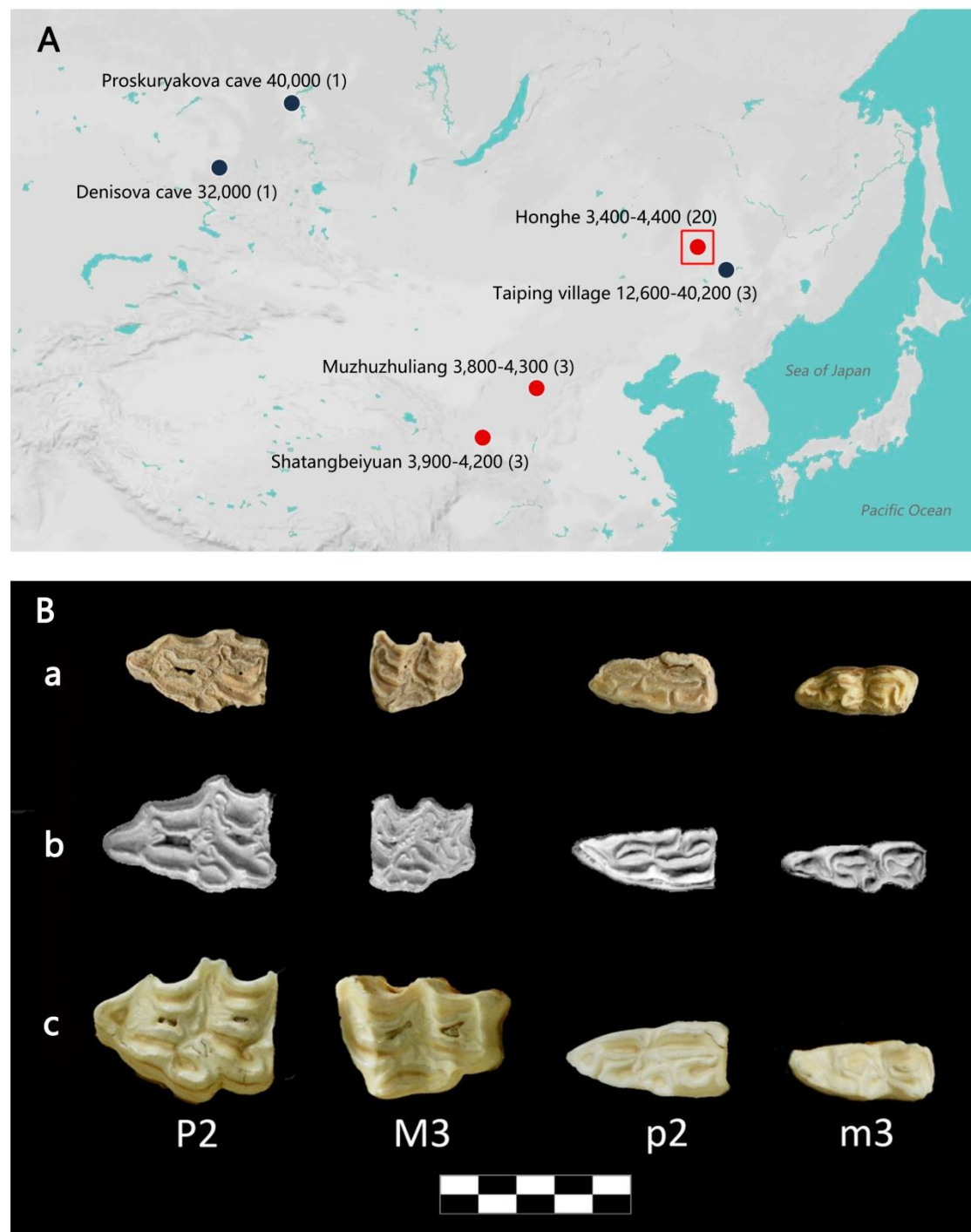
Figure 1. Sampling distribution and archaeological research of *E.* (*Sussemionus*)

ovodovi.   (**A**) *E.* (*Sussemionus*) *ovodovi* geographic range. The three red circles

indicate the archaeological sites analyzed in this study. The site (Honghe) that

delivered the complete genome sequence at 12.0-fold average depth-of-coverage

113 (HH06D) is highlighted with a square. The black circles indicate sites that provided

114 complete mitochondrial genome sequences in previous studies (Druzhkova et al.,

115 2017; L. Orlando et al., 2009; Vilstrup et al., 2013; Yuan et al., 2019). The temporal

116 range covered by the different samples analyzed is given in years before present (YBP)

117 and follows the name of each site. Numbers between parentheses indicate the number

118 of samples for which DNA sequence data could be generated. (**B**) *Facies masticatoria*

119 *dentis* of P2, M3, p2 and m3 for the *E. (Susseminous) ovodovi* samples of the Honghe

120 site analyzed here (a), *E. Sussemionus* (b), and *E. caballus* (c). Teeth from the right

121 side are shown, except for *E. Sussemionus*. The erupted teeth of the samples of the

122 Honghe site appear to be smaller than those of the *E. Sussemionus* specimen.

123 The following figure supplements are available for figure 1:

124 **Figure supplement 1.** Archaeological material investigated in this study.

125 **Figure supplement 2.** Aerial view of the Honghe site.

126

Taxonomic status

128 To assess whether the sequenced specimens belonged to the same taxonomic group or

129 comprised different species, we carried out a Principle Component Analysis (PCA)

130 including all the equine species sequenced at the genome level (depth of coverage ≥

131 1×) (Figure 2A and Figure 2—figure supplements 3 and 4). For this, we downloaded

132 11 previously-published equine genomes representing all extant species of equids and

133 the extinct quagga zebra (Huang et al., 2015; Jonsson et al., 2014; Kalbfleisch et al.,

134 2018; L. Orlando et al., 2013; Renaud et al., 2018) (Table S3). All the Chinese

135   specimens analyzed in this study were found to cluster together along the first two

136   PCA components, in a group that was distinct from all other equine species (Figure

137   2A and Figure 2—figure supplement 3) but closer to non-caballine equine species

138   than to the horse (Figure 2A). This suggested that they were all members of a unique

139   taxonomic group, most related to non-caballine equids.

140   Maximum likelihood (ML) phylogenetic analyses including the nearly-complete 17

141   mitochondrial genomes reported in this study (Table S1, depth of coverage no less

142   than 1×) confirmed their clustering with non-caballine equids, within a single

143   monophyletic group that also included five previously-characterized Sussemiones

144   specimens (Figure 2B and Figure 2—figure supplements 5 and 6). This grouping was

145   supported with maximal (100%) bootstrap support. This, and the PCA clustering,

146   indicated that the different excavation sites investigated in this study in fact all

147   provided specimens that belonged to the *E.* (*Sussemionus*) *ovodovi* species.

148   We also inferred maternal lineages of *E. ovodovi* using complete mitogenomes.

149   Phylogenetic analyses showed that the species were mainly divided into two major

150   haplogroups (A and B), of which most of the specimens were characterised by the

151   haplogroup B, including all previously reported sequences, whereas 2 Muzhuzhuliang

152   and 3 Honghe individuals belonged to the new haplogroup A (Figure 2B). Further

153   distinction could also be carried out in the two haplogroups (Figure 2B).

154   To further assess phylogenetic affinities, we used the two genomes characterized to

155   at least 3× average depth-of-coverage (HH04D and HH06D) to replace Sussemiones

156   within the equine phylogenetic tree. To achieve this, we used ML phylogenetic

8

157   reconstruction and an alignment of the coding sequences of the protein coding genes

158   (Figure 2C and Figure 2—figure supplement 7A). This showed that the Chinese

159   ancient specimens branched off before the radiation leading to modern asses and

160   zebras (Figure 2C). Similar tree topologies were recovered using whole-genome SNPs

161   by TreeMix (Pickrell & Pritchard, 2012) (Figure 2—figure supplements 8 and 9).

162   Combined with the analysis of the occlusal surface of the molars, in particular the

163   absence of the caballine notch, the shape of metacones and protocones, and the

164   reduced tooth size (Figure 1B), our analyses allowed us to conclude that the material

165   analyzed represented small specimens of the extinct *Equus* (*Sussemionus*) *ovodovi*.

166   This lineage, thus, survived in China during the Holocene, and until cal 3,477-4,481

167   cal BP, which is approximately ~8,300-9,300 years after the latest known specimen to

168   date (Druzhkova et al., 2017; L. Orlando et al., 2009; Vilstrup et al., 2013; Yuan et al.,
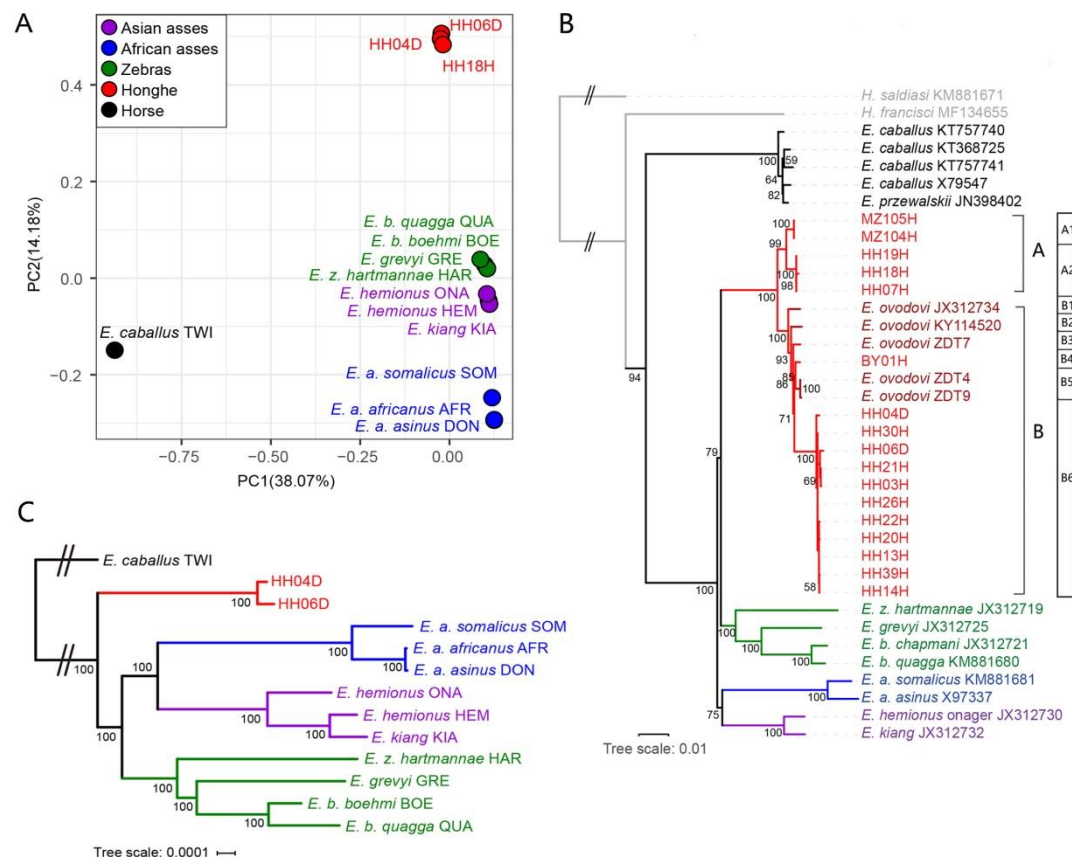
169   2019).

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Figure 2. Genetic relationships within the *Equus* genus. Honghe (HH),

Muzhuzhuliang (MZ) and Shatangbeiyuan (BY) specimens are shown in red, while

Asian asses, African asses, zebras and horses are shown in whereas purple, blue, green

and black, respectively. (**A**) PCA based on genotype likelihoods, including horses and

all other extant non-caballine lineages (16,293,825 bp, excluding transitions). Only

specimens whose genomes were sequenced at least to 1.0× average depth-of-coverage

are included. (**B**) Maximum likelihood tree based on 5 mitochondrial partitions

(representing a total of 15,399 bp). Previously published *E. ovodovi* sequences are

shown in deep red. The tree was rooted using *Hippidion saldiasi* and *Haringtonhippus*

francisci as outgroups (not shown). Node supports were estimated from 1,000

bootstrap pseudo-replicates and are displayed only if greater than 50%. The black line

10

183   indicates the mitochondrial haplogroup A and B. (**C**) Maximum likelihood tree based

184   on sequences of 19,650 protein-coding genes with specimens sequenced at least 3.0×

185   average coverage (representing 32,756,854 bp).

186   The following figure supplements are available for figure 2:

187   **Figure supplement 1.** DNA Damage patterns for HH06D.

188   **Figure supplement 2.** Error profiles of the 26 ancient genomes characterized in this

189   study.

190   **Figure supplement 3.** Principal Component Analysis (PCA) based on genotype

191   likelihoods using the horse reference genome.

192   **Figure supplement 4.** Principal Component Analysis (PCA) based on genotype

193   likelihoods using the donkey reference genome.

194   **Figure supplement 5.** RAxML-NG (GTR+GAMMA model) Maximum Likelihood

195   phylogeny of complete mitochondrial sequence data.

196   **Figure supplement 6.** Bayesian mitochondrial phylogeny based on 6 partitions and

197   using *Hippidion Saldiasi* as outgroup.

198   **Figure supplement 7.** Exome-based Maximum likelihood phylogeny rooted by the

199   horse lineage.

200   **Figure supplement 8.** Treemix analysis of based on genome-wide SNP data

201   conditioned on transversions using the horse reference genome.

202   **Figure supplement 9.** Treemix analysis of based on genome-wide SNP data

203   conditioned on transversions using the donkey reference genome.

204

11

Interspecies admixture and demographic modeling

205

206   Bifurcating trees fail to capture possible admixture events between lineages. Yet,

207   previous research has unveiled pervasive admixture within equids, even amongst

208   extant equids showing different chromosomal numbers (Jonsson et al., 2014). We thus

209   next assessed whether the genomic data showed evidence for gene flow between

210   Sussemiones and other non-caballine equids. To achieve this, we first applied

211   D-statistics (Soraggi, Wiuf, & Albrechtsen, 2018) to the genome sequence underlying

212   26 individual genomes and detected that *E. ovodovi* shared an excess of derived

213   polymorphisms with asses than relative to zebras (Figure 3—figure supplements 1 and

214   2). This suggested that at least one admixture event could have taken place between

215   Sussemiones and the ancestor of asses after their divergence from zebras.

216   We next leveraged the ancient genome characterized to high depth-of-coverage

217   (HH06D) to reconstruct the equine demographic history using G-PhoCS (Gronau,

218   Hubisz, Gulko, Danko, & Siepel, 2011). More specifically, we first selected members

219   of each equine lineage representing a total number of 10 genomes, and assumed that

220   the genus *Equus* emerged some 4.0-4.5 Mya, following previous estimates (L.

221   Orlando et al., 2013). G-PhoCS analysis confirmed previous analyses indicating that

222   the zebras and asses linages diverged ~2.0 Mya and that the deepest divergence within

223   zebras and asses took place prior to ~1.5 Mya (Jonsson et al., 2014) (Figure 3). It

224   revealed that the Sussemiones lineage diverged from the ancestor of extant

225   non-caballine equids ~2.3-2.7 Mya, in line with the fossil record (Eisenmann, 2010).

226   Allowing for migrations provided support for gene flow between Sussemiones and the

12

227   ancestor of asses and zebras (Figure 3). However, weak to no migrations were

228   detected between Sussemiones and extant equids (Table S6). Importantly, the

229   admixture between Sussemiones and the ancestor of asses seems to have been

230   stronger than that between Sussemiones and the ancestor of zebras, in line with the

231   results of D-statistics. G-PhoCS also supported the presence of significant

232   unidirectional gene-flow prior to ~2.3-2.7 Mya, from the horse branch into the

233   ancestral branch to all non-caballine equids, including Sussemiones (total migration

234   rate 2.2-9.2%, Table S7). This is consistent with previous HMMCoal analyses applied

235   to whole genome sequences of all extant equine species, which indicated significant

236   gene-flow between the deepest branches of the *Equus* phylogenetic tree until 3.4 Mya,

237   mostly from a caballine lineage into the ancestor of all non-caballine equids (Jonsson
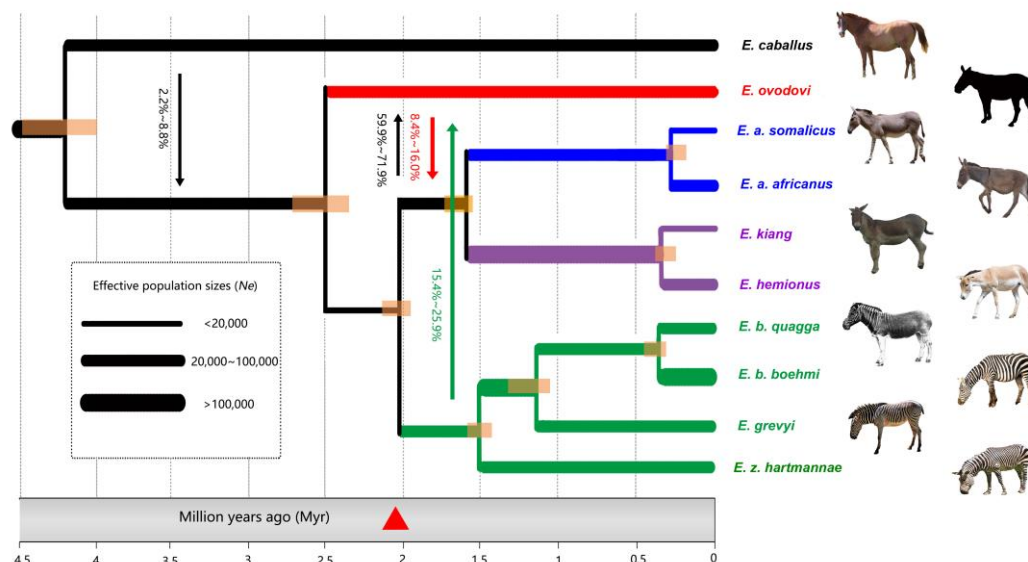
238   et al., 2014).

239

240

241   **Figure 3.** Demographic model for extinct and extant equine lineages as inferred by

242   G-PhoCS (Gronau et al., 2011)**.** Node bars represent 95% confidence intervals. The

13

243   width of each branch is scaled with respect to effective population sizes (N_e).

244   Independent N_e values were estimated for each individual branch of the tree, assuming

245   constant effective sizes through time. Migration bands and probabilities of migration

246   (transformed from total migration rates) are indicated with solid. The red triangle

247   indicates the earliest *Sussemionus* evidence found in the fossil record. (Images: *E.*

248   *caballus* by Infomastern, *E. a. somalicus* by cuatrok77, *E. kiang* by Dunnock_D, *E. a.*

249   *africanus* by jay galvin, *E. hemionus* by Cloudtail the Snow Leopard, *E. z.*

250   *hartmannae* by calestyo, *E. b. quagga* by Internet Archive Book Images, *E. b. boehmi*

251   by GRIDArendal, and *E. grevyi* by 5of7.)

252   The following figure supplements are available for figure 3:

253   **Figure supplement 1.** *D*-statistics in the form of (zebra, ass; *E. ovodovi*, outgroup),

254   using sequence alignments against the horse reference genome.

255   **Figure supplement 2.** *D*-statistics in the form of (zebra, ass; *E. ovodovi*, outgroup),

256   using sequence alignments against the donkey reference genome.

257   **Figure supplement 3.** NJ tree of selected samples based on 15,324 candidate 'neutral'

258   loci identified using sequence alignments against the horse reference genome.

259

Dynamic demographic profiles, heterozygosity and inbreeding levels

261   We next leveraged the high-coverage Sussemiones genome characterized here to

262   explore further the demographic dynamics of this lineage until its extinction. When

263   modeled as constant through time, population sizes in G-PhoCS indicated that most

264   lineages, including Sussemiones, consisted of small populations, excepting the

265   Burchell's zebra (Table S8). Pairwise Sequential Markovian Coalescent (PSMC)

266   analyses, however, provided us evidence for population size variation through time.

267   First, the Sussemiones demographic trajectory was found to diverge from that of other

268   non-caballine equids (specifically, *E. hemionus*) after ~2.0 Mya, confirming the

269   divergence date estimate retrieved by G-PhoCS (Table S8). Second, we found that the

270   Sussemiones demographic trajectory constantly increased during the last million year

271   but stay at a level which was lower than that of other lineages for a long time, until it

272   reached a peak between 74-84 kya. It was, then, followed by an approximately

273   45-fold collapse until 13 kya (Figure 4). The Sussemiones population size

274   experienced a 1.86-fold collapse between 35-42 kya, which is almost coincident with

275   the timing of the great human expansion to Eurasia (ie. 35-45 kya, (Henn,

276   Cavalli-Sforza, & Feldman, 2012)). The lineage maintained extremely reduced

277   population sizes through the Last Glacial Maximal (LGM, 19-26 kya) (Clark et al.,

278   2009) and the Holocene, until it finally became extinct.

279   Importantly, the sample sequenced to sufficient coverage (HH06D) showed

280   minimal heterozygosity and moderate inbreeding levels identified by the fraction of

281   the segments within ROH (Figure 5). Strikingly, this is true in spite of the increased

282   sequencing error rates of this genome, which likely inflate our estimates. The limited

283   population sizes and genetic diversity but not the enhanced inbreeding may have

284   limited the chances of survival of the species, ultimately leading to extinction.
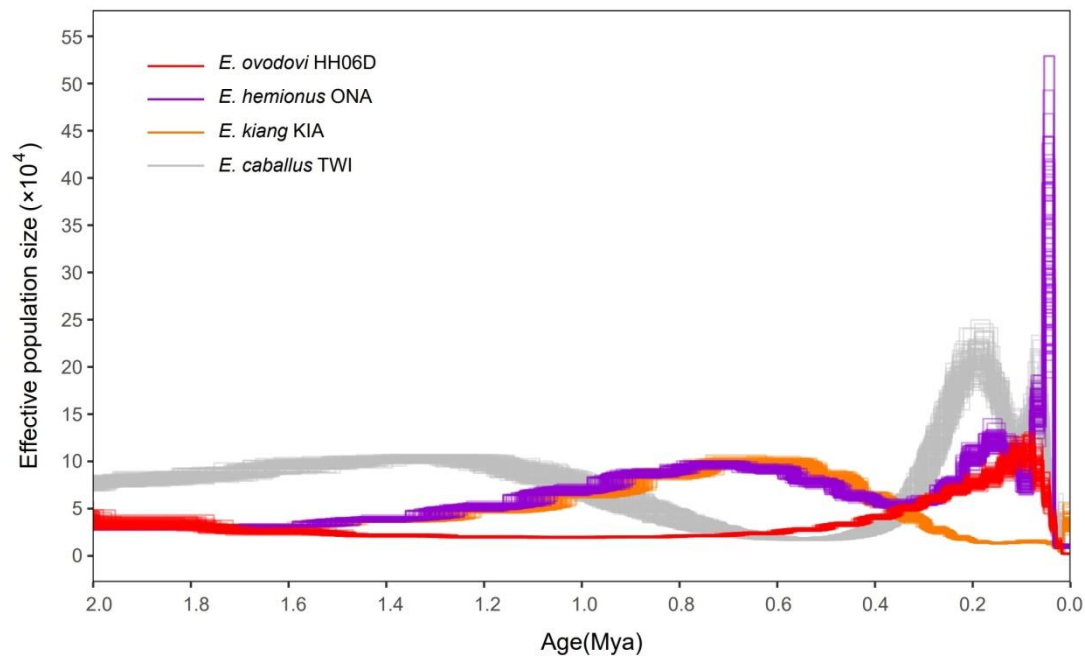
285

286

Figure 4. PSMC profiles (100 bootstrap pseudo-replicates) of four Eurasian equine

species (*E. ovodovi* HH06D, *E. caballus* TWI (Kalbfleisch et al., 2018), *E. hemionus*

ONA and *E. kiang* KIA) (Jonsson et al., 2014)**.** The y axis represents effective

population size (×10,000), and the x axis represents millions of years before present.

Faded lines show bootstrap values.

The following figure supplements are available for figure 4:

Figure supplement 1. PSMC bootstrap pseudo-replicates for samples with and

without transitions.

Figure supplement 2. Determining the uniform false-negative rate (uFNR) that was

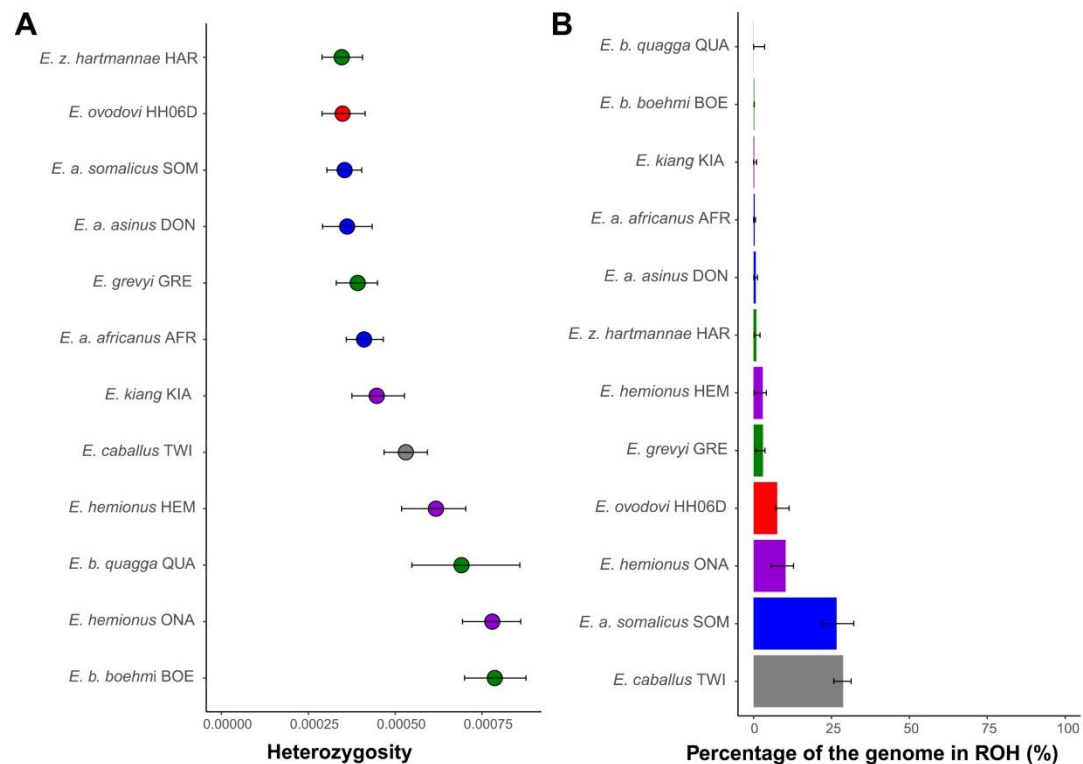necessary for PSMC scaling.

297

16

Figure 5. The Heterozygosity and Inbreeding levels of extinct and extant equine lineages. (**A**) Individual heterozygosity outside Runs-of-Homozygosity (ROH). (**B**) Fraction of the genome in ROH. Estimates were obtained excluding transitions and are shown together with their 95% confidence intervals. The colors mirror those from Figure 2.

The following figure supplements are available for figure 5:

Figure supplement 1. Heterozygosity rates outside Runs-of-Homozygosity (ROH) together with 95% confidence intervals.

Figure supplement 2. The fraction of the genome segments consisting of ROHs together with 95% confidence intervals.

Discussion

Phylogenetic placement of *Equus* (*Sussemionus*) *ovodovi*

312   In this study, we have characterized the first nuclear genomes of the now-extinct

313   equine lineage, *E.* (*Sussemionus*) *ovodovi*, the last surviving member of the subgenus

314   *Equus Sussemionus*. We demonstrated that this lineage survived in China well into the

315   Holocene with the most recent specimens analyzed dating to ~3,477-3,637 cal BP.

316   This is almost 9,000 years after the latest specimens previously documented in the

317   fossil record (Druzhkova et al., 2017; Vilstrup et al., 2013; Yuan et al., 2019). Our

318   work, thus, shows that *Sussemionus* represents the last currently known *Equus*

319   subgenus to become extinct. Our work also adds to the list of recently identified

320   members of the horse family that were still alive at the time horses and donkeys were

321   first domesticated, some ~5,500 years ago (Fages et al., 2019; Gaunitz et al., 2018;

322   Rossel et al., 2008). In contrast to those divergent members that were identified in

323   Siberia and Iberia and both belonged to the horse species (Fages et al., 2019; Schubert,

324   Jonsson, et al., 2014), Sussemiones members were most closely related to

325   non-caballine equids. This is in agreement with previous studies (Der Sarkissian et al.,

326   2015; Druzhkova et al., 2017; Heintzman et al., 2017; L. Orlando et al., 2009;

327   Vilstrup et al., 2013; Yuan et al., 2019), which could, however, not fully resolve the

328   exact phylogenetic placement of this species within non-caballine clade lineages as

329   topological tests based on mitochondrial genomes received low confidence support

330   (Der Sarkissian et al., 2015; Druzhkova et al., 2017; Heintzman et al., 2017; L.

331   Orlando et al., 2009; Vilstrup et al., 2013; Yuan et al., 2019). Our study solved this

332   question by reporting the first whole genome phylogeny of Sussemiones, which

333 confirmed with maximal bootstrap support this species as a unique basal lineage of

334 non-caballine equids.

335

Suitable habitat and geographic distribution

337 Previous zooarchaeological and environmental research indicated an ecological range

338 for Sussemiones overlapping with the grasslands located east of the Altay Mountains

339 and west of the Yenisei River during the Late Pleistocene (Khenzykhenova et al.,

340 2016; Malikov, 2016; Plasteeva, 2015; Shchetnikov, Klementiev, Filinov, & Semeney,

341 2015; Shunkov, 2018). Recent research also reported this species in northeastern

342 China (~12,600-40,200 YBP), where similar climatic and ecological conditions were

343 found at the time (Yuan et al., 2019). It could, thus, be speculated that Sussemiones

344 was adapted to an environment with moderately dry climatic conditions and steppe

345 landscapes (Yuan et al., 2019). However, our study identified Sussemiones in three

346 late Holocene sites from China that have mild and humid environmental conditions. In

347 addition, two distinct mitochondrial haplogroups from 22 individuals have been

348 defined from the six known sites, suggesting that Sussemiones had adapted to

349 different environmental regions. It also suggests that the species could adapt to a

350 wider variety of habitats than previously hypothesized, and rejects the contention that

351 the species became extinct as it could not survive in warmer climatic conditions

352 (Yuan et al., 2019).

353   Interestingly, the Sussemiones specimens identified in this study were excavated

354 from sites in northeastern China located at almost the same latitude as those

19

355   Sussemiones localities known so far from Russia, but also at lower latitudes (Figure

356   1A). This implies that the geographic range of *E. ovodovi* was larger than previously

357   expected and included at least Northern China and Southern Siberia. In the absence of

358   identified fossils from Mongolia, whether those two regions were in contact or

359   separated remains unknown. Further work is necessary to establish whether or not the

360   species survived in other pockets both within and outside China.

361

362   Demographic history with ancestral interspecific admixture

363   Our analyses reveal that the divergence between Sussemiones and the most recent

364   common ancestor of all extant non-caballine equids took place some ~2.3-2.7 Mya,

365   right before the divergence of zebras and asses. Post-divergence admixture events

366   with the lineage ancestral to asses and zebras on the one hand, and the lineage

367   ancestral to all extant zebras, were also identified (Figure 3 and Table S7). Our results,

368   thus, reveal non-caballine ancestral lineages occupying partly sympatric distributions

369   that were, consequently, different than those of their descendants, in which zebras are

370   restricted in Africa and Asian asses in Asia. Whether the admixture events identified

371   here directly involved the Sussemiones lineage or one (or more) ghost lineage(s)

372   closely related to Sussemiones requires further research.

373

374   Limited genetic diversity before extinction

375   The demographic profile of Sussemiones shows that after the peak of population size

376   culminating some ~74 kya, Sussemiones went through a slow and continuous decline

20

377 until 13 kya (Figure 4). This time period encompasses several major climate changes

378 (especially the LGM) and the great human expansion to Eurasia (~35-45 kyr BP)

379 (Henn et al., 2012). The effective size of Sussemiones populations that survived in

380 Northern China until at least ~3,500 years ago, remained extremely small, as indicated

381 by their extremely reduced heterozygosity levels compared to other extant and extinct

382 equine species, although extensive inbreeding was not detected (Figure 5). So

383 combined with a degree of inbreeding, the reduced genetic diversity available

384 ultimately resulted in the extinction of the lineage, in a process reminiscent of what

385 was previously described for the woolly mammoth (Palkopoulou et al., 2015).

386   In conclusion, our study clarifies the phylogenetic placement, speciation timing and

387 evolutionary history of the now-extinct *Equus Sussemionus* equine subgenus. This

388 group did not remain in reproductive isolation from other equine lineages, but

389 contributed to the genetic makeup of the ancestors of present-day Asiatic asses, while

390 receiving genetic material from the ancestors of African zebras. This supports

391 geographic distributions at least partly overlapping at the time, thus, not identical to

392 those observed today. The species demographic trajectory experienced a steady

393 decline from ~74 kya and during a period witnessing both important climatic changes

394 and the Great human expansion across Asia (Henn et al., 2012). It survived with

395 minimal genetic diversity the Pleistocene-Holocene transition, and for at least eight

396 millennia before it became extinct, which providing insights into the extinction of

397 large animals since Holocene.

398

Additional information

Additional files

Supplementary files

22

408   • Supplementary file 1. Supplementary tables that support the analysis and results

409   above.

410   • Transparent reporting form

411

Materials and Methods

Genome Sequencing

414   Prior to DNA extraction, the outer surface of the sample was cleaned with a brush.

415   The cleaned sample was subsequently cut into smaller pieces and soaked in 10%

416   bleach for 20 min, rinsed with ethanol and distilled water, and then UV-irradiated for

417   30 min on each side. Finally, powder was obtained by drilling with a dental drill

418   (Traus 204，Korea). Ancient DNA was extracted from the sample powder by using a

419   modified silica spin column method (Yang, Eng, Waye, Dudar, & Saunders, 1998) in

420   dedicated ancient DNA facilities at Jilin University (JLU). For each specimen, a total

421   of 200mg powder was added with 3.9ml EDTA (0.465mol/L) and placed in the

422   refrigerator at 4 ° C for 12 hours for decalcification, and then 0.1 ml Proteinase K

423   (0.4mg/mL) were added and incubated overnight in a rotating hybridization oven at

424   50℃ (220rpm/min). After centrifugation, the supernatant was transferred into an

425   Amicon® Ultra-4 centrifugal filter device (Merck Millipore Ltd, 10000 Nominal

426   Molecular Weight Limit), reduced to less than 100ul, and purified with QIAquick®

427   PCR Purification Kit (QIAGEN), according to the manual instructions.

428   Before preparation of DNA libraries, we first PCR targeted short fragments of the

429   mitochondrial hypervariable region to select those samples positive for the presence

23

430 of equine DNA (which was further confirmed through Sanger sequencing). For this,

431 we used the oligonucleotide primers L15473 5' –CTTCCCCTAAACGACAACAA–3'

432 and reverse primer H15692 5' –TTTGACTTGGATGGGGTATG–3' ; and forward

433 primer L15571 5' –AATGGCCTATGTACGTCGTG–3' and reverse primer H15772

434 5' –GGGAGGGTTGCTGATTTC–3'from (Dawei et al., 2007), and the amplification

435 conditions therein.

436 Double-stranded single-indexed libraries were prepared using NEBNext® Ultra^{TM}

437 II DNA Library Prep Kit for Illumina® (NEB #E7645S) and NEBNext® Multiplex

438 Oligos for Illumina® Index Primers Set 1 and 2 (NEB #E7335S, #E7500S), following

439 the manufacturer's instructions with minor modifications. Specifically, the extracted

440 DNA (50ul) were end repaired and A-tailed by adding 7µl of NEBNext Ultra II End

441 Prep Reaction Buffer and 3 µl of NEBNext Ultra II End Prep Enzyme Mix, and

442 incubated for 40 min at 20 °C and then 30 minutes at 65 °C. The adaptor was ligated

443 to the dA-tailed DNA fragments by adding 30ul of NEBNext Ultra II Ligation Master

444 Mix, 1ul of NEBNext Ligation Enhancer and 2.5ul of NEBNext Adaptor for Illumina

445 (Dilution 1:10), and incubated for 20 min at 20 °C. The adaptor was then linearized

446 by adding 3ul of USER™ Enzyme and performing an incubation for 15 min at 37°C.

447 The adaptor-ligated DNA were cleaned without size selection using the MinElute®

448 PCR Purification Kit (QIAGEN, Germany), following the instructions provided by

449 the manufacturer. PCR enrichment was performed by using 30ul of NEBNext Ultra II

450 Q5 Master Mix, 1ul of Index Primer, 1ul of Universal PCR Primer and 18ul of

451 adaptor-ligated DNA. PCR cycling conditions comprised an initial denaturation at

452 98°C for 30s, 14-16 cycles of 98°C for 10s, 65°C for 75s, and a final extension at

453 65°C for 5min. PCR amplified DNA libraries were purified using Agencourt AMPure

454 XP Beads, following the manufacturer's instructions, and Illumina sequencing was

455 performed on HiSeq X Ten platform using 150bp paired-end reads. Overall, we

456 sequenced a total of 28 DNA libraries and generated 2,727,843,803 read pairs.

457   All pre-PCR procedures were conducted in a dedicated ancient DNA laboratory at

458 JLU that is physically separated from the post-PCR laboratory. To remove potential

459 contaminant DNA, working areas and benches were frequently cleaned with bleach

460 and UV exposure. Lab experiments were carried out wearing full body suits,

461 facemasks, and gloves. To detect contamination, mock blank controls were included

462 in each experimental step, including DNA extraction, DNA library preparation and

463 PCR setup.

464

Data Processing

466 Sequencing reads were processed and aligned against the horse (EquCab3.0

467 (Kalbfleisch et al., 2018)) and donkey (Renaud et al., 2018) reference genomes using

468 the PALEOMIX pipeline (Schubert, Ermini, et al., 2014) with default parameters,

469 except that we followed the recommendations from (Schubert et al., 2012) and

470 disabled seeding. Briefly, paired-end (PE) reads longer than 25 nucleotides were

471 trimmed with AdapterRemoval v2.2 (Schubert, Lindgreen, & Orlando, 2016) and

472 aligned against the reference genomes using BWA (Li & Durbin, 2009), retaining

473 alignments with mapping qualities superior to 25. PCR duplicates were then removed

474   using Picard MarkDuplicates (http://broadinstitute.github.io/picard/). Finally, all

475   ancient and modern reads were locally realigned around indels using GATK

476   (McKenna et al., 2010).

477   Postmortem DNA damage and average sequencing error rates were determined

478   with mapDamage2.0 (Jonsson, Ginolhac, Schubert, Johnson, & Orlando, 2013)

479   (Figure 2—figure supplement 1) and ANGSD (Korneliussen, Albrechtsen, & Nielsen,

480   2014) (Figure 2—figure supplement 2), respectively. Further rescaling and trimming

481   procedures were implemented following (Gaunitz et al., 2018) to limit the impact of

482   remnant nucleotide mis-incorporations in subsequent analyses. For each of the DNA

483   libraries examined, the base composition of the position preceding read starts on the

484   horse reference genome showed an excess of Guanine and, to a lesser extent, of

485   Adenine residues (Figure 2—figure supplement 1). This is in line with depurination

486   driving post-mortem DNA fragmentation (Briggs et al., 2007). Additionally, error rate

487   estimates for each nucleotide substitution class indicated the predominance of C→T

488   and G→A mis-incorporations (Figure 2—figure supplement 2). Such

489   mis-incorporation rates were particularly inflated towards read ends, but not read

490   starts (Figure 2—figure supplement 1). This is in line with the DNA nucleotide

491   mis-incorporation profiles expected for the type of DNA library constructed

492   (Seguin-Orlando et al., 2015), and indicates Cytosine deamination at 5'-overhanging

493   ends as the most prominent post-mortem DNA degradation reactions (Jonsson et al.,

494   2013).

495 GATK HaplotypeCaller was used to obtain individual gvcf files with

496 "--minPruning 1 --minDanglingBranchLength 1" to increase sensitivity. Then we

497 employed GATK GenotypeGVCFs for genotyping with the option

498 "--includeNonVariantSites" in order to retain non-variant loci. The vcf files were

499 further filtered in TreeMix and G-PhoCS analysis.

500

Principal component analysis (PCA)

502 The genotype likelhood framework implemented in ANGSD helped mitigate various

503 error rates in ancient and modern genomes. Using EquCab3 (Kalbfleisch et al., 2018)

504 as the reference genome, ANGSD was run using the following options:

505 "-only_proper_pairs 1 -uniqueOnly 1 -remove_bads 1 -minQ 20 -minMapQ 25 -C 50

506 -baq 1 -skipTriallelic 1 -GL 2 -SNP_pval 1e-6 -rmTrans 1". This provided a dataset

507 consisting of a total of 16,293,825 transversions when the horse was included, and

508 10,094,431 transversions when the horse was excluded (i.e. when analyses were

509 restricted to non-caballine genomes only). In these analyses, only specimens

510 sequenced to an average depth of coverage $\geq 1\times$ were retained. Principal Component

511 Analyses were carried out using the PCAngsd package (Meisner & Albrechtsen, 2018)

512 (Figure 2A). To assess the impact of potential reference bias, all analyses were

513 repeated after mapping the sequence data against the donkey reference (Figure

514 2—figure supplement 4).

515

Phylogenetic inference

517 **Mitochondrial phylogeny**

518 Cleaned reads were mapped against the mitochondrial genome (Genbank accession no.

519 NC_001640), following the same procedure as when mapping against the nuclear

520 genome. Samples showing an average depth-of-coverage <1× were disregarded,

521 leaving a total of 17 individuals for further analyses. After removing duplicates,

522 consensus mitochondrial sequences were generated using ANGSD (-doFasta 2

523 -doCounts 1 -setMinDepth 3 -uniqueOnly 1 -remove_bads 1 -minQ 25 -minMapQ 25).

524 Multiple alignment was performed together with the comparative mtDNA sequences

525 downloaded from GenBank (Table S4) using MUSCLE v3.8.31 (Edgar, 2004), with

526 default parameters. The alignments were then split into six partitions (1st, 2nd and 3rd

527 codon positions, rRNA, tRNA, and control region).

528   Two Maximum Likelihood (ML) trees based on all 6 partitions and excluding the

529 control region (positions 15,469-16,660 of the horse reference mitochondrial genome)

530 were both reconstructed using RAxML-NG v.0.9.0 (A. M. Kozlov, Darriba, Flouri,

531 Morel, & Stamatakis, 2019) with GTR+GAMMA substitution model. A total of 1,000

532 bootstrap pseudo-replicates were carried out to assess node robustness (Figure

533 2—figure supplement 5). BEAST 2.5.1.0 (Bouckaert et al., 2019) was used to perform

534 Bayesian phylogenetic reconstruction and to estimate split times. The six partitions

535 described above were used, for which the best substitution model was determined

536 using modelgenerator (version 0.85, (Keane, Creevey, Pentony, Naughton, &

537 McLnerney, 2006)) and a Bayesian Information Criterion. We applied together with

538 the Coalescent Constant Population model and a strict LogNormal correlated

539   molecular clock for 50 million generations (sampling frequency = 1 every 1,000).

540   Convergence was assessed visually using Tracer v1.6 and posterior date estimates

541   were retrieved using 10% as burn-in. The final consensus tree was produced using

542   TreeAnnotator 2.5.1.0 (Drummond & Rambaut, 2007) and plotted using ITOL

543   (Letunic & Bork, 2016) (Figure 2—figure supplement 6).

544

545   **Autosomal phylogeny**

546   As for autosomes, we reconstructed a (ML) phylogenetic tree as implemented

547   in the PALEOMIX phylo pipeline for phylogenomic reconstructions (Schubert,

548   Ermini, et al., 2014). This analysis was based on the coding sequence (CDS) of

549   protein-coding genes annotated in EquCab3.0, partitioning data according to 1st, 2nd

550   and 3rd codon positions. Maximum Likelihood phylogenetic inference was performed

551   using ExaML v3.0.21 (Alexey M. Kozlov, Aberer, & Stamatakis, 2015) and RAxML

552   v8.2.12 (Stamatakis, 2014), under the GAMMA substitution model with 100 bootstrap

553   pseudo-replicates (Figure 2C and Figure 2—figure supplement 7A). We also repeated

554   the same procedure after mapping against the donkey reference genome and got the

555   same topology (Figure 2—figure supplement 7B).

556   Additionally, we extracted biallelic single nucleotide polymorphisms (SNPs) from

557   the dataset generated in Section 5 using bcftools v1.9 (Li et al., 2009). Both variant

558   datasets obtained following mapping against the horse and donkey reference genomes

559   were used in this analysis to rule out reference bias. We applied filters composed of

560   minimum phred-scaled quality score quality (QUAL) = 20, sites for all individuals

29

561   below 2 or twice the mean coverage, and allowed up to three individuals with missing

562   data per site. After disregarding transitions, a total of 18,803,101 (mapping against

563   horse genome) and 19,459,070 (mapping against donkey genome) transversions were

564   finally used as input for TreeMix (Pickrell & Pritchard, 2012) with parameters "-k 500

565   -root TWI", and considering an increasing number of migrations edges (0≤m≤3;

566   Figure 2—figure supplements 8 and 9, Table S5).

567

Admixture analyses with *D*-statistics

569   *D*-statistics were calculated to investigate potential introgression between *E. ovodovi*

570   and other non-caballines (Figure 3—figure supplement 1) using the doAbbababa2

571   programme in ANGSD (Soraggi et al., 2018). Individuals were grouped by their

572   respective species. D-statistics were computed in the form (((H1, H2), H3), Outgroup)

573   considering only the autosomal sites from bam files mapping against the horse

574   reference with the following options: "-minQ 20 -minMapQ 25 -remove_bads 1

575   -only_proper_pairs 0 -uniqueOnly 1 -baq 1 -C 50". The horse reference genome was

576   used as the Outgroup. H1 and H2 denoted any non-caballine genomes except *E.*

577   *ovodovi* while H3 denoted the *E. ovodovi*. Confidence intervals were estimated

578   applying a jackknife procedure and 5-Mb windows. Z-scores with absolute values

579   higher than 3 were considered to be statistically significant. To eliminate the bias of

580   the reference genome, we also rerun the same analysis using sequence alignments

581   against the donkey reference genome (Figure 3—figure supplement 2).

582

G-PhoCS demographic model

Data preparation and filtering

In order to model the equine evolutionary history, we selected a total of 10 individuals

representing each individual lineage and used their high-coverage genomes as input

for G-PhoCS (Gronau et al., 2011). Genotypes were called by GATK and candidate

'neutral' loci were identified by applying the following filters:

(1) The simple repeats track available for the reference genome was obtained from

Ensembl v99 release; corresponding regions were masked.

(2) All exons of protein-coding genes were discarded together with their 10 kb

flanking regions; this was done based on the GTF format annotation file of the

reference genome available from Ensembl v99 Genome Browser.

(3) We identified conserved noncoding elements (CNEs) using phastCons scores

(based on the 20-way Conservation track provided for the mammal clade according to

the genomic coordinates of the human reference) downloaded from the Table Browser

of UCSC. All CNEs and their 100 bp flanking regions were masked, using liftOver to

convert human genome coordinates into EquCab3.0 horse genome coordinates.

(4) Exons of noncoding RNA genes together with their one kilobase flanking

regions were removed, based on the annotations available for the reference genome.

(5) Gaps in the reference genome were disregarded.

Besides the various hard filters described above, regions/sites likely to (1) be

enriched for misaligned bases, and to (2) have high false negative rates during read

alignment or variant detection were masked as missing data. Different individuals

605   may be treated differently depending on the result of genotyping in Section 5

606   depending on the presence of (1) indels, (2) triallelic sites, (3) positions with depth of

607   coverage twice the mean depth recorded for each individual, and; (4) transition sites.

608   We selected 1 kb loci located with minimum inter-locus distance of 30 kb from the

609   intervals that pass all the criteria described above. Then consensus sequences were

610   generated for each individual from the vcf file generated in Section 5 using bcftools

611   'consensus' command, with IUPAC codes indicating heterozygous genotypes

612   (--iupac-codes) and "N" representing masked sites (--mask and --missing 'N').

613   Finally, we excluded contiguous intervals if the total amount of missing bases was

614   greater than 50% of the region length, resulting in a final collection of 15,324 loci

615   using the horse reference genome (autosomes only). Neighbor-joining trees were

616   constructed to confirm the topology before the inferring the population divergence

617   (Figure 3—figure supplement 3).

618

619   **MCMC setup**

620   We used default global settings (Gronau et al., 2011), including a Gamma prior

621   distribution ($\alpha= 1$, $\beta= 10,000$) for all mutation-scaled population sizes (θ) and a

622   Gamma prior distribution ($\alpha= 0.002$, $\beta= 0.00001$) for all mutation-scaled migration

623   rate (m). The initial parameter value of mutation-scaled divergence times (τ) was first

624   set individually for each population. Then we ran ~100,000-200,000 iteration tests

625   and manually evaluated the convergence by checking the achieve acceptance ratios (*ie*.

626   accept if around 30-70%) or using Tracer v1.6

627   (http://tree.bio.ed.ac.uk/software/tracer/). For each test, we updated the input of the

628   initial τ and all fine-tuned parameters based on previous results to get the appropriate

629   value. The final results in Figure 3 are based on 500,000 MCMC iterations.

630

631   **Parameter calibration**

632   We assumed an average generation time (g) of 8 years, considering the mutation

633   rate μ (per year) could be variable when using different sequences. The coalescent

634   time of the *Equus* (4.0-4.5 Mya) (L. Orlando et al., 2013) was used to bound the

635   parameter μ. Effective population sizes (Ne) and divergence times (T) were estimated

636   by calibrating θ and τ parameter using g and μ (Table S6), given by: $Ne = \theta/(4\mu g)$ and

637   $T = \tau/\mu$ (Gronau et al., 2011).

638

639   **Inferring gene flow**

640   Total migration rates (M) were estimated by a mutation-scaled version (m) given by:

641   $M = m\tau_m$, where τ_m is the mutation-scaled time span of the migration band. We then

642   converted such rates, M , into a probability of migration using the formula: $p = 1\text{-}e^{-M}$

643   (where p is the probability of gene flow), according to the method presented in

644   (vonHoldt et al., 2016).

645   The migration model implemented in G-PhoCS makes it possible to detect gene

646   flow between any two lineages by introducing migration bands manually to the

647   demographic model. However, it remains difficult to detect weak migration events.

648   Additionally, scenarios including a large number of migration bands can lead to

33

649   spurious results. To address this, we first inferred a demographic model with no

650   migration bands, and then introduced several migration bands corresponding to five

651   independent scenarios (Table S6). A significant migration band was considered

652   supported if both the 95% Bayesian credible interval of total migration rate (M) did

653   not include 0 and the mean value of M was estimated to be greater than 0.03.

654   Settings for the migration bands between extant caballines are based on previous

655   research (Jonsson et al., 2014). The significant migration band from horse to the

656   non-caballine ancestor were identified (Table S6), in line with previous work (Jonsson

657   et al., 2014). However, no other non-negligible ($M > 3\%$) migration bands was found

658   in our analyses (Table S6).

659   We then tried to estimate the migration events between *E. ovodovi* and other

660   branches. We added all possible migration bands between *E. ovodovi* and

661   non-caballine branches into the demographic model except the migration bands

662   between *E. ovodovi* and the ancestor of non-caballines, as the model is often

663   underpowered to infer migration between sister populations. All of the migration

664   bands were separated into four demographic models. Only three migration bands were

665   shown significant (Table S6).

666   Finally, the total four migration bands were combined into one demographic model

667   (Table S7) and compared the estimates to the one including no migration (Table S8).

668

669   **Demographic trajectories with PSMC**

670   **PSMC analyses**

34

671   In order to reconstruct the past demographic dynamics of the *E. ovodovi* lineage, we

672   applied the PSMC algorithm (version 0.6.5-r67) (Li & Durbin, 2011) to the sample

673   HH06D (12.0×) , as well as three other Eurasian equine species (*E. caballus* TWI, *E.*

674   *hemionus* ONA and *E. kiang* KIA).

675   We first obtained the diploid consensus sequences after mapping against the horse

676   genome for the autosomes of each specimens using bcftools 'mpileup' command and

677   the 'vcf2fq' command from vcfutils.pl with the following filters: mapping quality $\geq$

678   25; adjust mapping quality =50; minimum depth-of-coverage = 8; maximum

679   depth-of-coverage $\leq$ 99.5% quantile of the coverage distribution; minimum RMS

680   mapping quality = 10; filtering window size of indels = 5.

681   After filtering the bases with Phred quality scores strictly lower than 35, we ran

682   PSMC with following command: 'psmc -N25 -t15 -r5 -p "4+25*2+4+6" '. Calibration

683   was carried out using a generation of 8 years and mutation rate of 7.242×10^{-9} per

684   generation per site, following previous work (Jonsson et al., 2014). However, as for

685   the mis-incorporation pattern and high error rate of HH06D (Figure 2—figure

686   supplements 1 and 2), we also performed analyses without transitions using mutation

687   rates of 2.3728×10^{-9} that was obtained assuming that the most recent common

688   ancestor of living equine species emerged 4 Mya (L. Orlando et al., 2013).

689   We found a great expansion of HH06D in the past 50,000 years when retaining

690   transitions but not when conditioning on transversions (Figure 4—figure supplement

691   1). The former is thus likely spurious and at least partly driven by severe post-mortem

692   DNA damage signatures in the sequence data. We therefore only used the latter when

693   considering the ancient HH06D specimen.

694

695   **False negative rate correction**

696   The HH06D genome (12.0×) was corrected assuming a uniform false-negative rate

697   (uFNR) following (L. Orlando et al., 2013), as the average depth-of-coverage is lower

698   than the recommended 20×. To identify the correction value of uFNR for HH06D, we

699   randomly down-sampled reads of SOM genome (21.0×) using DownsampleSam

700   function of Picard Tools to down-scale sequence data to the same average

701   depth-of-coverage as that obtained for HH06D. This indicated that a value of 0.22 was

702   the most suitable uFNR value for rescaling the HH06D PSMC profile (Figure

703   4—figure supplement 2A). The KIA and the ONA genomes, which also showed

704   limited coverage, were also rescaled following the same procedure (Figure 4—figure

705   supplement 2B-C). Finally, PSMC confidence intervals were assessed from 100

706   bootstrap pseudo-replicates (Figure 4).

707

708   **Heterozygosity Inference and Inbreeding**

709   Global heterozygosity rates and inbreeding levels were inferred for high coverage

710   individuals (>10×) using ROHan (Renaud, Hanghoj, Korneliussen, Willerslev, &

711   Orlando, 2019) with default parameters, except that transitions were excluded

712   (--tvonly) (Figure 5—figure supplement 1). To limit the impact of remnant

713   mis-incorporations, we used the attached estimateDamage.pl script to estimate

714   damage for all ancient samples prior to heterozygosity computation. Inbreeding was

715   co-estimated together with genome-wide heterozygosity levels from the total ROH

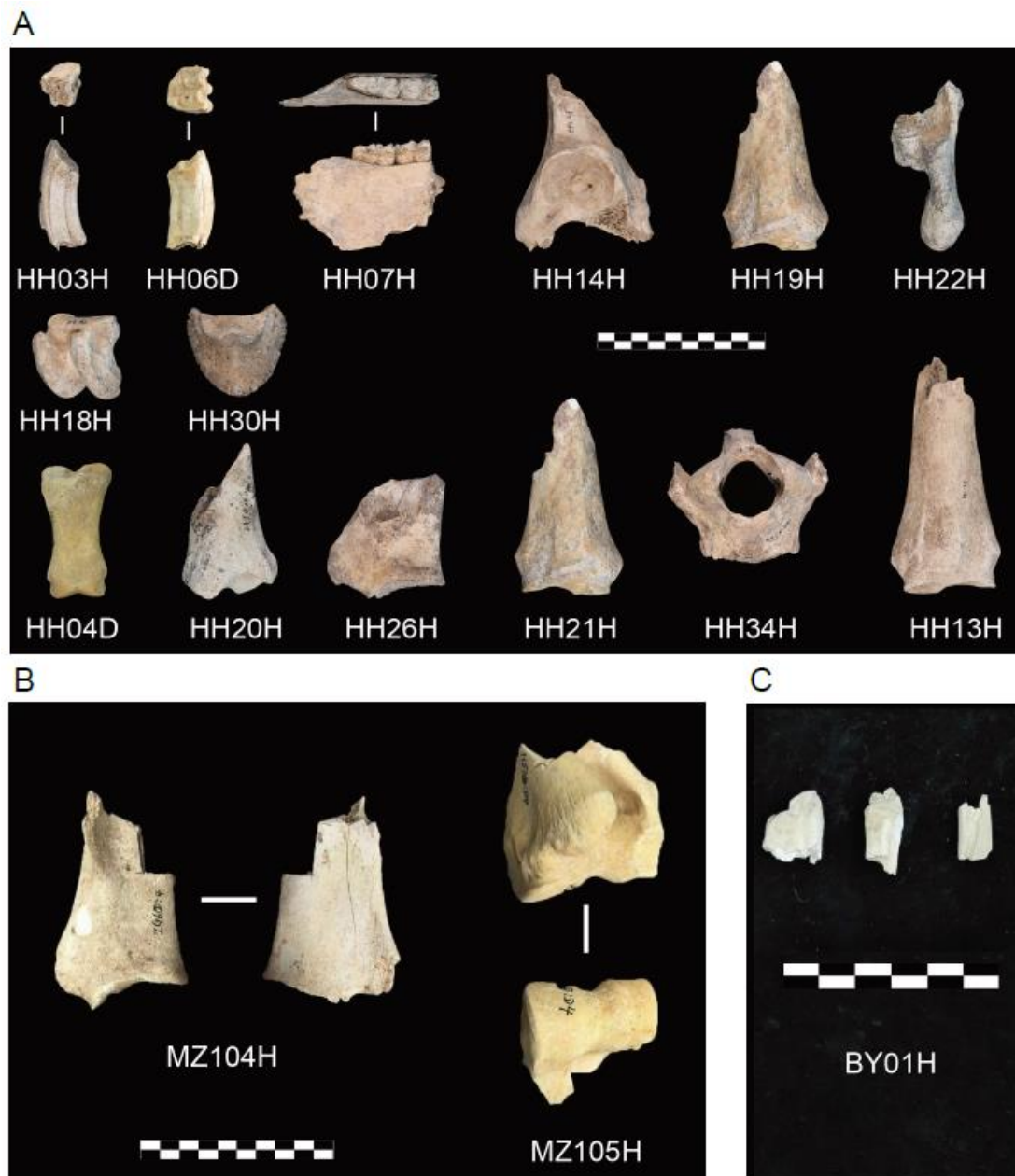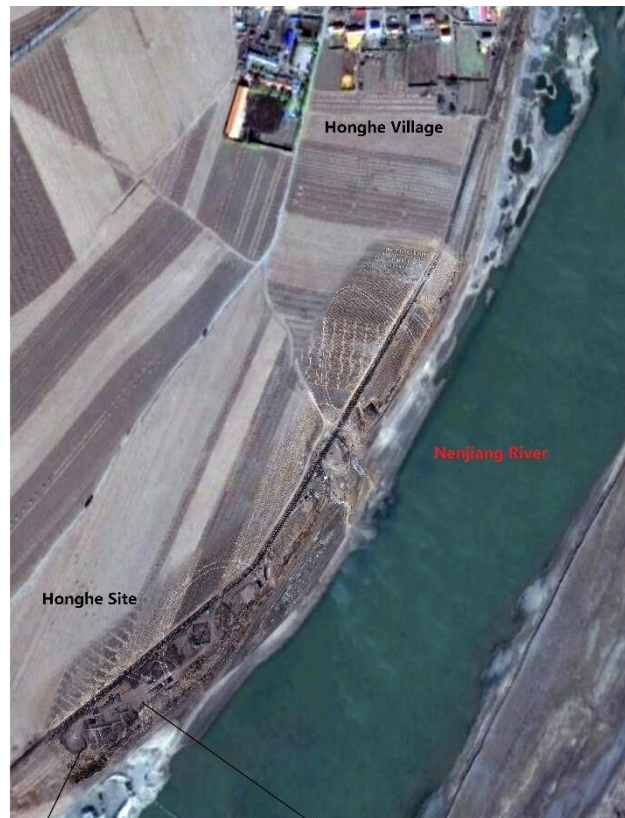716   length (Figure 5—figure supplement 2).

717

718

Figure 1—figure supplement 1. Archaeological material investigated in this study.

(A) Honghe (HH), (B) Muzhuzhuliang (MZ) and (C) Shatangbeiyuan (BY).

721

38

722

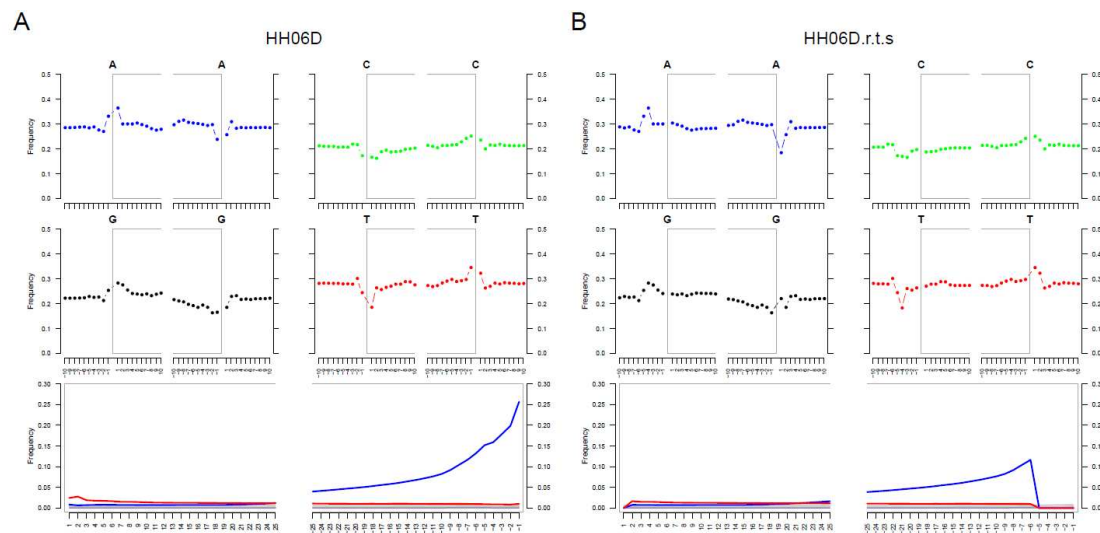**Figure 1—figure supplement 2.** Aerial view of the Honghe site.

724

Figure 2—figure supplement 1. DNA Damage patterns for HH06D. (**A**) Before

rescaling and trimming and (**B**) after rescaling and trimming the region comprising

the five first and last nucleotides sequenced.
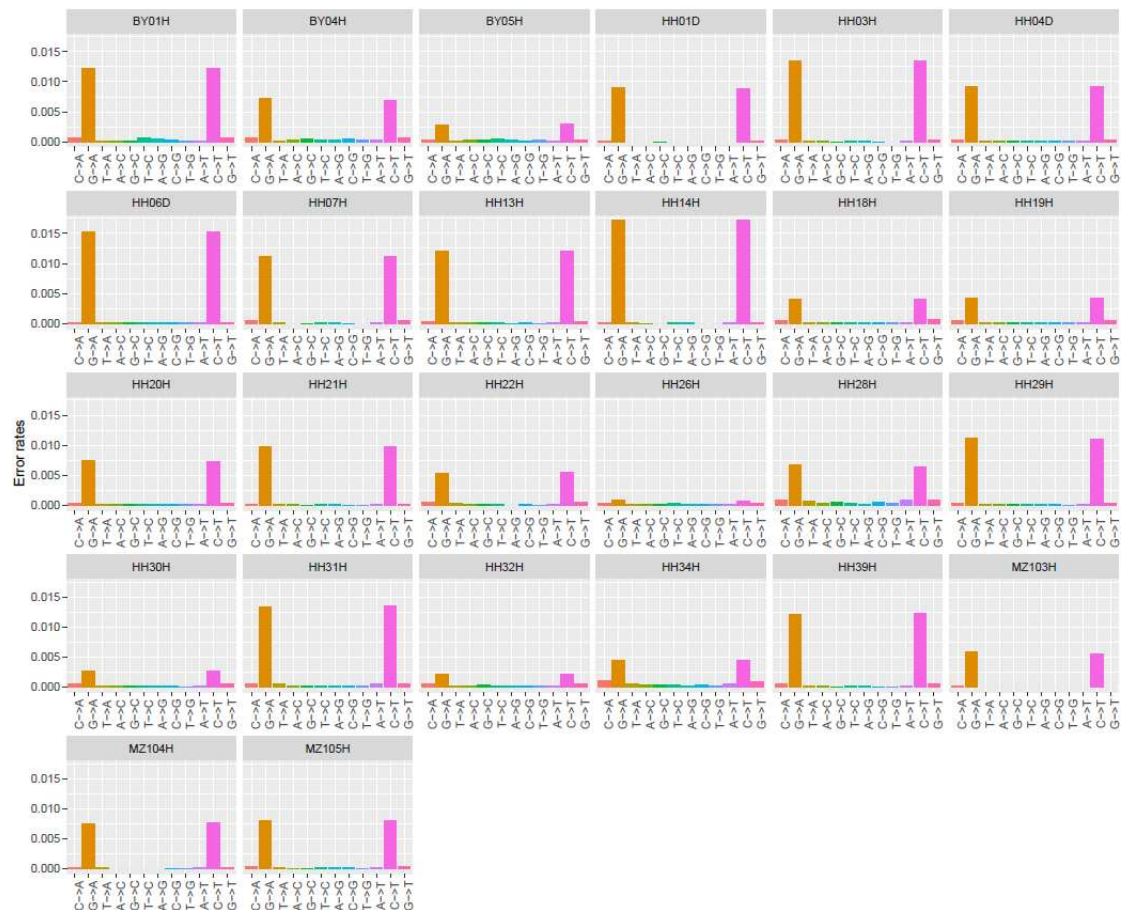
Figure 2—figure supplement 2. Error profiles of the 26 ancient genomes

characterized in this study. After trimming and rescaling, reads showing mapping

quality scores inferior to 25 and bases showing quality scores inferior to 20 were

disregarded.
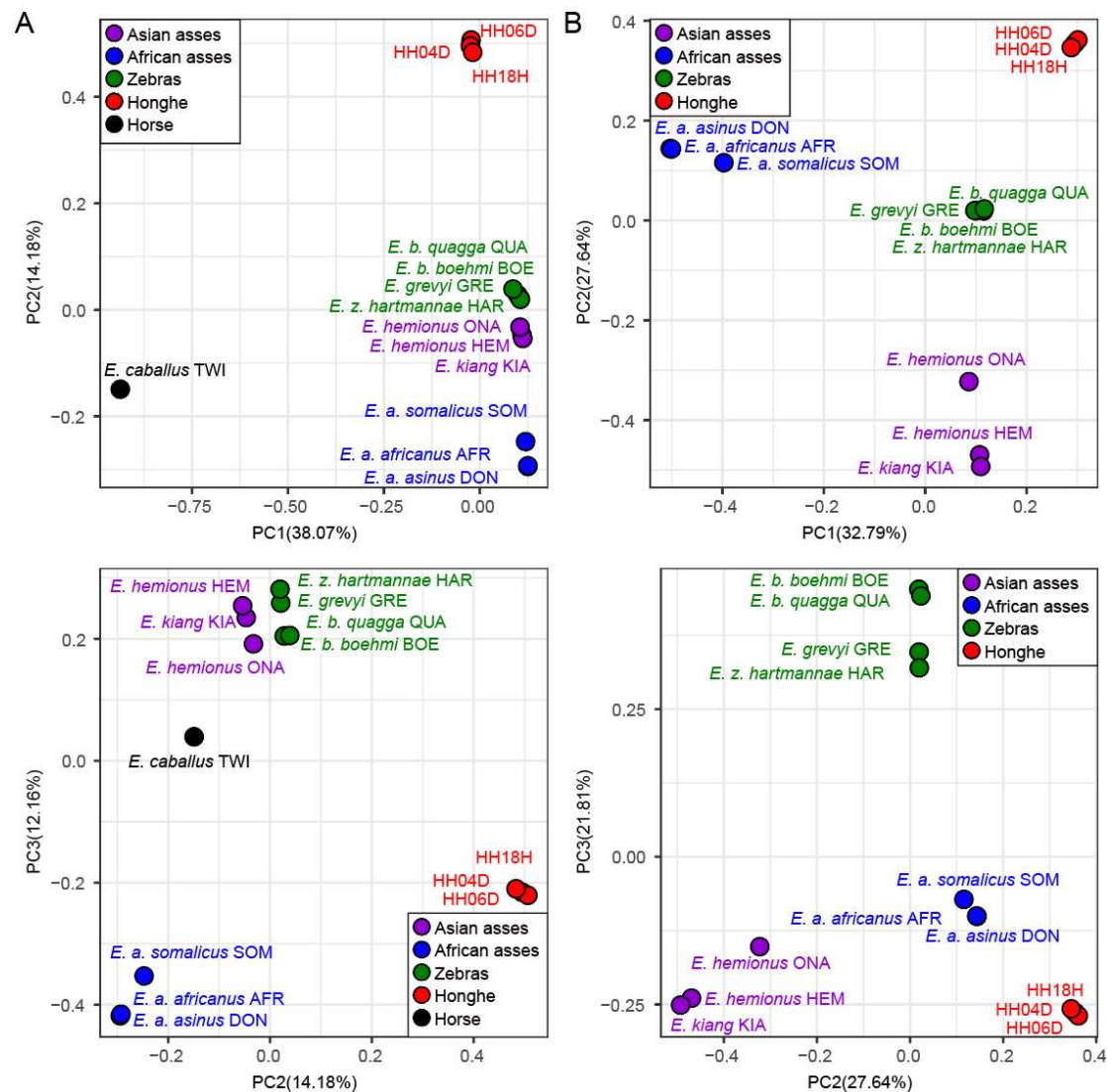
41

736

Figure 2—figure supplement 3. Principal Component Analysis (PCA) based on

genotype likelihoods using the horse reference genome. (**A**) Including and (**B**)

excluding the outgroup individual underlying the horse reference genome (TWI)

(Kalbfleisch et al., 2018). Sequence data were aligned against the horse reference
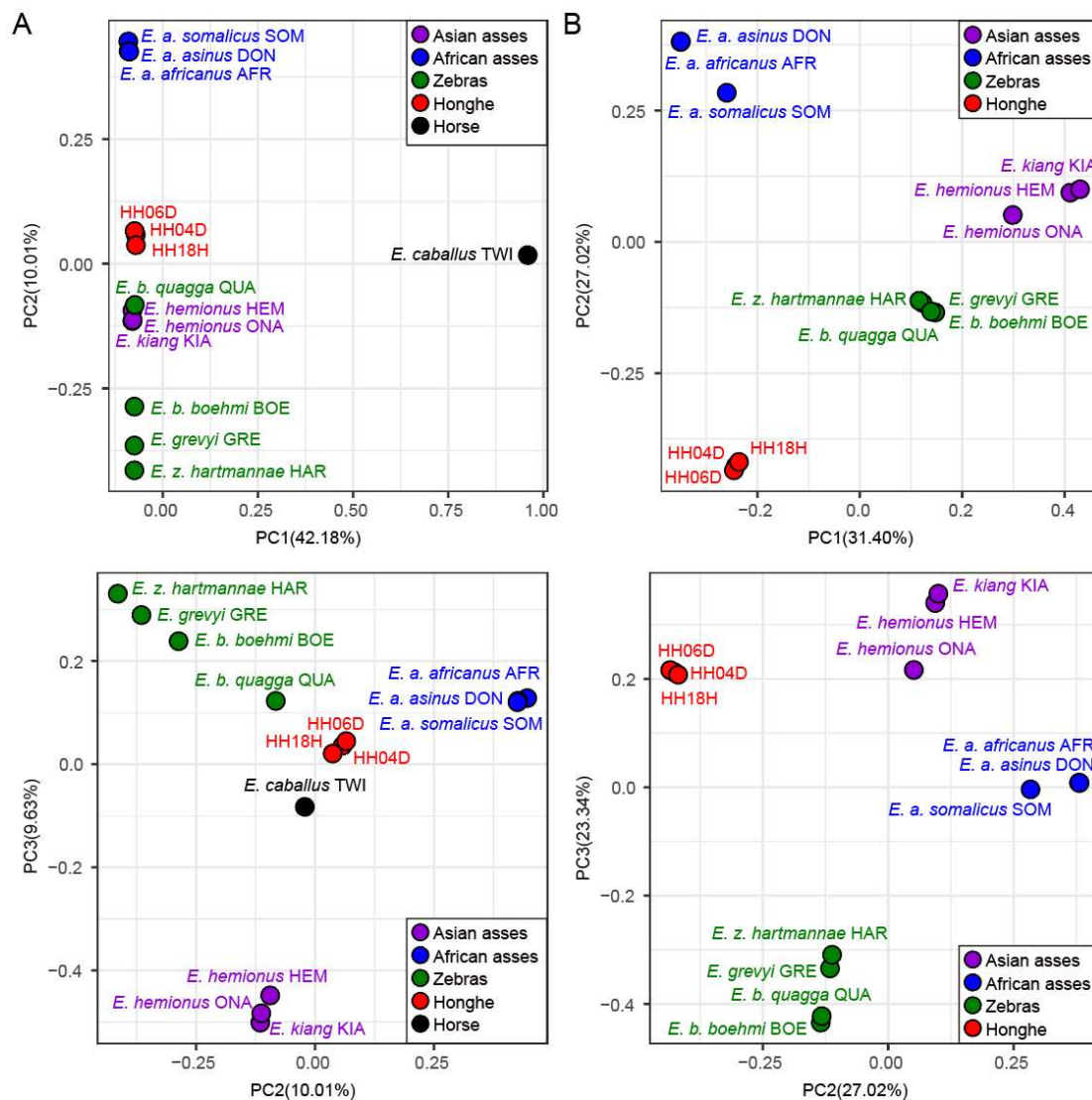
genome (Kalbfleisch et al., 2018).

742

Figure 2—figure supplement 4. Principal Component Analysis (PCA) based on genotype likelihoods using the donkey reference genome. (**A**) Including and (**B**) excluding the outgroup individual underlying the horse reference genome (TWI) (Kalbfleisch et al., 2018). Sequence data were aligned against the donkey reference genome (Renaud et al., 2018).
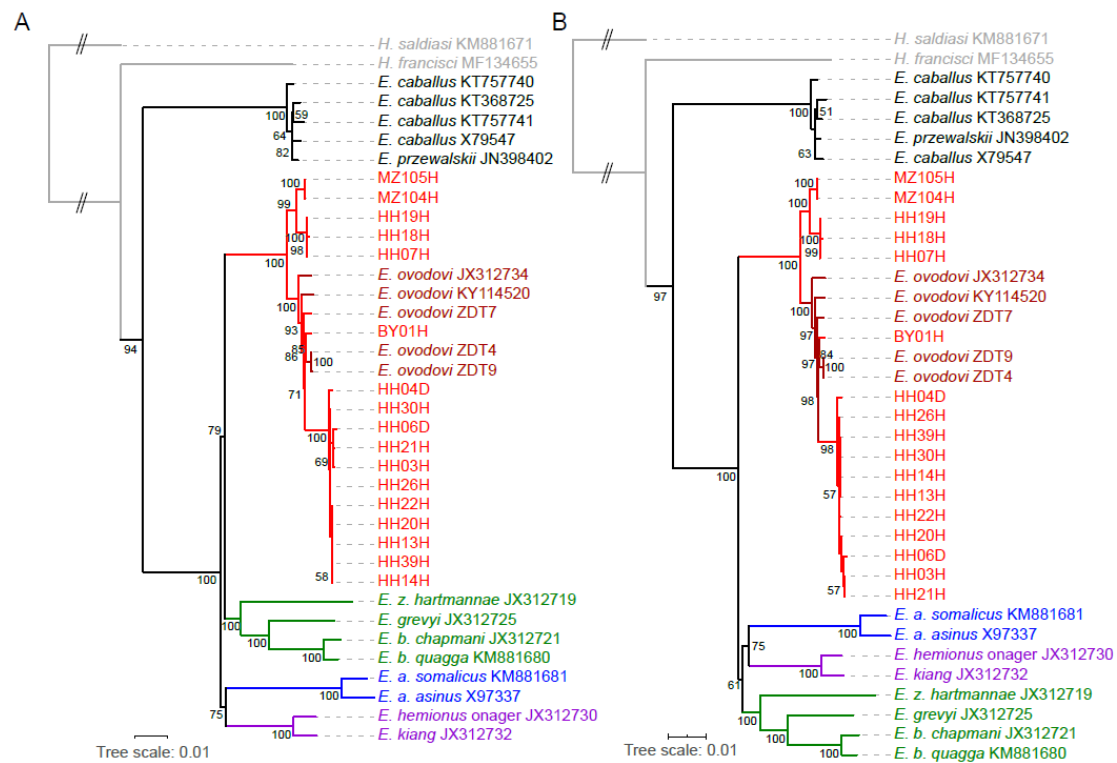
Figure 2—figure supplement 5. RAxML-NG (GTR+GAMMA model) Maximum Likelihood phylogeny of complete mitochondrial sequence data. (**A**) Including the control region. (**B**) Excluding the control region. Node support was estimated from 1,000 bootstrap pseudo-replicates and the tree was manually rooted using *Hippidion Saldiasi*.
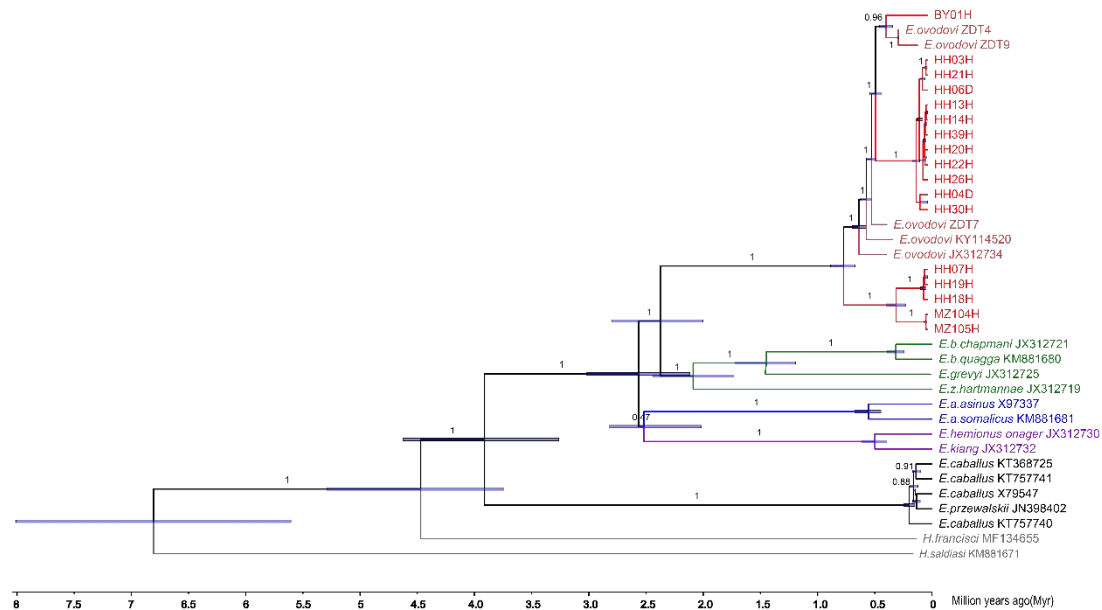
Figure 2—figure supplement 6. Bayesian mitochondrial phylogeny based on 6 partitions and using *Hippidion Saldiasi* as outgroup. The tree was reconstructed using a total number of 50 million MCMC states in BEAST (sampling frequency = 1 every 10,000, burn-in = 10%). The substitution models applied to the six sequence partitions were the TrN+I+G model (1st codon position = 3,802 sites), the TrN+I model (2nd codon position = 3,799 sites), the GTR+I+G model (3rd codon position = 3,799 sites), the HKY+I model (transfer RNAs = 1,517 sites), the TrN+I+G model (ribosomal RNAs = 2,556 sites) and the HKY+I+G model (control region = 1,192 sites).
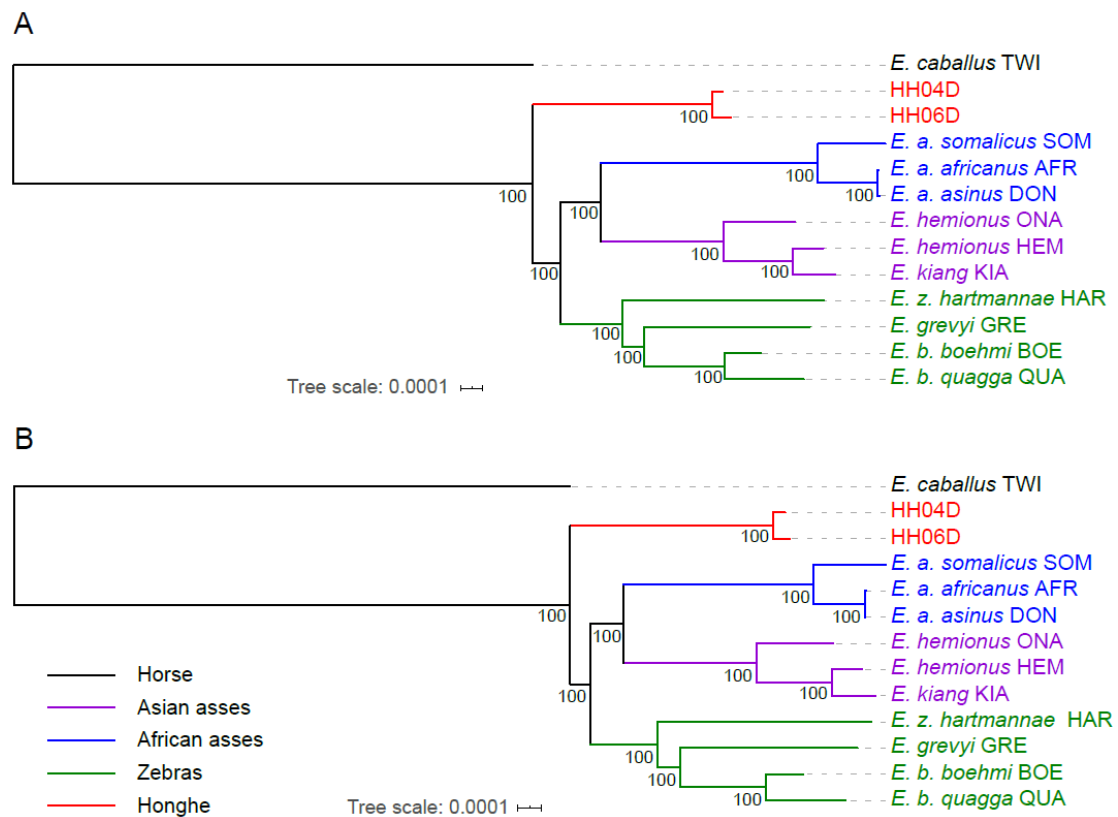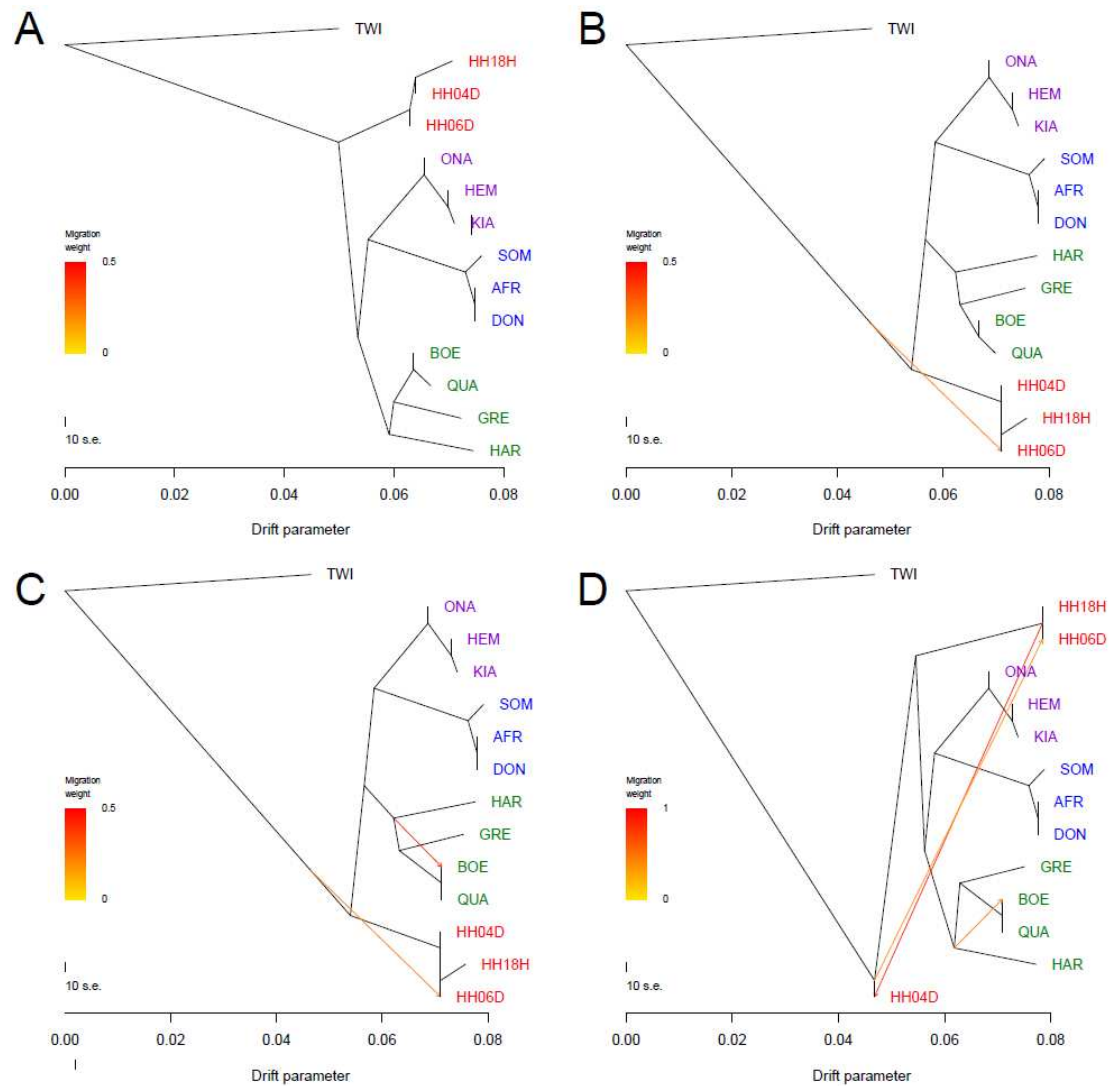
45

Figure 2—figure supplement 7. Exome-based Maximum likelihood phylogeny rooted by the horse lineage. (**A**) Using sequence alignments against the horse reference genome (Kalbfleisch et al., 2018). (**B**) Using sequence alignments against the donkey reference genome (Renaud et al., 2018). Node supports were estimated from 100 bootstrap pseudo-replicates.
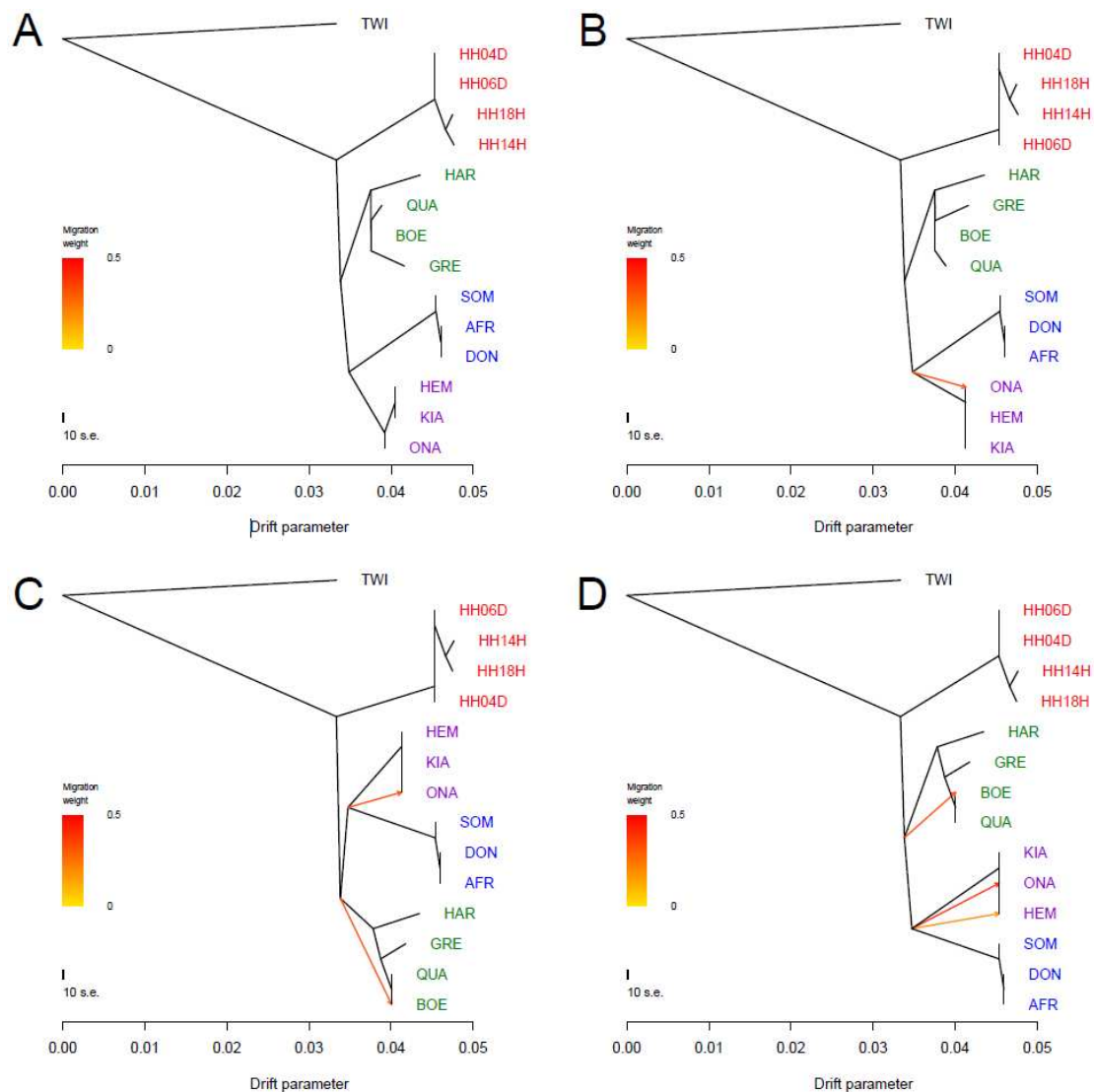
Figure 2—figure supplement 8. Treemix analysis of based on genome-wide SNP

data conditioned on transversions using the horse reference genome. Sequence data

were mapped against the horse reference genome (Kalbfleisch et al., 2018). A total of

0 to 3 migration edges were considered. The result of each analysis is shown in panels

(**A**) to (**D**), respectively. Considering additional migration edges did not improve the

variance explained by the TreeMix model (Table S5).

782

Figure 2—figure supplement 9. Treemix analysis of based on genome-wide SNP

data conditioned on transversions using the donkey reference genome. Sequence data

were mapped against the donkey reference genome (Renaud et al., 2018). A total of 0

to 3 migration edges were considered. The result of each analysis is shown in panels

(**A**) to (**D**), respectively. Considering additional migration edges did not improve the

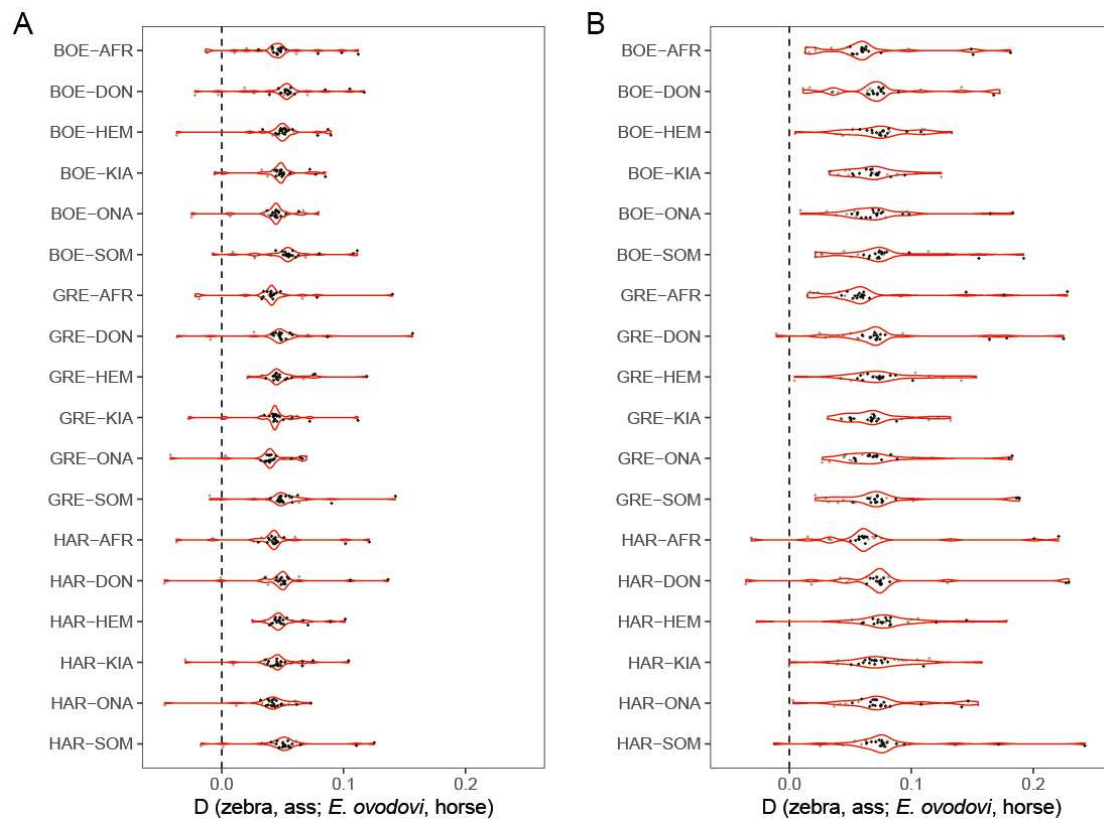variance explained by the TreeMix model (Table S5).

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Figure 3—figure supplement 1. *D*-statistics in the form of (zebra, ass; *E. ovodovi*,

outgroup), using sequence alignments against the horse reference genome.

Significantly positive D-statistics are indicative of an excess of shared derived

polymorphisms between *E.* (*Sussemionus*) *ovodovi* and extant assess, which is

compatible with admixture between both lineages. (**A**) Including transitions. (**B**)
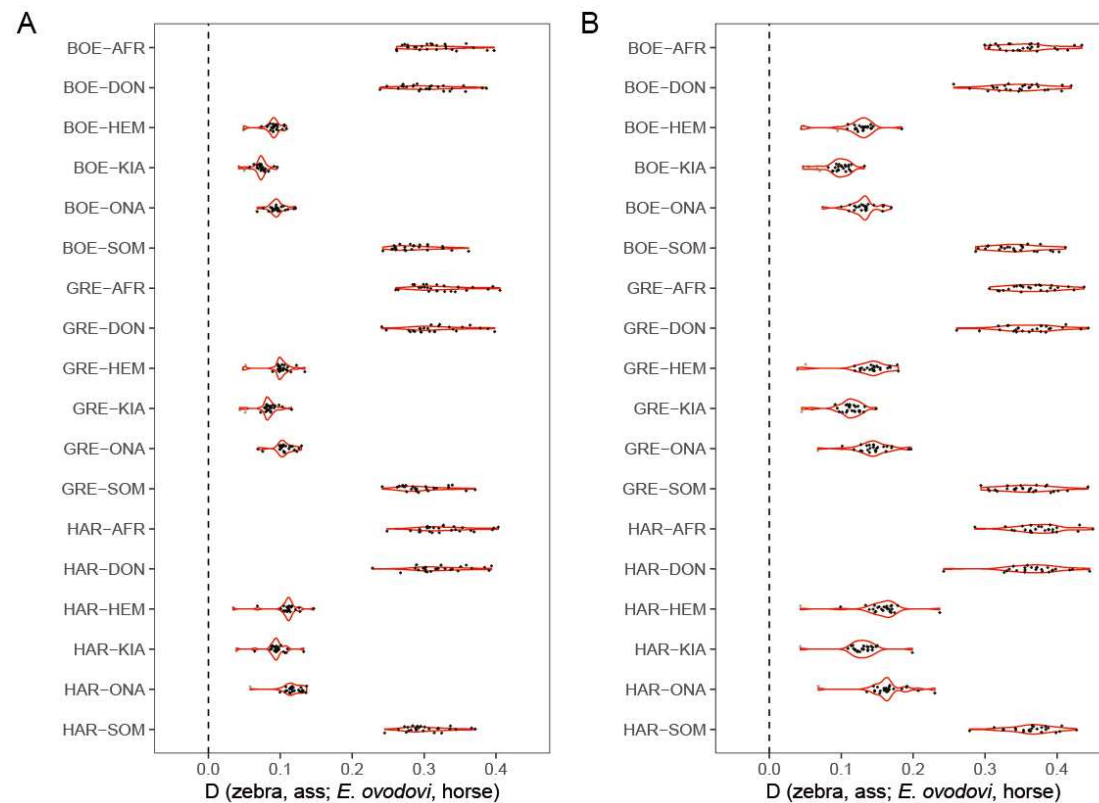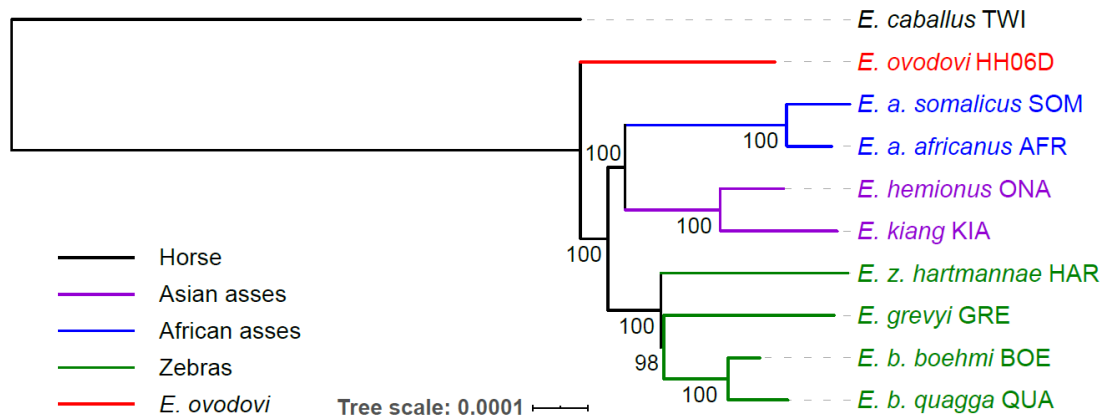
Excluding transitions.

798

799 **Figure 3—figure supplement 2.** *D*-statistics in the form of (zebra, ass; *E. ovodovi*,

800 outgroup), using sequence alignments against the donkey reference genome.

801 Significantly positive D-statistics are indicative of patterns of shared derived

802 polymorphisms between *E.* (*Sussemionus*) *ovodovi* and extant assess, which is

803 compatible with admixture between both lineages. (**A**) Including transitions. (**B**)

804 Excluding transitions.

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Figure 3—figure supplement 3. NJ tree of selected samples based on 15,324 candidate 'neutral' loci identified using sequence alignments against the horse reference genome (detailed in **Data preparation and filtering**). Node supports were assessed from 1,000 bootstrap pseudo-replicates.
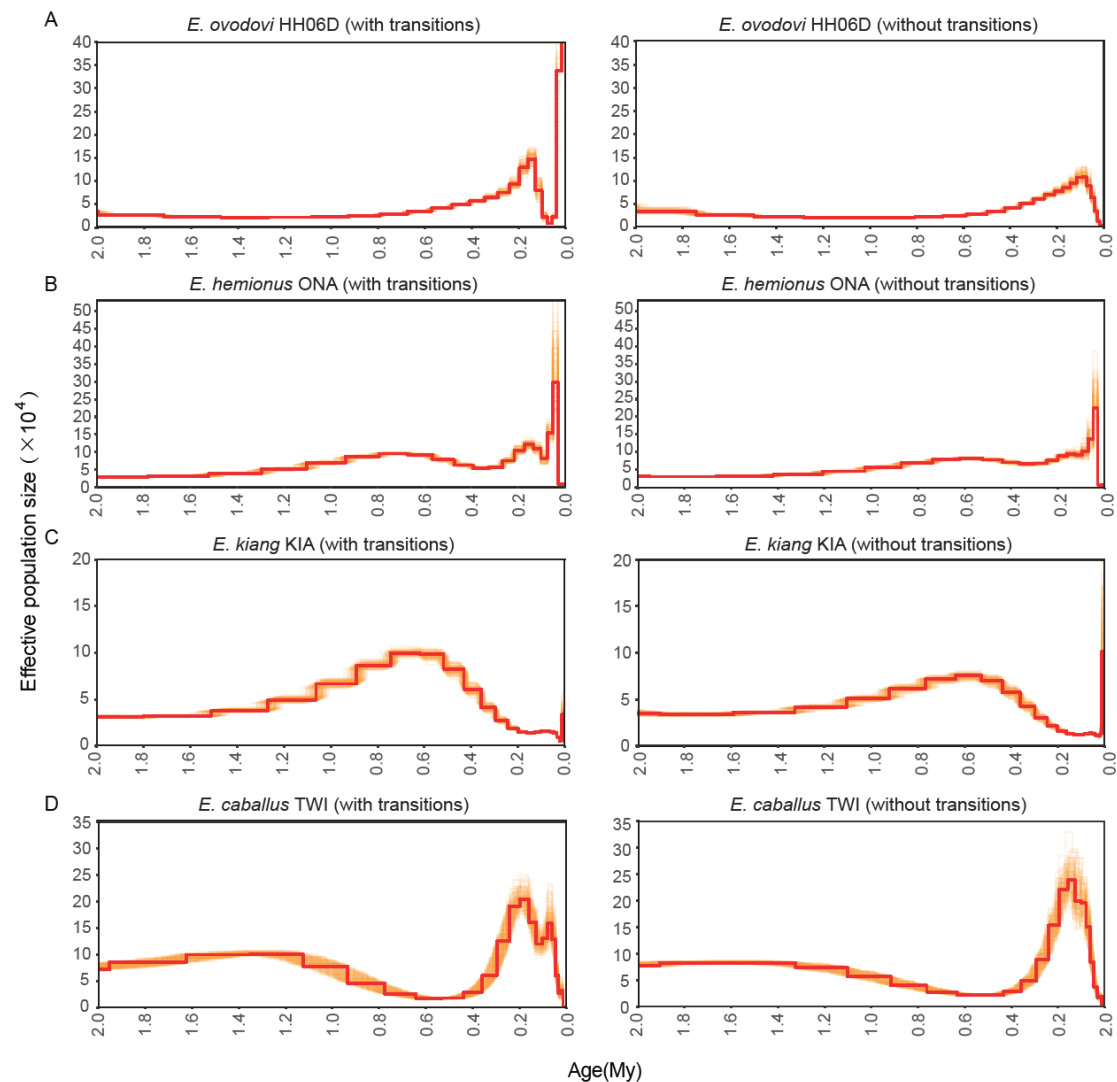
812

Figure 4—figure supplement 1. PSMC bootstrap pseudo-replicates for samples with

(left) and without (right) transitions. (**A**) HH06D, (**B**) ONA, (**C**) KIA and (**D**) TWI.

The *E. ovodovi* genome still included, even after rescaling, a significant proportion of

nucleotide mis-incorporations pertaining to post-mortem DNA damage. This resulted

in the presence of an excessive fraction of singleton mutations along this lineage, and

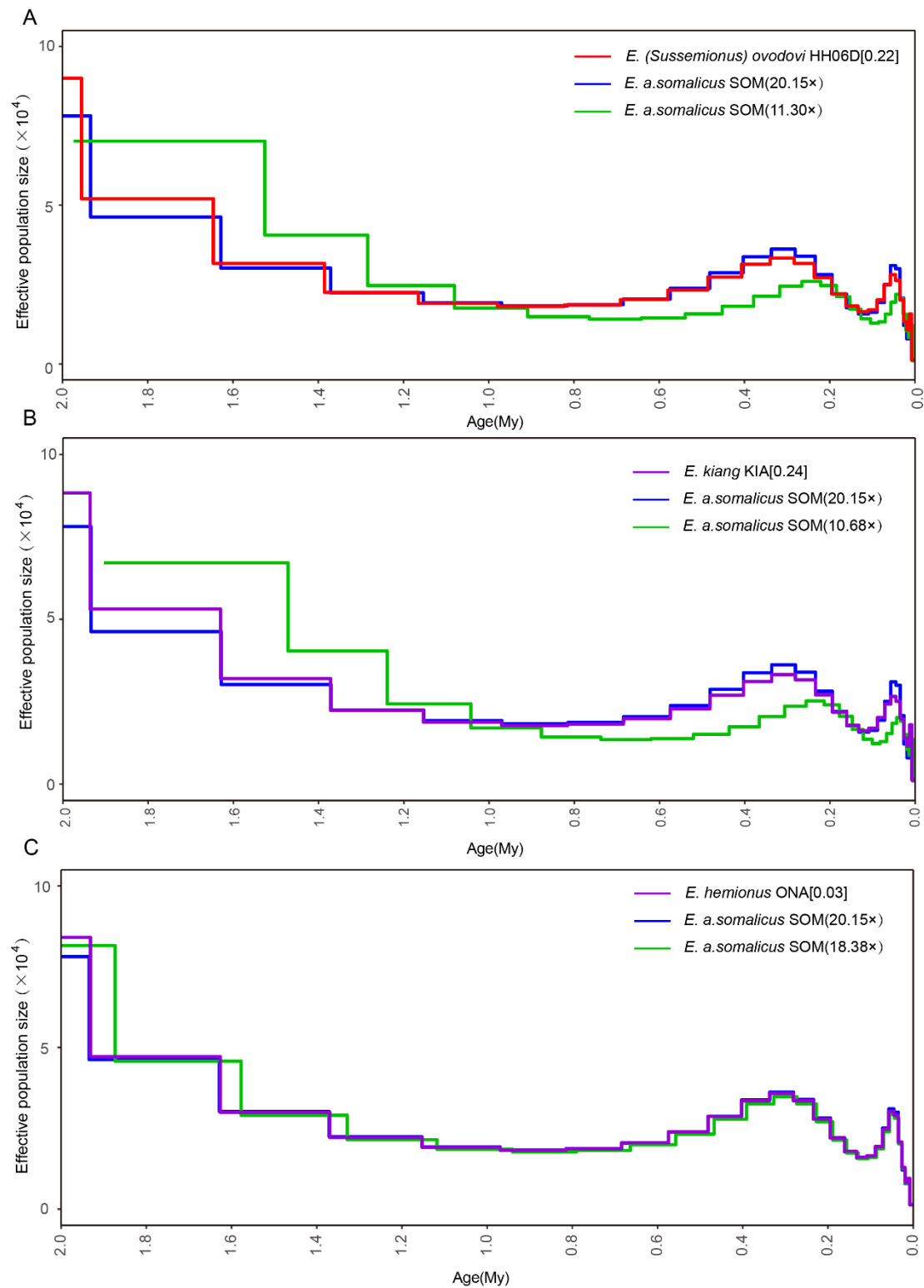the artefactual expansion observed in the most recent time range.

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Figure 4—figure supplement 2. Determining the uniform false-negative rate (uFNR)

that was necessary for PSMC scaling. (**A**) HH06D (11.30×), (**B**) KIA (10.68×) and

(**C**) ONA (18.38×).

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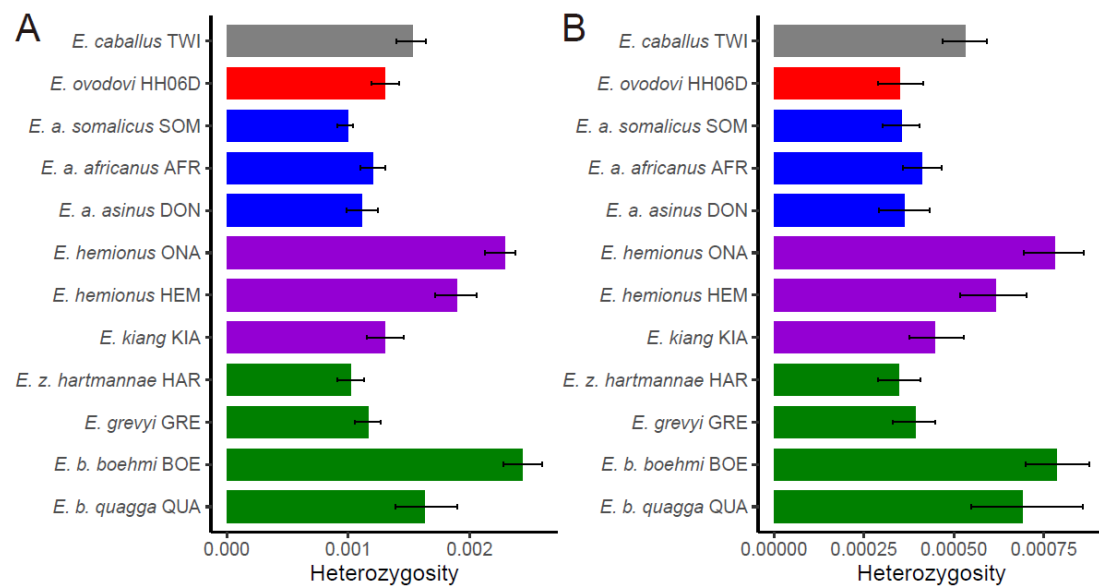**Figure 5—figure supplement 1.** Heterozygosity rates outside Runs-of-Homozygosity

827 (ROH) together with 95% confidence intervals. (**A**) Including transitions and (**B**)
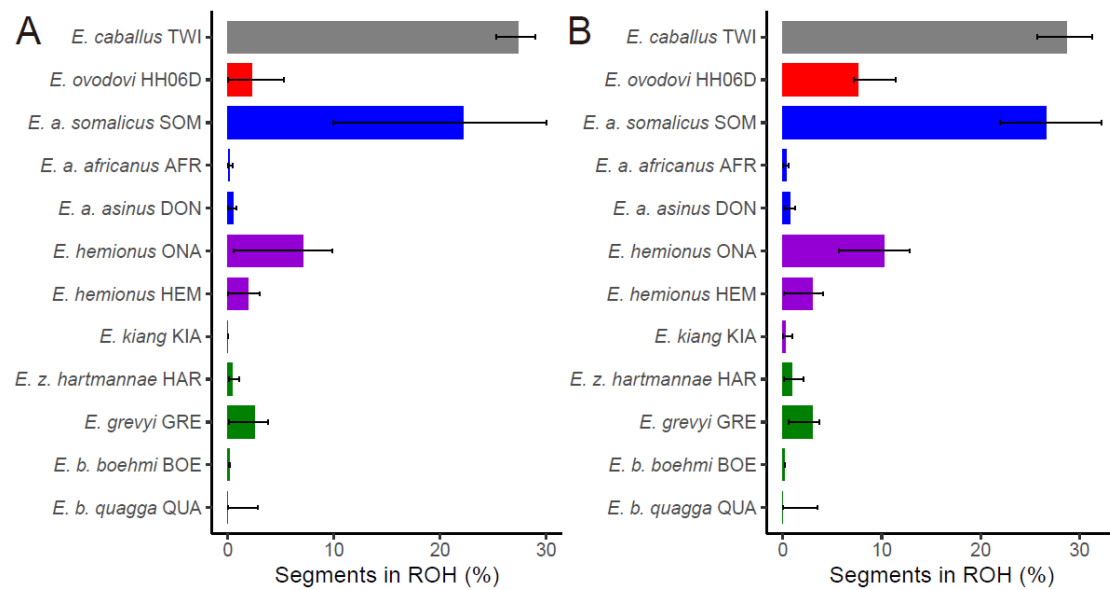
828 excluding transitions.

829

Figure 5—figure supplement 2. The fraction of the genome segments consisting of ROHs together with 95% confidence intervals. (**A**) Including transitions and (**B**) excluding transitions.

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