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1 **The genetic identity of the earliest human-made hybrid animals, the kungas of Syro-**
2 **Mesopotamia**

3 Short title: Ancient DNA of Bronze Age equid hybrids

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20 **Abstract:** Before the introduction of domestic horses in Mesopotamia in the late third millennium
21 BCE, contemporary cuneiform tablets and seals document intentional breeding of highly valued
22 equids called kungas, for use in diplomacy, ceremony and warfare. Their precise zoological
23 classification, however, has never been conclusively determined. Morphometric analysis of equids
24 uncovered in rich Early Bronze Age burials at Umm el-Marra, Syria, placed them beyond the ranges
25 reported for other known equid species. We sequenced the genomes of one of these ~4,500-year-old
26 equids, together with an ~11,000-year-old Syrian wild ass (hemippe) from Göbekli Tepe and two of
27 the last surviving hemippes. We conclude that kungas were F1 hybrids between female domestic
28 donkeys and male hemippes, thus documenting the earliest evidence of hybrid animal breeding.

29

30 **One-Sentence Summary:** Ancient DNA identifies elite equids in Bronze Age Mesopotamia as the
31 first human-bred hybrid animals, the enigmatic kungas

32

33 INTRODUCTION

34 In the third millennium BCE, urbanized, socially-stratified and literate societies appeared for the
35 first time in Syria and northern Mesopotamia (1, 2). Part of this “second act” of the urban revolution
36 was the breeding and employment of an equid of high status and prestige designated a “kunga.” The
37 precise taxonomical determination of the kunga and its identification in the archaeological record
38 have been uncertain until now. Third millennium BCE cuneiform clay tablets from Syro-
39 Mesopotamia describe several equids, using the generic term ANŠE associated with various
40 logographs. Of these, the so-called “kunga”, was represented by the cuneiform signs ANŠE.BARxAN
41 (3, 4) (Fig. 1A). Texts from the Diyala region in Mesopotamia and the kingdom of Ebla in the Levant
42 state that the prices for these equids were considerable, costing up to six times the price of a donkey
43 (5). References for these valuable equids are found in multiple clay tablets (3, 4) (Fig. 1A) such as
44 those detailing fodder expenses, e.g., barley for the equids of the god Shara and the deified king

45 Shulgi from Umma (6), and dowries for royal marriages (7). Large-sized male kungas were used to
46 pull the vehicles of “nobility and gods” (6), and their size and speed made them more desirable than
47 asses for the towing of four-wheeled war wagons (8), which predate horse-pulled chariots. Smaller-
48 sized males and female kungas were used in agriculture, where they were frequently reported pulling
49 ploughs (4, 9). Kunga foals were seldom born within the urban centers of Sumer and Syria, and Ebla
50 purchased young kungas almost exclusively from what may have been the principal breeding-center
51 at Nagar (modern Tell Brak), in northern Mesopotamia, whose rulers also provided them as gifts to
52 the elites of allied territories (3). Presumed kungas featured prominently on royal seals throughout
53 the region (10), and images of these hybrids likely appear on both the “war” and “peace” panels of
54 the standard of Ur, a Sumerian artefact excavated from the royal cemetery in the ancient city of Ur
55 (in modern-day Iraq). In one of the first depictions (2600 BCE) of a military expedition in human
56 history, warriors stand on four-wheeled war wagons, each drawn by a team of unspecified equids
57 (Fig. 1B). An example of the rein ring featured in this image has been found in a royal grave at Ur
58 (Fig. 1C), decorated with a small statue of a non-caballine equid, either a kunga or hemione. Kunga
59 use and traditions decreased and eventually vanished following the introduction of domestic horses
60 in the region (9, 11). Early references to horses in cuneiform writing coincides with the Third Dynasty
61 of Ur (late 3rd millennium BCE), where they are referred to as anše-zi-zi and later anše-kur-ra (equids
62 of the mountain) (6, 8). An introduction of domestic horses in Mesopotamia by the end of the third
63 millennium is also supported by paleogenetic data illustrating their late arrival in Anatolia around
64 2000 BCE, presumably through the Caucasus (12).

65 While the symbol for kunga (ANŠE.BARxAN) is used to describe a hybrid equid, the
66 unambiguous assignment of this term to a species is difficult and controversially discussed. Some
67 authors even argue that the kunga referred only to wild caught Persian onagers (also known as Iranian
68 onagers; *E. hemionus hemionus*, a subspecies of the Asiatic wild ass) rather than hybrid animals (3,
69 6), although the difficulty in taming modern onagers, which are reportedly less tractable than zebras

70 (13), does not support this interpretation. One of the likely parents of the kunga is the donkey (*E.*
71 *africanus asinus*), thought to be present in Sumer from at least the late 4th millennium BCE (8). The
72 identity of the other parent, however, remains unclear. Another equid attested since the Early Dynastic
73 period I (ca. 2800 BCE) is the anše-edin-na, literally translated as “equid of the desert”. This animal
74 was hunted for its meat and hide, but never used as a draught animal. The anše-edin-na is broadly
75 considered to be a type of onager (4, 8), although it is impossible to say whether it refers to the Persian
76 onager (*E. hemionus onager*) or to the Syrian wild ass, or hemippe (*E. hemionus hemippus*) (8),
77 sometimes also named “Syrian onager”. Described as a light, swift animal (14), the hemippe was the
78 smallest of all modern equids until the subspecies went extinct early in the 20th century (11). It has
79 been argued that in addition to its untamable, aggressive nature (14), its diminutive size made it an
80 unlikely candidate for use in breeding kungas (3 and references therein). Some authors considered
81 the non-donkey parent to be a horse (discussed in 4, 9).

82 In the elite burial complex of Tell Umm el-Marra (2600-2200 BCE), possibly belonging to the
83 ancient city of Tuba, 55 km east of Aleppo in modern-day northern Syria (Fig. 2), men and women
84 were interred with ceramics, bronze and silver vessels, bronze weapons and tools, and personal
85 ornaments made of bronze, silver, gold and lapis lazuli (15). Within this royal burial complex,
86 complete skeletons of 25 male equids and bones from 6 additional animals were buried separately
87 from humans, either in a sequence of pits or in their own mud-brick structures (15), akin to the 3000
88 BCE donkey burials at Abydos, Egypt (16). While some animals were interred after natural deaths,
89 more than half appear to have been deliberately killed for burial in the complex. Morphometric values
90 obtained from these bones indicate that these animals constitute a population outside of the typical
91 ranges of horses, asses, and onagers, and it has been proposed that these skeletons represent hybrids,
92 presumably kungas (17 and Supplementary Materials). In absolute size, the skeletons are closer to
93 hemiones, but are more robust; commonly-used slenderness indices suggest greater affinities with
94 asses than with hemiones. The leg characteristics of hemiones, responsible for a speed exceeding that

95 of horses, is retained in these animals, suggesting that they were also fast (18). Interestingly,
96 discrepancies in wear between the incisors and cheek teeth of some of the equids indicate that the
97 animals were foddered and not commonly grazed (17), features that would have been expected on the
98 skeletons of the equids depicted on the standard of Ur, whose lip- or nose-rings would have made
99 grazing difficult (Fig 1B). These animals would have been stronger and faster than donkeys, and must
100 have been more tamable than hemiones (19).

101 Taxonomic classification of equids uncovered in tombs across Mesopotamia (Ur, Kish, Lagash—
102 now al-Hiba, Abu Salabikh, Tell Madhhur) is often controversial (for discussion see e.g., 5, 8, 20,
103 21). The degree of variation within ancient populations is not fully known, and the degree of variation
104 between individuals within a population – especially of domesticated animals – is large, making it
105 difficult to differentiate between *E. africanus* and *E. hemionus* using solely bone morphological and
106 metrical characteristics (for discussion see 22).

107 In order to clarify whether the burials of Tell Umm el-Marra indeed contained the remains of the
108 politically and symbolically important hybrids referred to in numerous cuneiform tablets as kunga,
109 and in order to determine the taxonomic status of those animals, we investigated the genomes in
110 samples from the skeletons of the equid installations at Umm el-Marra, an equid sample from the
111 Early Neolithic site of Göbekli Tepe (Turkey), as well as the last survivors of the Syrian wild ass
112 conserved in the Natural History Museum of Vienna.

113 **RESULTS AND DISCUSSION**

114 **Analysis of the maternal and paternal lineages of the Umm el-Marra equids**

115 An initial PCR screening of equid samples from Umm el-Marra showed that DNA was extremely
116 poorly preserved in these bones owing to the hot climate in Syria, detrimental to long-term DNA
117 preservation, and the poor condition of the bones (phalanges, sternum) available for study (Fig. S1).

118 Therefore, we combined shotgun nuclear DNA sequencing with highly sensitive PCR, targeting
119 taxonomically informative regions of both uniparental markers: mitochondrial DNA and the Y-
120 chromosome. To better pinpoint the genetic identity of the parental species, we increased the available
121 Y-chromosome data by sequencing regions from additional populations of both modern and 19th and
122 20th century museum samples of hemiones and donkeys, for which the mitochondrial sequences were
123 previously generated (23). Short, overlapping PCR products suited to the degraded DNA of the
124 samples were designed to amplify a highly diagnostic mitochondrial control region fragment (324
125 base pairs long), including the site of a well-characterized 28 base pair-deletion exclusive to hemiones
126 (23), and three separate regions of the Y chromosomal DNA (in total 168 base pairs long)
127 encompassing four single nucleotide polymorphisms (SNPs), which we show to be diagnostic
128 between *E. ferus* (*caballus* and *przewalskii*), *E. africanus*, and *E. hemionus*.

129 The full targeted mitochondrial sequence was successfully amplified from two out of the six
130 individual equids tested from Umm el-Marra. At every position divergent between *E. ferus*, *E.*
131 *africanus*, and *E. hemionus*, both of these sequences contained the *E. africanus* specific bases and
132 lacked the 28 base pair-deletion specific to *E. hemionus* (23). The maternal lineage of these equids
133 thus unambiguously belongs to *E. africanus* as visible in a median joining network (24) (Fig. 3A).
134 All three Y-chromosome fragments were successfully amplified from these same two Umm el-Marra
135 individuals. Within these three regions, four diagnostic positions differentiate *E. africanus* (T/G/T/A)
136 from *E. hemionus* (C/A/G/G), two of which also differentiate *E. ferus* (C/G/G/A) from either *E.*
137 *hemionus* or *E. africanus*. At each diagnostic position, the equids from Umm el-Marra were found to
138 have the *E. hemionus*-specific base, and no diagnostic position of any product contained the *E.*
139 *africanus*-specific base (table S1). The hemione-specific Y-SNPs were also confirmed previously in
140 diverse hemiones from archaeological samples from the Caucasus, museum specimens from Tibet
141 and Syria and present-day specimens from the Gobi in Mongolia (23) (Fig. 3B and table S1).

142 In addition to the *E. hemionus* diagnostic positions, both Umm el-Marra sequences contained two
143 additional Y-chromosome SNPs observed only in the two hemippes from the 19th and 20th centuries
144 analyzed herein (Fig. 3B), one of them being the last known member of the subspecies. This animal
145 had been caught in the deserts north of Aleppo in 1911 and had been kept in the Schönbrunn Zoo in
146 Vienna until its death in 1929 (see Fig. S2 for images of two of the hemippes used in this study).
147 Thus, the Umm-el-Marra equids harbor the maternal lineage of the domestic donkey and the paternal
148 lineage of the Syrian wild ass, suggesting they could be F1 hybrids, since interspecific equid hybrids
149 are generally sterile, or poorly fertile.

150 **Analysis of the nuclear genomes of Umm el-Marra and Göbekli Tepe equids and the last** 151 **Syrian wild asses**

152 To further establish the hybrid identity of these equids, we sequenced a subset of the nuclear
153 genome of the best preserved Umm-El-Marra equid bone (table S5). In addition, we established the
154 genome sequence of the extinct Syrian hemippe by sequencing a ca. 11,000-year-old wild ass from
155 the early Neolithic site of Göbekli Tepe, present-day Turkey, representing the first temple (25), and
156 two 19th century specimens from the Schönbrunn Zoo (table S5). These four newly generated
157 genomes were compared to six modern horse (26), six domestic donkey (27), three Mongolian
158 khulans (27, 28) (an *E. hemionus* subspecies from the Gobi), two kiang genomes (27, 29) (*E.*
159 *hemionus kiang* or *E. kiang*), and one Persian onager genome (29) (an *E. hemionus* subspecies from
160 Iran), (table S4). A set of 15.5 million SNPs residing outside of repeated sequences and being variable
161 in the modern genome equid panel was used for calling the ancient genomes (see Supplementary
162 Methods and table S5). Even though the best Umm-el-Marra extract contained only 0.18%
163 endogenous DNA, we could obtain 45.6K SNPs, 40.4K of which were shared with either hemippe.
164 Of these, 15.2K SNPs (37%) were shared with both of the two best-covered hemippe genomes. First,
165 we performed principal component analysis (PCA) to compare (a) the hemione and donkey genomes
166 (Figs. 4A, S4A), or (b) hemione, donkey and horse genomes (Figs. S4B-E). Identical results were

167 obtained whether we used the 15.2K SNPs shared between the Umm-el-Marra equid (UMM9), the
168 Göbekli Tepe and the 1864 hemippe, or whether we used the 4.1 million SNPs shared between the
169 Göbekli Tepe and the 1864 hemippe genome and projected the UMM9 equid onto the PCA (compare
170 Figs.4A and S4A, S4B and S4D, S4C and S4E), showing that the 15.2K SNPs obtained allowed
171 robust characterization of the status of the Umm-el-Marra equid. When only donkey and hemione
172 genomes are used, PC1 separates donkeys from hemiones and PC2 separates the hemiones (Figs. 4A
173 and S4A). The most differentiated in PC2 are the Persian onager and the kiang zoo specimens. The
174 Mongolian khulan and the kiang from neighboring regions in China are very closely related and
175 overlapping in the PCA, which is in accordance with the shared mitochondrial lineages we reported
176 previously that led us to question the specific taxonomic status of the kiang as a separate species (23)
177 (see also the phylogenetic trees of the mitogenomes and the genomes in Figs. S6 and S7). The two
178 modern hemippes as well as the Göbekli Tepe sample overlap as well (the lower coverage 1892
179 hemippe was projected) and are located at an intermediate position in PC2. The Göbekli Tepe sample,
180 a mare, is clearly an ancient hemippe as observed from the phylogenetic trees constructed from both
181 mitochondrial and nuclear genomes (Figs. S6-S7). The UMM9 equid falls exactly halfway between
182 the donkeys and the hemippes. When the PCA also includes the horses, PC1 differentiates the horse
183 from the non-caballine equids, PC2 separates the donkeys from the hemiones, and PC3 separates the
184 hemiones in a similar way as PC2 does when horses are not included (Figs. S4B-E). In all analyses,
185 the results for the UMM9 equid illustrate an intermediate position between the donkeys and hemippes.
186 The PCA analyses thus indicate that the UMM9 genome is a 50% mixture of donkey and hemippe.

187 We further explored this outcome through ADMIXTURE analysis (30) (Fig. 4B). A 4-population
188 model separates horses, donkeys, Mongolian khulans and kiangs from onagers and the two best-
189 covered hemippes. The UMM9 equid is modelled as an admixture of equal proportion between
190 donkey and hemippe/onager (Fig. 4B). Likewise, when considering the 4738 UMM9 equid SNPs for

191 which all six donkeys differ from all hemippes that have the position covered, the UMM9 equid
192 harbors SNPs corresponding to roughly half of those specific to each putative parent (Fig. 4C).

193 Finally, a bifurcating tree with gene flow analysis was performed using *treemix* (31) (Fig. 4D).
194 For the reference equids, genomic tree topology is similar in both the full mitogenome and genome
195 tree topology obtained with different methods (Figs. S6-S7). The Persian onager and the hemippes
196 are closely related, and the Mongolian khulans and the Tibetan kiangs are even more closely related.
197 In this respect, the genetic distances between the various hemiones correspond to the geographic
198 distances between their native range (onager: Iran; hemippe: Syria; Mongolian khulan: Mongolia;
199 kiang: Tibet). The UMM9 equid is represented on the tree as related to the donkey, but the residuals
200 between the hemippe and the UMM9 equid are high and a gene flow event from the hemippe to the
201 UMM9 equid best describes the phylogeny (see also Fig. S5). These results demonstrate the
202 sufficiency of the Umm el-Marra SNPs to determine the phylogenetic relationships between the
203 equids. The tree also placed the Umm el-Marra sample halfway between the asses and the hemippes
204 (Fig. 4), which meets expectations when dealing with F1 hybrids of these species. Evidence from Y-
205 chromosome analysis indicates that the Syrian hemippe rather than the Persian onager was used to
206 father the Umm el-Marra equids, whereas the mitochondrial DNA reveals that a donkey contributed
207 the maternal genome. That the Umm el-Marra equids were in fact F1 hybrids and not back-crossed
208 hybrids is also supported by the relative hybrid sterility between donkeys and horses, as well as
209 experiments in the 1940s crossing female donkeys with male hemiones, the Turkmenian kulans (*E.*
210 *hemionus kulan*), which produced sterile offspring (32).

211 Expectedly, the 19th-20th century hemippes, representing some of the last survivors of the
212 subspecies are genetically similar, whereas the ~11,000-year-old Göbekli Tepe hemippe is more
213 divergent (Fig. S7). We also noted that the divergence between the three sequenced hemippes is much
214 larger than that observed between the six domestic donkeys (Fig. S7). The higher diversity between
215 the sequenced hemippe genomes versus between the donkey genomes suggests that the donkey

216 mother of the UMM9 equid is more closely related to present-day donkeys than the Syrian hemippe
217 father of the UMM9 equid is to the other sequenced hemippes. This difference, albeit small, may
218 account for the slightly higher affinity of the UMM9 equid to present-day domestic donkeys that is
219 visible on Figs. 5 B & D. Both the genomic phylogenetic tree (Fig. S7) and the PCA analyses (Figs.
220 4B & S4) indicate that differentiation between the donkey genomes is low, far less than between the
221 various present-day hemiones, presumably because donkeys went through a major bottleneck,
222 possibly upon domestication and translocation to southwest Asia outside the range of the ancestors
223 of donkeys. The observation that the ~4500-year-old UMM9 equid appears more closely related to
224 present-day donkeys than to the last hemippe that disappeared a century ago suggests that the
225 bottleneck of the donkey population had already taken place by the third millennium BCE.

226 It has been noted that the Syrian wild ass (hemippe), whose range once extended across the Levant,
227 was the smallest form of modern equids (18). Both historical specimens analyzed in this study stood
228 ca. 100 cm at the shoulder (14) (Fig. S2). In contrast, the hybrids of Umm el-Marra were estimated
229 to average 130 cm at the shoulder (17). Regarding this difference in size, previous work had recovered
230 mitochondrial haplotypes from larger-sized Bronze Age equids recovered from Tell Munbaqa,
231 situated in northern Syria east of Umm el-Marra, as well as from three historical hemippe samples
232 dating from the mid-19th to early 20th century. Both the larger ancient and smaller more recent
233 animals were shown to cluster together in a single, separate mitochondrial clade (23). The Göbekli
234 Tepe wild asses were on average even slightly larger than those of roughly contemporaneous Tell
235 Mureybet (10th-9th millennia BCE) and 3rd-2nd millennia BCE Tell Munbaqa, two sites located in the
236 direct vicinity of Umm el-Marra (22), (Fig. 2). It was concluded, therefore, that the small Syrian wild
237 ass was likely to have been a dwarfed descendant of a genetically continuous population of larger,
238 more robust animals populating Syria in the third millennium BCE and earlier (23). The genomic
239 analyses of both ancient and historical hemippes in the present study support this earlier finding.
240 Indeed, no dwarf form has ever been reported from Late Pleistocene and Holocene sites in

241 Mesopotamia (33) or Anatolia (34). Nearly 2,000 years after the equid burials of Umm el-Marra,
242 sixth century BCE palace reliefs featuring hunted hemiones from Nineveh (in modern-day northern
243 Iraq) show already relatively small animals (Figs. 1D & S8).

244 **CONCLUSION**

245 The genomic results from the rare equid burials at the elite mortuary complex of Umm el-Marra
246 confirm earlier hypotheses based on morphological data that these animals are indeed hybrids (8, 9,
247 37) and, given their interment in high-status tombs, are most likely identical with the valuable kungas
248 frequently mentioned in cuneiform texts and depicted in images and royal seals throughout
249 Mesopotamia. This study now offers a firm zoological classification of the historical kunga as an F1
250 cross between a female donkey and a male Syrian wild ass, or hemippe, putting to rest past
251 speculations regarding the taxonomic identification of the BAR x AN. We further show that the 3rd
252 millennium BCE ancestors of the hemippe were likely larger than those first described by European
253 travelers visiting Syria in the 19th century. Our study also presents the earliest known case in human
254 history of inter-species hybridization practiced by Early Bronze Age breeders at sites such as Nagar
255 (Tell Brak) (Fig. 2), to generate animals famous for their power, both physical and symbolic, in
256 ancient warfare and diplomacy. This result also deepens our insight into the economic and political
257 relationships between contemporary royal households of Greater Mesopotamia, and the dynamics by
258 which these social elites fostered distant alliances. It also increases our understanding of the ways in
259 which the earliest stratified urban societies of the Middle East developed and maintained their
260 positions of authority. In this respect, genomic characterization of additional equids from comparable
261 contexts, particularly from Nagar, may help clarify the scale of hybrid breeding in 3rd millennium
262 BCE Mesopotamian societies prior to the introduction of domestic horses.

263

264 **Materials & Methods**

265 **Sample description**

266 Bone remains from equid skeletons dated between ca. 2550 and 2300 BCE and excavated in 2006 at
267 Tell Umm el-Marra, a Bronze Age elite cemetery in northern Syria (15, 17, 35) were sampled for
268 ancient DNA analysis. Further descriptions of the samples are given in the Supplementary Materials
269 and photos of the samples from the two individuals from which sufficient DNA was recovered appear
270 in Fig. S1. A petrous bone excavated from a layer dated between 9500-8300 BCE from the site of
271 Göbekli Tepe in southeast Turkey (25) and Supplementary Material) was also analyzed in the present
272 study. Furthermore, we analyzed two samples of the extinct *Equus hemionus hemippus* originating
273 from the desert of Aleppo in Syria and kept in the zoo of Schönbrunn, Vienna, Austria, a tooth from
274 the NMW 6048/ST345 specimen and a hair and skin sample from the NMW1308/B4690 specimen,
275 corresponding to animals who died in the Schönbrunn zoo in 1864 and 1892, respectively (36) (Fig.
276 S2). Finally, hair of a male Somalian ass (*E.africanus somaliensis*) from the “Réserve Africaine de
277 Sigean” (Sigean, France) was provided for the analysis of the Y chromosome by Dr. Elodie Trunet
278 (Sample “As.Somalie”).

279 **aDNA Extraction, Amplification, and Sequencing**

281 Hair samples were added to 1.5 mL hair digestion buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl,
282 40 mM DTT, 3 mM CaCl₂, 2% N-lauryl sarcosyl, 250 µg/mL proteinase K) and incubated 4-24 hours
283 at 50°C, shaken 300 RPM. Solutions were then pelleted and the supernatant was purified using a
284 QIAquick Gel extraction kit (Qiagen, Hilden, Germany) according to instructions.

285 DNA from archaeological bone samples was extracted, purified and prepared for either qPCR or
286 sequencing in the ancient DNA lab described previously (37–39). Bone cleaning and treatment
287 protocols were as described previously (12, 23). Briefly, after removal of the surface with a razor’s
288 blade or surface-cleaning with bleach the bones were either sawed using a flame-sterilized diamond-
289 disc of a Dremel Fortiflex (Dremel Europe, The Netherlands) and ground to fine powder in a 6775

290 Freezer/Mill®Spex SamplePrep in liquid nitrogen or drilled at low speed with a flame-sterilized bit.
291 The dense pyramidal part of the petrous bone GT64 was isolated using a flame-sterilized diamond-
292 disc of a Dremel and then ground to fine powder in a 6775 Freezer/Mill®Spex SamplePrep in liquid
293 nitrogen. Half of the GT64 powder was treated with diluted hypochlorite (1/20) and both halves
294 washed with phosphate buffer according to Korlević et al., 2015 (40). DNA extraction was performed
295 by incubating the bone powder at 37°C for 48 to 90 hours in either 1 to 10 mL extraction buffer A
296 (0.5 M EDTA, 0.25 M PO₄³⁻ pH 8.0, 0.14 M β-mercaptoethanol) or in twice 1 mL extraction buffer
297 B (0.5 M EDTA, 0.05% Tween20, 250 µg/mL Proteinase K, 0.14 M β-mercaptoethanol) that were
298 pooled prior to purification. Samples were purified using silica membrane spin-columns (QIAquick
299 Gel Extraction kit) with a vacuum manifold (Qiagen) and 25 mL extenders (Qiagen) as described
300 (38) (41), as well as with either the 5M guanidine HCl, 40% isopropanol (5M40) buffer as described
301 in Dabney et al (42), or the 2M guanidine HCl, 70% isopropanol (2M70) buffer as described in Glocke
302 and Meyer (43). The elution was performed twice in 25 µl 10 mM Tris-HCl pH 8.0, 0.05% Tween-
303 20 (EBT) made from gamma-irradiated water (8 kGy).

304 Purified DNA was amplified by qPCR, the extract making up 5-20% total volume (10-20µl per
305 reaction). Inhibition characteristics were determined for failed samples indicating possible inhibition,
306 and once optimal dilutions were determined, qPCR was attempted again. To protect against cross-
307 contamination, the UQPCR method was used (37, 39, 44), in which uridine was substituted for
308 thymidine in all PCRs, and incubation with uracil N-glycosylase (UNG, extracted from *G. morhua*;
309 Biotec Marine Biochemicals, Norway) was performed prior to each reaction. Mock extracts were
310 included with each extraction and amplified to control for contamination. qPCR reactions varied
311 slightly depending on the sample, but a typical reaction included 1.77µL of LC FastStart DNA
312 MasterPLUS mix1b and 0.23uL of either FastStart DNA MasterPLUS mix1b, mix1a, or FastStart
313 Taq (Roche Applied Science, Mannheim, Germany), a final concentration of 1µM of each primer and
314 1U per reaction of UNG in 10µL total volume. Primers were obtained from Sigma-Aldrich (St. Louis,
315 USA). Mitochondrial primers were designed to amplify 357 base pairs of the hyper variable region

316 of *E. africanus* and *E. hemionus* mitochondria using short, overlapping fragments (table S3). Y-
317 chromosome primers were designed to amplify three short sections of Y-chromosome DNA
318 containing the target SNPs (table S1-S3). Several modifications of these primers were designed to
319 increase sensitivity of qPCRs by minimizing the likelihood of primer-dimers and artifacts, and
320 increasing primer efficiency. A list of primers used and product sizes is given in table S3. qPCR was
321 performed using Lightcycler 1.5 or Lightcycler 2 (Roche Applied Science, Mannheim, Germany).
322 qPCR programs varied depending on primer requirements and product length, but a typical program
323 involved UNG incubation at 37°C for 15 minutes, followed by polymerase activation at 95°C for 5
324 minutes, then 2-step cycles of denaturation at 95°C for 10 seconds, then primer annealing and
325 extension at 62°C for 40 seconds, and finally a temperature increase of 0.1°C/1second from 62°C to
326 95°C with continuous fluorescence measurement to generate melt-curves of the products. Products
327 were purified by a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and both strands were
328 sequenced by capillary electrophoresis at Eurofins/MWG Operon (Ebersberg, Germany) using an
329 ABI 3730xl DNA Analyzer (Life Technologies). Samples that yielded sequence results for the Y-
330 chromosome are shown in table S1. An average of one non-template control (NTC) was run for every
331 6.6 samples (including mocks). No DNA was amplified in either NTCs or mocks, demonstrating that
332 no detectable equid DNA was introduced during sample preparation or was present in reagents.
333 Samples from the two individuals from Umm-el-Marra with the best preserved DNA identified via
334 qPCR (UMM4 and UMM9) were selected for shotgun sequencing, and 24 double-stranded libraries
335 using dual-barcodes were prepared from DNA purified from three or four different areas of each bone
336 using the protocol described in Massilani, et al., 2016 (45). Seven of these libraries were treated with
337 UDG to reduce the presence of cytosine deamination damage in the resulting sequences.
338 Double-stranded libraries of the two *E. h. hemippus* (hemippe) museum specimens were constructed
339 using the NxSeq ampFREE Low DNA Library kit (Lucigen, Middleton, WI, USA) following the
340 protocol and the modifications described in Bennett, et al., 2019 (46). Barcodes were added during
341 an amplification reaction using dual-barcoded single-stranded library adapters (47) as primers, rather

342 than those in the kit, where 20 μ l of eluted library was added to 25 μ l OneTaq 2x Master Mix (Roche)
343 and 0.6 μ M of each adapter for 50 μ l total volume, and amplified with the following protocol: 5
344 minutes 95°C, 30 cycles of 30 seconds 95°C, 30 seconds 60°C, 45 seconds 68°C, followed by a 5
345 minute cycle at 68°C. A library for a hair and skin sample belonging to specimen NMW5493/B 3625
346 was constructed using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (NEB, Ipswich,
347 MA, USA).

348 Libraries from the Göbekli Tepe petrous bone GT64 extracts were constructed using the NEBNext®
349 Ultra™ II DNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) after a pretreatment with
350 USER enzyme mix (NEB, Ipswich, MA, USA).

351 Dual-barcoded libraries were then purified and size-selected using NucleoMag beads (Macherey-
352 Nagel) for two rounds of purification following the supplied protocol at a ratio of 1.3x beads per
353 reaction volume and eluted in 30 μ l EBT.

354 All libraries were quantified with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific), Bioanalyzer
355 2100 (Agilent, Santa Clara, CA, USA) and by qPCR. Screening by shotgun sequencing of Umm-el-
356 Marra samples and of the two hemippe samples was performed on an Illumina MiSeq system using
357 a v3 reagent kit for 2x75 cycles. The libraries constructed from sample SP345, which came from a
358 molar belonging to specimen NMW6048/ST345 (1864 hemippe), a hair and skin sample belonging
359 to specimen NMW1308/B4690 (1892 hemippe), and the two libraries from the Umm-el-Marra 9
360 (UMM9) sample purified using the 5M40 and 2M70 buffers were selected for deep genomic
361 sequencing.

362 Sequencing of the 1864 hemippe genome was performed on an Illumina NextSeq using a NextSeq
363 500/550 High Output Kit v2 (2x75 cycles). The custom sequencing primer CL72 (47) was substituted
364 for the read 1 primer sequencing steps, which is compatible with the single-stranded adapters used
365 for these samples. Sequencing of the 1892 hemippe and of two of the UMM9 libraries was performed
366 first on an Illumina MiSeq using a v3 reagent kit for 2x75 cycles and then on an Illumina Novaseq

367 6000 using a S2 flow cell for 2x50 cycles. Sequencing of the GT64 libraries was performed on a
368 Novaseq 6000 using a S4 flow cell for 2x75 cycles.

369 **Paleogenetic data analyses**

370 Sequences from PCRs were manually curated, assembled and aligned using the Geneious software
371 suite (48). Median-joining network analysis (24) was performed on mitochondrial sequences covering
372 357 bp of the hyper variable region generated in this study by PCR combined with those previously
373 reported (23) (accession numbers given in table S2), and Y-chromosome sequences generated in this
374 study combined with those publicly available (samples and sources shown in table S1). Maximum
375 likelihood (ML) analyses of the complete mitochondria of the two hemippes combined with donkey
376 and hemione complete mitochondria after deletion of the 11-bp-tandem repeat in the HVR were
377 computed using RAxML (49) with a GTR nucleotide substitution model, a gamma-distributed rate
378 of variation among sites with four rate categories, and invariant sites (i.e., GTR-GAMMA-I) (Fig.
379 S6). We used 100 bootstraps to estimate node robustness.

380 **Genomic analyses**

381 Fastq reads from six modern horse genomes (24), six domestic donkey genomes (25), three
382 Mongolian khulan genomes (25, 26) (an *E. hemionus* subspecies from the Gobi), two kiang genomes
383 (25, 27) (*E. hemionus kiang* or *E. kiang*), and one Persian onager genome (27) (an *E. hemionus*
384 subspecies from Iran), (table S4), were trimmed with cutadapt (v1.18) (50) and aligned to the *E.*
385 *caballus* reference genome (eqCab2.0) using the BWA (v0.7.17) (51) *mem* program. PCR duplicates
386 were removed using Picard MarkDuplicates (v2.20.0) (52), and reads aligning to the reference
387 genome with mapping quality score below 30 were removed using samtools 1.9 (53).

388 *SNPs selection:* We curated the 36 million biallelic variant list used to differentiate equids in the
389 Zonkey workflow (54) to filter out variants found in repeated sequences using a EqCab2 genome
390 repeat mask downloaded from the UCSC browser <https://genome.ucsc.edu/cgi-bin/hgTables> . The
391 rationale for this filtration was that these variants would be less reliably called using the short,

392 damaged reads typical of ancient DNA libraries, in particular when mapping reads from a non-
393 caballine equid to the horse reference genome because these genomes are expected to differ markedly
394 in repeat location and sequence variability. This filtration reduced the variant list to 22 million. We
395 then called variants from this list on the modern equid genomes using bcftools (v1.9; (55)) mpileup -
396 B -q30 -Q30 and bcftools call -m. The vcf file was imported in plink (v1.9; (56)), and filtered to
397 include only SNPs, removing invariant and multiallelic positions. The final curated list contains 15.5
398 million SNPs.

399 Shotgun reads for the Umm-el-Marra (UMM9) and the Göbekli Tepe (GT64) samples were merged
400 with leeHom (57) using the ancientdna option, while the historical hemippe reads were trimmed with
401 cutadapt (v1.18) (50). Fragments smaller than 28 bp were discarded, and the remaining reads were
402 aligned to the *E. caballus* reference genome (eqCab2.0) using the BWA (v0.7.17) (51) aln program
403 with parameters “-n 0.01 -l 0” followed by samse (UMM9 and GT64) or sampe (hemippe). PCR
404 duplicates were removed using picard MarkDuplicates (v2.20.0)(52), and reads aligning to the
405 reference genome below a mapping quality score of 20 and a length of 28 bp were removed. To
406 reduce the increase in spurious alignments from shorter reads described in (58), mapped reads less
407 than 35 bp containing indels were also removed using an awk script. The ancient nature of the UMM9
408 and GT64 sequences was confirmed by analyzing the damage profile using mapDamage2 (59) of
409 libraries generated from extracts not treated with USER-enzyme (Fig. S3). To remove the C->T
410 mutations at the end of the molecules that escaped the USER treatment (Fig. S3), the base quality
411 was rescaled at the last 2 bases using mapDamage2 (59). Since all Umm el-Marra samples had very
412 low levels of endogenous equid DNA (zero to 0.18% of reads), they were additionally aligned to the
413 human and bovine genome reference sequence (GRCh37 and ARS_UCD1.2 respectively). Only
414 libraries that had at least 5-fold more reads mapping to the horse than to the cow or human genome
415 when a seed length of 18 was used during bwa aln mapping were kept. Summaries of the sequencing
416 results are given in table S5. Hemippe reads were additionally aligned to the kiang mitochondrial
417 genome (NC_016061.1) (60) using bwa aln and bwa mem. The resulting *E. h. hemippus*

418 mitochondrial genomes had a mean coverage of 52x (1864 hemippe), 40x (1892 hemippe) and 51x
419 (Göbekli Tepe GT64). Complete mitogenome sequences were generated by consensus calling of the
420 bases using Geneious (48). To obtain a full-length mitogenome, gaps were filled using both targeted
421 PCR data of the HVR (23) and by analyzing, at the boundary of the gaps, the soft clipped reads
422 resulting from mapping with bwa mem rather than bwa aln.

423 Nuclear SNPs were called from the Umm-el-Marra and hemippe bam files using the samtools (55)
424 *mpileup* command with the following parameters: -B -A -Q20 and specifying only the 15.5 million
425 SNP positions described above. Calling and selection of a single allele for all heterozygous sites were
426 performed using pileupCaller (61). This resulted in 6.8 million shared positions between extant equids
427 and the 1864 hemippe, 2.2 million shared with the 1892 hemippe, 10.9 million with the Göbekli Tepe
428 GT64 sample, and 45,604 with the Umm-el-Marra 9 sample (table S5).

429 Principal component analysis (PCA) was performed using EIGENSOFT SmartPCA (v16000) (62,
430 63) by projecting the samples with partial coverage onto eigenvectors calculated from all shared
431 positions of well covered equids (projectlsq: YES). For the PCA represented in Figs. 4A and S4 D-
432 E, we used the 15.2K SNPs shared between UMM9 and both the GT64 and the 1864 hemippe, and
433 only the 1892 hemippe was projected. For the PCA represented in Figs. S4 A-C, we used the 4.1M
434 SNPs shared between the extant equids used and both the GT64 and the 1864 hemippe, with both the
435 UMM9 and the 1892 hemippe being projected. Admixture (v1.3.0) (64) was used to estimate ancestry
436 of the six horses, six donkeys, three Mongolian khulans, two kiangs, the onager, the 1864 and the
437 11,000 BP GT64 hemippe and UMM9 using the 15.2K SNPs shared between UMM9 and both the
438 GT64 and the 1864 hemippe, and a four population model (K=4). Fig. 4B represents the admixture
439 bar graph obtained in 70% of the 30 iterations (90% showed the UMM9 sample as a 1:1 admixture
440 of onager/hemippe and donkey). The bifurcating tree with gene flow was performed using treemix
441 (31) with the 40.4K SNPs shared between UMM9 and either of the three hemippes, and considering
442 the following equid groups (number of individuals): Horse (6), Donkey (6), Hemippe (3), UMM9 (1),
443 Onager (1), Mongolian khulan (3) and Kiang (2). Horses were used as the outgroup and sample size

444 correction was disabled. The tree obtained with one migration/admixture event is represented in Fig.
445 4D and the residuals are plotted in Fig. S5. From these 40.4K SNPs, we identified those where all
446 donkeys are identical and all hemippes that have the corresponding positions covered are identical
447 and distinct from the donkeys (4738 SNPs) and counted the SNPs where the UMM9 SNPs are
448 identical to either the donkey- or the hemippe-specific SNPs. These counts are represented in Fig.
449 4C.

450 The genome phylogeny shown in Fig. S7 was obtained using the 738.5K SNPs shared between all
451 three hemippes and equids, after calculation of the pairwise distance matrix between all equids using
452 plink (56) and construction of the phylogenetic tree using fastme with nni optimization (65).

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610

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633 **Author contributions**

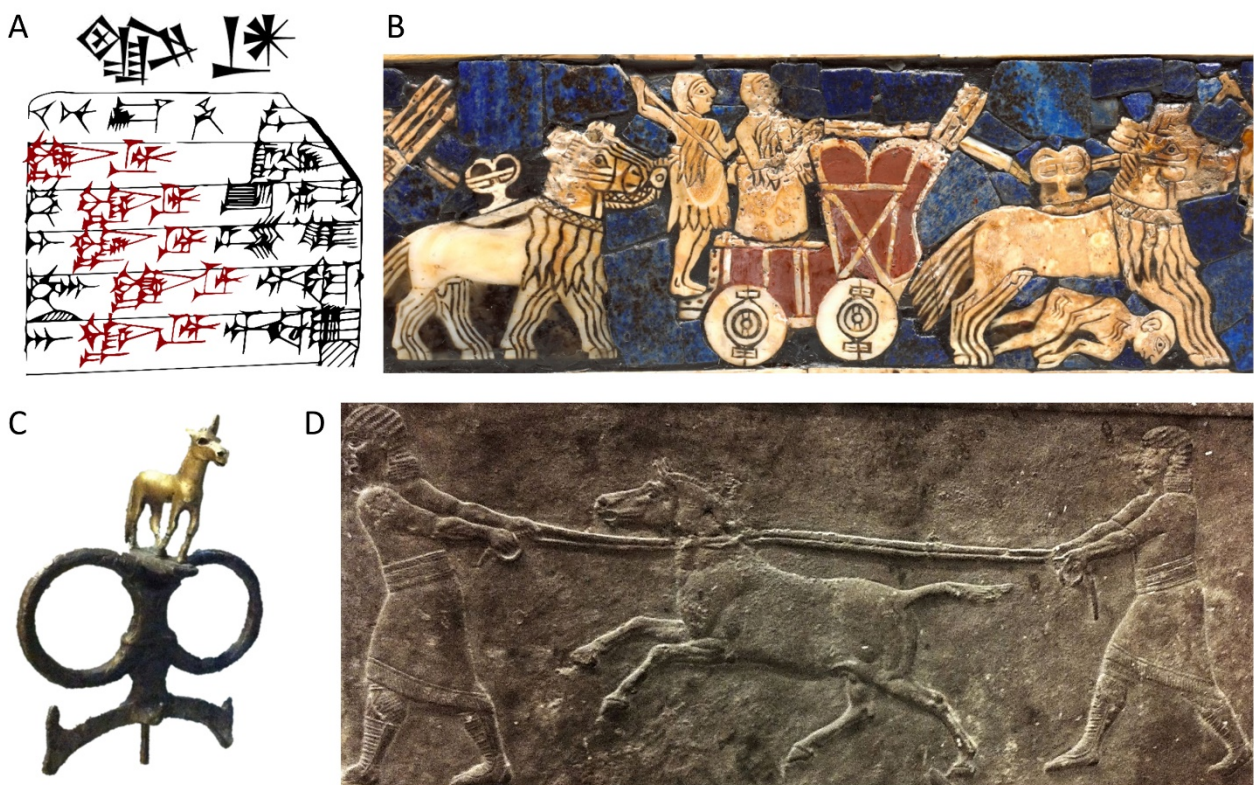
634 EMG and JW initiated the project; EMG and TG conceptualized and supervised the project; JW, JP
635 and GS provided material; EAB, WB, SC, EMG and TG performed the laboratory work; EAB, TG
636 and EMG analyzed the data; EAB, TG and EMG wrote the paper with input from JP and GS.

637 **Competing Interests.** The authors declare that they have no competing financial interests that might
638 have influenced the work described in this manuscript.

639 **Data availability**

640 Sequence data generated for this study are available from EBI European Nucleotide Archive
641 PRJEB47929. Syrian wild ass (hemippe) mitochondrial sequences are available on Genbank
642 MN990427, OK393913 and OK393914.

643 **Legends to Figures**

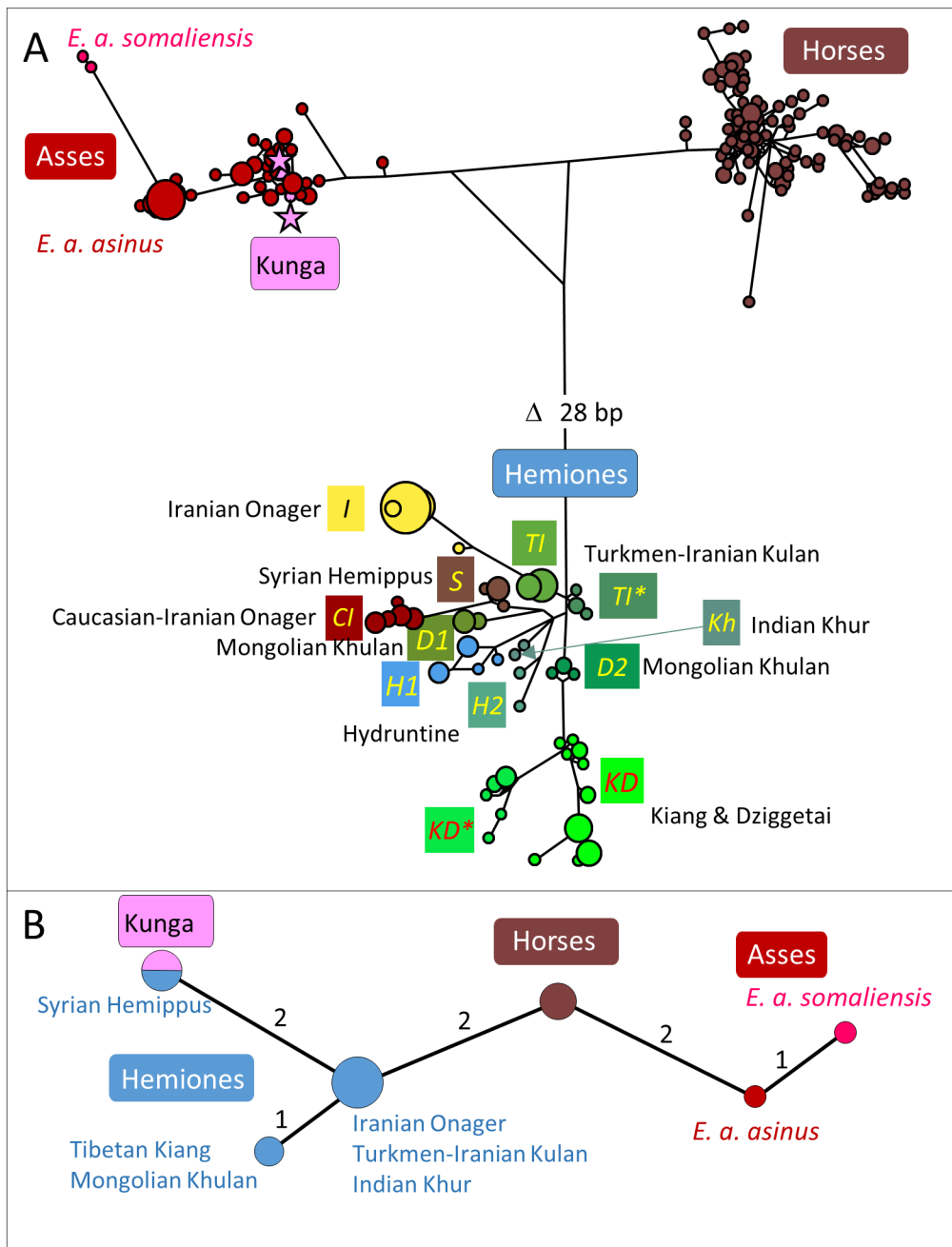


645 **Figure 1.** Iconographic and textual depiction of the kunga. (A) Third millennium BCE cuneiform
646 signs for the kunga (ANŠE.BAR×AN) above a photo and drawing of a clay tablet from UrIII
647 Girsu/Lagaš (British Museum BM23836) featuring multiple occurrences, highlighted in the
648 juxtaposed drawing. The first two lines read, “transmitted barley plots of 1 bur 6 iku (=8.64 ha) in
649 area, (for the keeping of) ANŠE.BARxAN — equids of the king” (drawing and translation courtesy

650 ©K. Maekawa). (B) Detail from the Standard of Ur shows an equid team pulling a four-wheeled
 651 wagon in battle (photo: ©The British Museum Images). (C) An image of a rein-ring with decorative
 652 equid from a royal grave at Ur, contemporary and similar to those visible in the Standard of Ur. (D)
 653 Nineveh panel: “hunting wild asses” (645-635 BCE) (British Museum, London). Fig. S8 shows
 654 additional panels attesting that the equids depicted are non-caballine. C and D, British Museum,
 655 London, photos: E. Andrew Bennett.

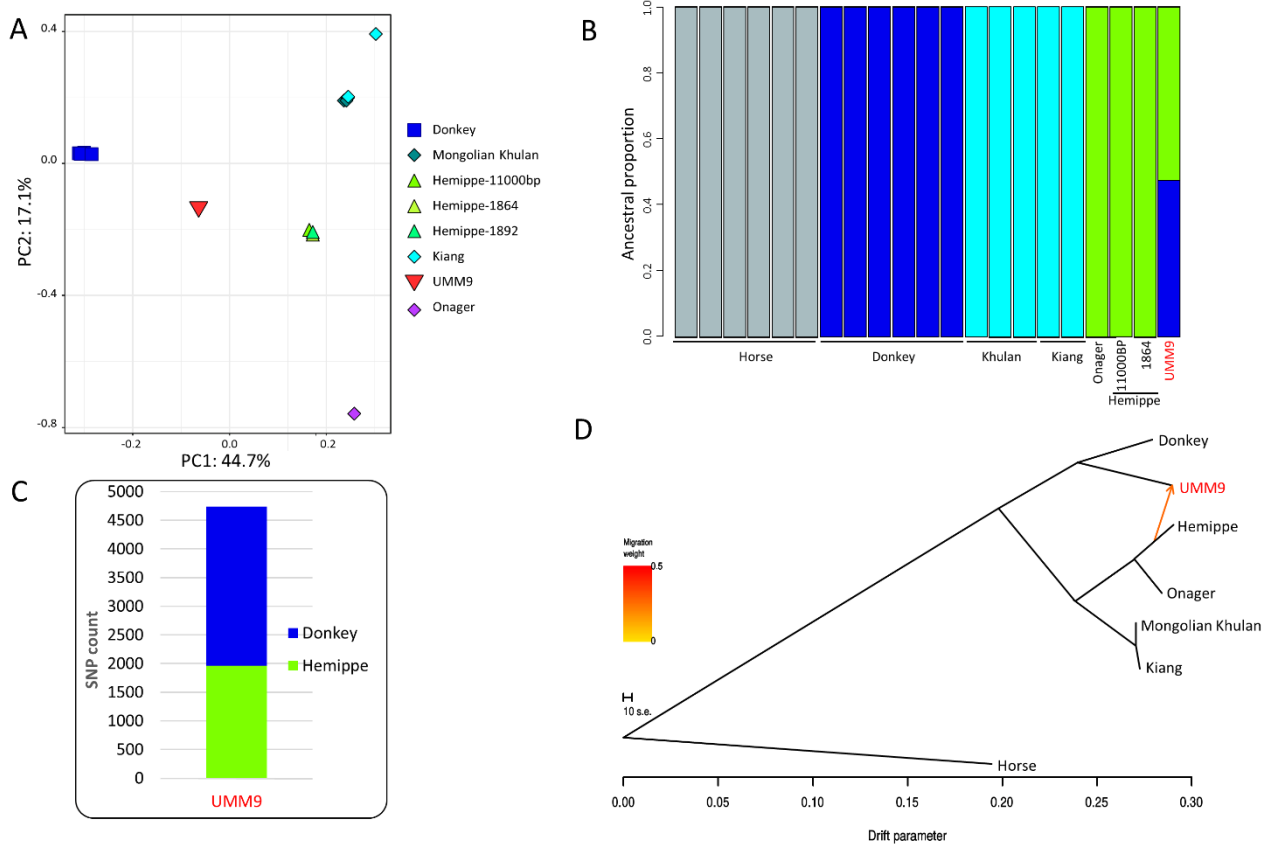


657 **Figure 2.** Map of 3rd millennium BCE Syro-Mesopotamia showing the major historical and
 658 archaeological sites (modified from Wikipedia
 659 https://fr.m.wikipedia.org/wiki/Fichier:Syrie_3mil_aC.svg). The insert shows a representative equid
 660 burial in Umm-el-Marra. Photo ©G. Schwartz.



662 **Figure 3.** Median joining network of equid sequences. (A) 324 bp of the hypervariable region of
 663 mitochondrial DNA from 278 individuals belonging to Asiatic wild asses (*E. hemionus* subspecies
 664 (23)), to horses (*E. f. caballus* and *E. f. przewalskii*) and to African asses (*E. africanus asinus* and *E.*
 665 *africanus somaliensis*). The position of the sequences obtained from the Umm el-Marra samples are
 666 indicated with pink stars. The *E. hemionus* mitogenome clades (I, TI, TI*, CI, H1, H2, D1, D2, Kh,
 667 KD, KD*) are as defined previously (23). (B) 168 bp of three different fragments of the Y-

668 chromosome of equids (asses, horses, hemiones). The position of the sequences obtained from the
 669 Umm-el-Marra samples are indicated in pink.



671 **Figure 4:** Genome-scale analyses reveal that the UMM9 equid shares equal ancestry from donkey
 672 and hemippe. (A) PCA plots of non-caballine equids. 15.2K SNPs shared between UMM9 the ca.
 673 11,000-year-old Göbekli Tepe sample and the 1864 hemippe were used and only the 1892 hemippe
 674 was projected. (B) Admixture (30) analysis modeling four populations based on 15.2K SNPs shared
 675 between UMM9 and the higher-coverage Göbekli Tepe and 1864 hemippe (used in the analysis). (C)
 676 Counts of the UMM9 equid SNPs that are identical to either hemippe or donkey using the 4738 SNPs
 677 where all donkeys are identical and differ from the hemippes (of 40.4K total SNPs shared between
 678 the UMM9 equid and either of the three hemippes). (D). Bifurcating tree of equids with gene flow
 679 performed using treemix (31). The 40.4K SNPs shared between UMM9 and either of the three
 680 hemippe were used. Each equid group is represented by the following numbers of individuals: Horse

681 (6), Donkey (6), Hemippe (3), UMM9 equid (1), Onager (1), Mongolian khulan (3) and Kiang (2).
682 Horses were used as the outgroup and sample size correction was disabled. The tree obtained with
683 one gene flow event is represented. The residuals with no or one gene flow event are plotted in Fig.
684 S5.

685 **Supplementary Material**

686 This PDF file includes:

687 Supplementary Materials & Methods

688 Supplementary Text: Archaeozoological analysis of the Equids of Umm el-Marra

689 Figs. S1 to S8

690 Tables S1-S5