GENETICS

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

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Before the introduction of domestic horses in Mesopotamia in the late third millennium BCE, contemporary cuneiform tablets and seals document intentional breeding of highly valued equids called kungas for use in diplomacy, ceremony, and warfare. Their precise zoological classification, however, has never been conclusively determined. Morphometric analysis of equids uncovered in rich Early Bronze Age burials at Umm el-Marra, Syria, placed them beyond the ranges reported for other known equid species. We sequenced the genomes of one of these ~4500-year-old equids, together with an ~11,000-year-old Syrian wild ass (hemippe) from Göbekli Tepe and two of the last surviving hemippes. We conclude that kungas were F1 hybrids between female domestic donkeys and male hemippes, thus documenting the earliest evidence of hybrid animal breeding.

INTRODUCTION

In the third millennium BCE, urbanized, socially stratified, and literate societies appeared for the first time in Syria and northern Mesopotamia (*1*, *2*). Part of this "second act" of the urban revolution was the breeding and employment of an equid of high status and prestige designated a "kunga." The precise taxonomical determination of the kunga and its identification in the archaeological record have been uncertain until now. Third millennium BCE cuneiform clay tablets from Syro-Mesopotamia describe several equids, using the generic term ANŠE associated with various logographs. Of these, the so-called kunga was represented by the cuneiform signs ANŠE.BARxAN (Fig. 1A) (*3*, *4*). Texts from the Diyala region in Mesopotamia and the kingdom of Ebla in the Levant state that the prices for these equids were considerable, costing up to six times the price of a donkey (*5*). References for these valuable equids are found in multiple clay tablets (*3*, *4*) (Fig. 1A) such as those detailing fodder expenses, e.g., barley for the equids of the god Shara and the deified king Shulgi from Umma (*6*), and dowries for royal marriages (*7*). Large-sized male kungas were used to pull the vehicles of "nobility and gods" (*6*), and their size and speed made them more desirable than asses for the towing of four-wheeled war wagons (*8*), which predate horse-pulled chariots. Smaller-sized male and female kungas were used in agriculture, where they were frequently reported pulling ploughs (*4*, *9*). Kunga foals were seldom born within the urban centers of Sumer and Syria, and Ebla purchased young kungas almost exclusively from what may have been the principal breeding center at Nagar (modern Tell Brak), in northern Mesopotamia, whose rulers also provided them as gifts to the elites of allied territories (*3*). Presumed kungas featured prominently on royal seals throughout the region (*10*), and images of these hybrids likely appear on both the "war" and "peace" panels of the standard of Ur, a Sumerian artifact excavated from the royal cemetery in the ancient city of Ur (in modern-day Iraq). In one of the first depictions (2600 BCE) of a military expedition in human history, warriors stand on four-wheeled war wagons, each drawn by a team of unspecified equids (Fig. 1B). An example of the rein ring featured in this image has been found in a royal grave at Ur (Fig. 1C), decorated with a small statue of a noncaballine equid, either a kunga or hemione. Kunga use and traditions decreased and eventually vanished following the introduction of domestic horses in the region (*9*, *11*). Early references to horses in cuneiform writing coincide with the Third Dynasty of Ur (late third millennium BCE), where they are referred to as anše-zi-zi and later anše-kur-ra (equids of the mountain) (*6*, *8*). An introduction of domestic horses in Mesopotamia by the end of the third millennium is also supported by paleogenetic data illustrating their late arrival in Anatolia around 2000 BCE, presumably through the Caucasus (*12*).

While the symbol for kunga (ANŠE.BARxAN) is used to describe a hybrid equid, the unambiguous assignment of this term to a species is difficult and controversially discussed. Some authors even argue that the kunga referred only to wild caught Persian onagers (also known as Iranian onagers; *Equus hemionus hemionus*, a subspecies of the Asiatic wild ass) rather than hybrid animals (*3*, *6*), although the difficulty in taming modern onagers, which are reportedly less tractable than zebras (*13*), does not support this interpretation. One of the likely parents of the kunga is the donkey (*Equus africanus asinus*), thought to be present in Sumer from at least the late fourth millennium BCE (*8*). The identity of the other parent, however, remains unclear. Another equid attested since the Early Dynastic period I (ca. 2800 BCE) is the anše-edin-na, literally translated as "equid of the desert." This animal was hunted for its meat and hide, but never used as a draught animal. The anše-edin-na is broadly considered to be a type of onager (*4*, *8*), although it is impossible to say whether it refers to the Persian onager (*Equus hemionus onager*) or to the Syrian wild ass, or hemippe (*Equus hemionus hemippus*) (*8*), sometimes also named "Syrian onager." Described as a light, swift animal (*14*), the hemippe was the smallest of all modern equids until the subspecies went extinct early in the 20th century (*11*). It

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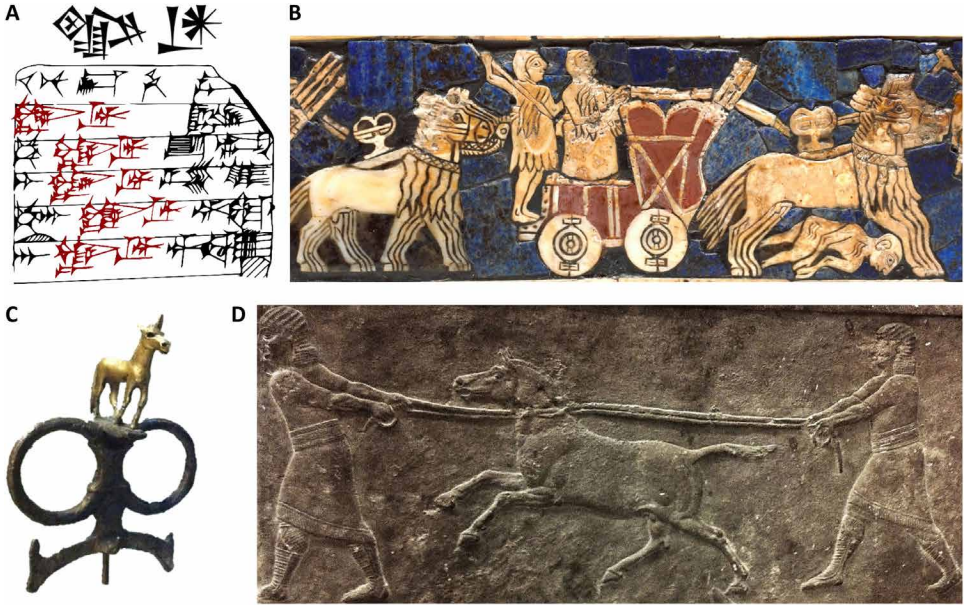

Fig. 1. Iconographic and textual depiction of the kunga. (**A**) Third millennium BCE cuneiform signs for the kunga (ANŠE.BARxAN) above a photo and drawing of a clay tablet from Urlll Girsu/Lagaš (British Museum BM23836) featuring multiple occurrences, highlighted in the juxtaposed drawing. The first two lines read "transmitted barley plots of 1 bur 6 iku (=8.64 ha) in area, (for the keeping of) ANŠE.BARxAN — equids of the king" (drawing and translation courtesy of K. Maekawa). (**B**) Detail from the Standard of Ur shows an equid team pulling a four-wheeled wagon in battle (photo credit: The British Museum Images). (**C**) Image of a rein ring with decorative equid from a royal grave at Ur, contemporary and similar to those visible in the Standard of Ur. (**D**) Nineveh panel: "hunting wild asses" (645 to 635 BCE) (British Museum, London). Figure S8 shows additional panels attesting that the equids depicted are noncaballine. (C and D) British Museum, London; photo credit: E. Andrew Bennett.

has been argued that in addition to its untamable, aggressive nature (*14*), its diminutive size made it an unlikely candidate for use in breeding kungas [(*3*) and references therein]. Some authors considered the nondonkey parent to be a horse [discussed in (*4*, *9*)].

In the elite burial complex of Tell Umm el-Marra (2600 to 2200 BCE), possibly belonging to the ancient city of Tuba, 55 km east of Aleppo in modern-day northern Syria (Fig. 2), men and women were interred with ceramics, bronze, and silver vessels; bronze weapons and tools; and personal ornaments made of bronze, silver, gold, and lapis lazuli (*15*). Within this royal burial complex, complete skeletons of 25 male equids and bones from six additional animals were buried separately from humans, either in a sequence of pits or in their own mud-brick structures (*15*), akin to the 3000 BCE donkey burials at Abydos, Egypt (*16*). While some animals were interred after natural deaths, more than half appear to have been deliberately killed for burial in the complex. Morphometric values obtained from these bones indicate that these animals constitute a population outside of the typical ranges of horses, asses, and onagers, and it has been proposed that these skeletons represent hybrids, presumably kungas [(*17*) and Supplementary Materials]. In absolute size, the skeletons are closer to hemiones, but are more robust; commonly used slenderness indices suggest greater affinities with asses than with hemiones. The leg characteristics of hemiones, responsible for a speed exceeding that of horses, is retained in these animals, suggesting that they were also fast (*18*). Discrepancies in wear between the incisors and cheek teeth of some of the equids indicate that the animals were foddered and not commonly grazed (*17*), features that would have been expected on the skeletons of the equids depicted on the standard of Ur, whose lip or nose rings would have made grazing difficult (Fig. 1B). These animals would have been stronger and faster than donkeys and must have been more tamable than hemiones (*19*).

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

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

RESULTS AND DISCUSSION

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

An initial polymerase chain reaction (PCR) screening of equid samples from Umm el-Marra showed that DNA was extremely poorly preserved in these bones owing to the hot climate in Syria, detrimental to long-term DNA preservation, and the poor condition of the bones (phalanges and sternum) available for study (fig. S1). Therefore, we combined shotgun nuclear DNA sequencing with

Fig. 2. Map of third millennium BCE Syro-Mesopotamia showing the major historical and archaeological sites (modified from Wikipedia https://fr.m.wikipedia.org/wiki/Fichier:Syrie_3mil_aC.svg). The insert shows a representative equid burial in Umm el-Marra. Photo credit: G. Schwartz.

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

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

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

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

To further establish the hybrid identity of these equids, we sequenced a subset of the nuclear genome of the best preserved Umm el-Marra equid bone (table S5). In addition, we established the genome sequence of the extinct Syrian hemippe by sequencing a ca. 11,000-year-old wild ass from the early Neolithic site of Göbekli Tepe, present-day Turkey, representing the first temple (*25*), and two 19th century specimens from the Schönbrunn Zoo (table S5). These four newly generated genomes were compared to six modern horse (*26*), six domestic donkey (*27*), three Mongolian khulans (an *E. hemionus* subspecies from the Gobi) (*27*, *28*), two kiang genomes (*E. h. kiang* or *E. kiang*) (*27*, *29*), and one Persian onager genome (an *E. hemionus* subspecies from Iran) (*29*) (table S4). A set of 15.5 million SNPs residing outside of repeated sequences and being variable in the modern genome equid panel was used for calling the ancient genomes (see Supplementary Materials and Methods and table S5). Although the best Umm el-Marra extract contained only 0.18% endogenous DNA, we could obtain 45.6K SNPs, 40.4K of which were shared with either hemippe. Of these, 15.2K SNPs (37%) were shared with both of the two best-covered hemippe

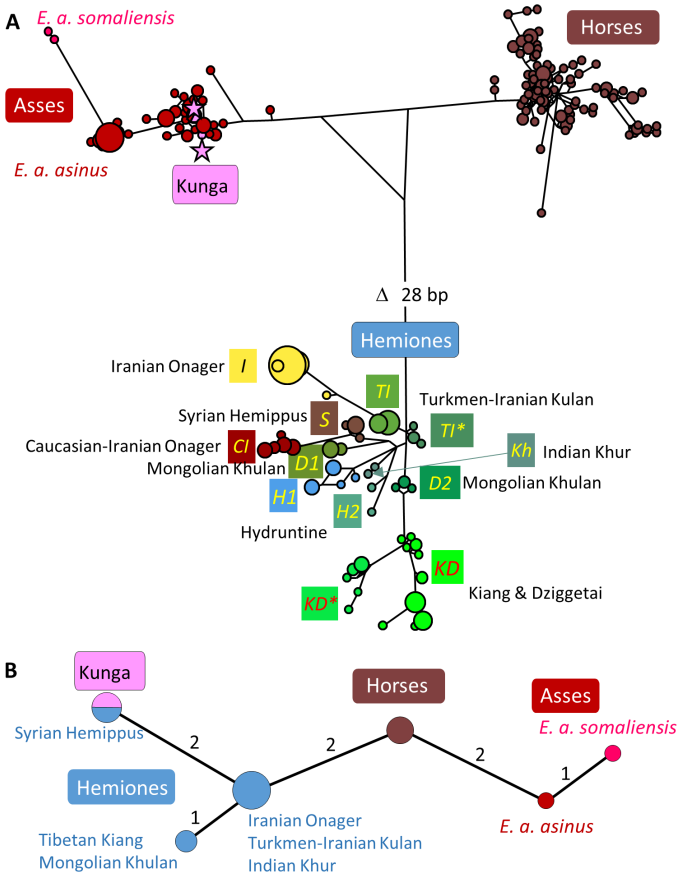

Fig. 3. Median-joining network of equid sequences. (**A**) Hypervariable region (324 bp) of mitochondrial DNA from 278 individuals belonging to Asiatic wild asses [*E. hemionus* subspecies (*23*)], to horses (*E. f. caballus* and *E. f. przewalskii*), and to African asses (*E. a. asinus* and *E. a. somaliensis*). The position of the sequences obtained from the Umm el-Marra samples is indicated with pink stars. The *E. hemionus* mitogenome clades (I, TI, TI*, CI, H1, H2, D1, D2, Kh, KD, and KD*) are as defined previously (*23*). (**B**) Three different fragments (168 bp) of the Y chromosome of equids (asses, horses, and hemiones). The position of the sequences obtained from the Umm el-Marra samples is indicated in pink.

genomes. First, we performed principal components analysis (PCA) to compare (i) the hemione and donkey genomes (Fig. 4A and fig. S4A) or (ii) hemione, donkey, and horse genomes (fig. S4, B to E). Identical results were obtained whether we used the 15.2K SNPs shared between the Umm el-Marra equid (UMM9), the Göbekli Tepe, and the 1864 hemippe, or whether we used the 4.1 million SNPs shared between the Göbekli Tepe and the 1864 hemippe genome and projected the UMM9 equid onto the PCA [compare Fig. 4A and fig. S4 (A to E)], showing that the 15.2K SNPs obtained allowed robust characterization of the status of the Umm el-Marra equid. When only donkey and hemione genomes are used, PC1 separates donkeys from hemiones and PC2 separates the hemiones (Fig. 4A and fig. S4A). The most differentiated in PC2 are the Persian onager and the kiang zoo specimens. The Mongolian khulan and the kiang from neighboring regions in China are very closely related and overlapping in the PCA, which is in accordance with the shared mitochondrial lineages we reported previously that led us to question the specific taxonomic status of the kiang as a separate species (*23*) (see also the phylogenetic trees of the mitogenomes and the genomes in figs. S6 and S7). The two modern hemippes and the Göbekli Tepe sample overlap as well (the lower coverage 1892 hemippe was projected) and are located at an intermediate position in PC2. The Göbekli Tepe sample, a mare, is an ancient hemippe as observed from the phylogenetic trees constructed from both mitochondrial and nuclear genomes (figs. S6 and S7). The UMM9 equid falls exactly halfway between the donkeys and the hemippes. When the PCA also includes the horses, PC1 differentiates the horse from the noncaballine equids, PC2 separates the donkeys from the hemiones, and PC3 separates the hemiones in a similar way as PC2 does when horses are not included (fig. S4, B to E). In all analyses, the results for the UMM9 equid illustrate an intermediate position between the donkeys and hemippes. The PCA analyses thus indicate that the UMM9 genome is a 50% mixture of donkey and hemippe.

We further explored this outcome through ADMIXTURE analysis (Fig. 4B) (*30*). A four-population model separates horses, donkeys, Mongolian khulans, and kiangs from onagers and the two best-covered hemippes. The UMM9 equid is modeled as an admixture of equal proportion between donkey and hemippe/onager (Fig. 4B). Likewise, when considering the 4738 UMM9 equid SNPs for which all six donkeys differ from all hemippes that have the position covered, the UMM9 equid harbors SNPs corresponding to roughly half of those specific to each putative parent (Fig. 4C).

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

Expectedly, the 19th to 20th century hemippes, representing some of the last survivors of the subspecies, are genetically similar, whereas the ~11,000-year-old Göbekli Tepe hemippe is more divergent (fig. S7). We also noted that the divergence between the three sequenced hemippes is much larger than that observed between the six domestic donkeys (fig. S7). The higher diversity between the sequenced hemippe genomes versus between the donkey genomes suggests that the donkey mother of the UMM9 equid is more closely related to present-day donkeys than the Syrian hemippe father of

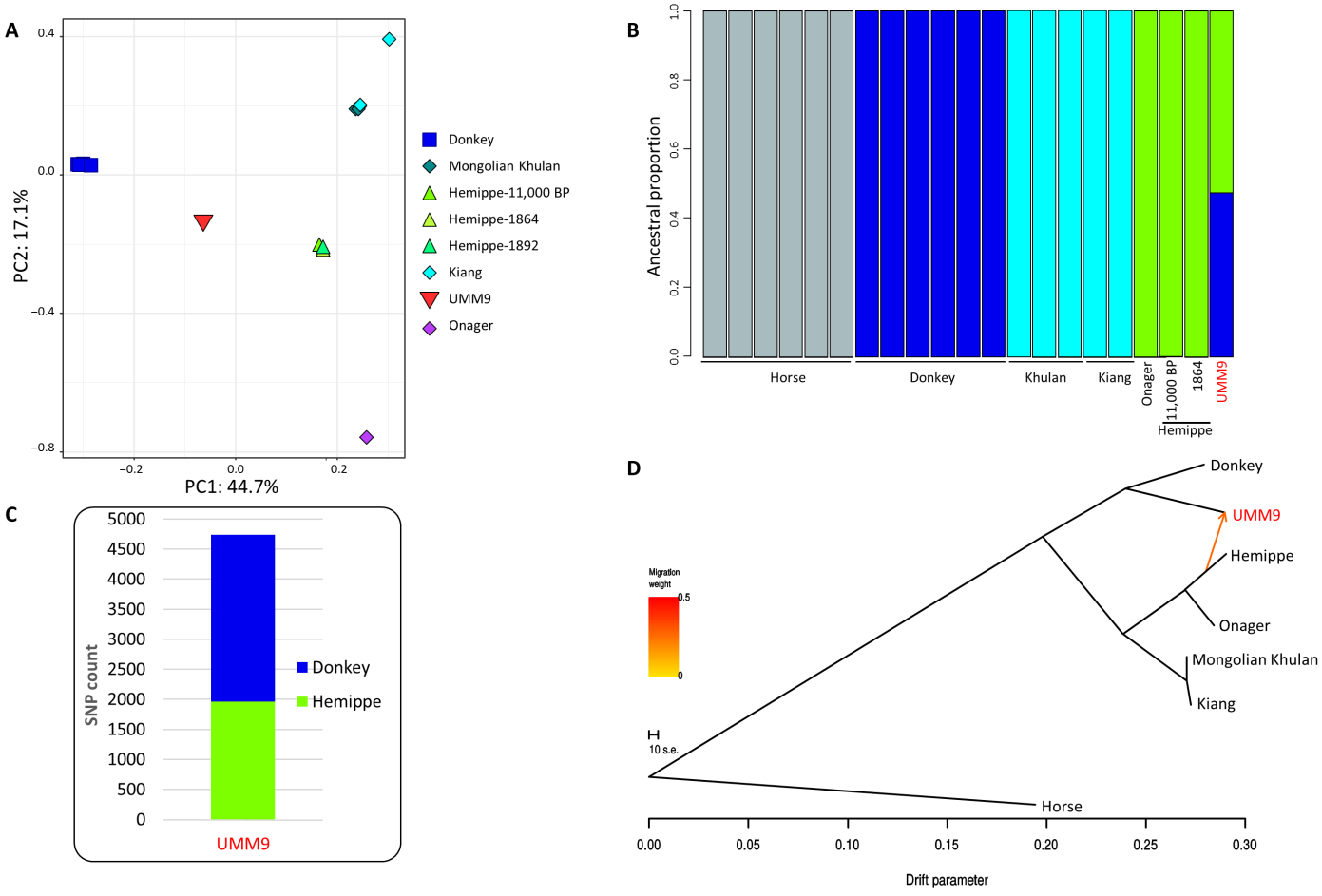

Fig. 4. Genome-scale analyses reveal that the UMM9 equid shares equal ancestry from donkey and hemippe. (**A**) PCA plots of noncaballine equids. The 15.2K SNPs shared between UMM9, the ca. 11,000-year-old Göbekli Tepe sample, and the 1864 hemippe were used, and only the 1892 hemippe was projected. (**B**) Admixture (*30*) analysis modeling four populations based on 15.2K SNPs shared between UMM9 and the higher-coverage Göbekli Tepe and 1864 hemippe (used in the analysis). (**C**) Counts of the UMM9 equid SNPs are identical to either hemippe or donkey using the 4738 SNPs, where all donkeys are identical and differ from the hemippes (of 40.4K total SNPs shared between the UMM9 equid and either of the three hemippes). (**D**) Bifurcating tree of equids with gene flow performed using treemix (*31*). The 40.4K SNPs shared between UMM9 and either of the three hemippes were used. Each equid group is represented by the following numbers of individuals: horse (six), donkey (six), hemippe (three), UMM9 equid (one), onager (one), Mongolian khulan (three), and kiang (two). Horses were used as the outgroup, and sample size correction was disabled. The tree obtained with one gene flow event is represented. The residuals with no or one gene flow event are plotted in fig. S5.

the UMM9 equid is to the other sequenced hemippes. This difference, albeit small, may account for the slightly higher affinity of the UMM9 equid to present-day domestic donkeys that is visible on fig. S5 (B and D). Both the genomic phylogenetic tree (fig. S7) and the PCA analyses (Fig. 4B and fig. S4) indicate that differentiation between the donkey genomes is low, far less than between the various present-day hemiones, presumably because donkeys went through a major bottleneck, possibly upon domestication and translocation to southwest Asia outside the range of the ancestors of donkeys. The observation that the ~4500-year-old UMM9 equid appears more closely related to present-day donkeys than to the last hemippe that disappeared a century ago suggests that the bottleneck of the donkey population had already taken place by the third millennium BCE.

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

Pleistocene and Holocene sites in Mesopotamia (*33*) or Anatolia (*34*). Nearly 2000 years after the equid burials of Umm el-Marra, sixth century BCE palace reliefs featuring hunted hemiones from Nineveh (in modern-day northern Iraq) show already relatively small animals (Fig. 1D and fig. S8).

To conclude, the genomic results from the rare equid burials at the elite mortuary complex of Umm el-Marra confirm earlier hypotheses based on morphological data that these animals are hybrids (*8*, *9*, *17*) and, given their interment in high-status tombs, are most likely identical with the valuable kungas frequently mentioned in cuneiform texts and depicted in images and royal seals throughout Mesopotamia. This study now offers a firm zoological classification of the historical kunga as an F1 cross between a female donkey and a male Syrian wild ass, or hemippe, putting to rest past speculations regarding the taxonomic identification of the BARxAN. We further show that the third millennium BCE ancestors of the hemippe were likely larger than those first described by European travelers visiting Syria in the 19th century. Our study also presents the earliest known case in human history of interspecies hybridization, which was practiced by Early Bronze Age breeders at sites such as Nagar (Tell Brak) (Fig. 2), to generate animals famous for their power, both physical and symbolic, in ancient warfare and diplomacy. This result also deepens our insight into the economic and political relationships between contemporary royal households of Greater Mesopotamia, and the dynamics by which these social elites fostered distant alliances. It also increases our understanding of the ways in which the earliest stratified urban societies of the Middle East developed and maintained their positions of authority. In this respect, genomic characterization of additional equids from comparable contexts, particularly from Nagar, may help clarify the scale of hybrid breeding in third millennium BCE Mesopotamian societies before the introduction of domestic horses.

MATERIALS AND METHODS

Sample description

Bone remains from equid skeletons dated between ca. 2550 and 2300 BCE and excavated in 2006 at Tell Umm el-Marra, a Bronze Age elite cemetery in northern Syria (*15*, *17*, *36*), were sampled for ancient DNA analysis. Further descriptions of the samples are given in the Supplementary Materials, and photos of the samples from the two individuals from which sufficient DNA was recovered appear in fig. S1. A petrous bone excavated from a layer dated between 9500 and 8300 BCE from the site of Göbekli Tepe in southeast Turkey [(*25*) and Supplementary Materials] was also analyzed in the present study. Furthermore, we analyzed two samples of the extinct *E. h. hemippus* originating from the desert of Aleppo in Syria and kept in the zoo of Schönbrunn, Vienna, Austria, a tooth from the NMW6048/ST345 specimen and a hair and skin sample from the NMW1308/B4690 specimen, corresponding to animals who died in the Schönbrunn zoo in 1864 and 1892, respectively (fig. S2) (*14*). Last, hair of a male Somalian ass (*E. africanus somaliensis*) from the "Réserve Africaine de Sigean" (Sigean, France) was provided for the analysis of the Y chromosome by E. Trunet (sample "As.Somalie").

Ancient DNA extraction, amplification, and sequencing

Hair samples were added to 1.5 ml of hair digestion buffer [100 mM tris-HCl (pH 8.0), 100 mM $NaCl_2$, 40 mM dithiothreitol, 3 mM $CaCl_2$, 2% *N*-lauryl sarcosyl, and proteinase K (250 μg/ml)] and incubated 4 to 24 hours at 50°C, shaken at 300 rpm. Solutions were then pelleted, and the supernatant was purified using a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) according to instructions.

DNA from archaeological bone samples was extracted, purified, and prepared for either quantitative PCR (qPCR) or sequencing in the ancient DNA laboratory described previously (*35*, *37*, *38*). Bone cleaning and treatment protocols were as described previously (*12*, *23*). Briefly, after removal of the surface with a razor blade or surface cleaning with bleach, the bones were either sawed using a flame-sterilized diamond disc of Dremel Fortiflex (Dremel Europe, The Netherlands) and grounded to fine powder in 6775 Freezer/MillSpex SamplePrep in liquid nitrogen or drilled at low speed with a flame-sterilized bit. The dense pyramidal part of the petrous bone GT64 was isolated using a flame-sterilized diamond disc of a Dremel and then grounded to fine powder in 6775 Freezer/MillSpex SamplePrep in liquid nitrogen. Half of the GT64 powder was treated with diluted hypochlorite (1:20), and both halves were washed with phosphate buffer according to Korlević *et al.* (*39*). DNA extraction was performed by incubating the bone powder at 37°C for 48 to 90 hours either in 1- to 10-ml extraction buffer A [0.5 M EDTA, 0.25 M PO_4^{3-} (pH 8.0), and 0.14 M ß-mercaptoethanol] or in twice 1-ml extraction buffer B [0.5 M EDTA, 0.05% Tween 20, proteinase K (250 μg/ml), and 0.14 M ß-mercaptoethanol] that was pooled before purification. Samples were purified using silica membrane spin columns (QIAquick Gel Extraction kit) with a vacuum manifold (Qiagen) and 25 ml of extenders (Qiagen) as described (*37*, *40*), as well as with either the 5 M guanidine HCl, 40% isopropanol (5M40) buffer as described by Dabney *et al.* (*41*) or the 2 M guanidine HCl, 70% isopropanol (2M70) buffer as described by Glocke and Meyer (*42*). The elution was performed twice in 25 μl of 10 mM tris-HCl (pH 8.0) and 0.05% Tween 20 (referred to as EBT) made from gamma-irradiated water (8 kGy).

Purified DNA was amplified by qPCR, the extract making up 5 to 20% total volume (10 to 20 μl per reaction). Inhibition characteristics were determined for failed samples indicating possible inhibition, and once optimal dilutions were determined, qPCR was attempted again. To protect against cross-contamination, the UQPCR [uracil *N*-glycosylase (UNG)–coupled quantitative PCR] method was used (*35*, *38*, *43*), in which uridine was substituted for thymidine in all PCRs, and incubation with UNG (extracted from *Gadus morhua*; Biotec Marine Biochemicals, Norway) was performed before each reaction. Mock extracts were included with each extraction and amplified to control for contamination. qPCRs varied slightly depending on the sample, but a typical reaction included 1.77 μl of LC FastStart DNA MasterPLUS mix1b; 0.23 μl of either FastStart DNA MasterPLUS mix1b, mix1a, or FastStart Taq (Roche Applied Science, Mannheim, Germany); a final concentration of 1 μM of each primer; and 1 U per reaction of UNG in 10-μl total volume. Primers were obtained from Sigma-Aldrich (St. Louis, USA). Mitochondrial primers were designed to amplify 357 bp of the hypervariable region (HVR) of *E. africanus* and *E. hemionus* mitochondria using short, overlapping fragments (table S3). Y-chromosome primers were designed to amplify three short sections of Y-chromosome DNA containing the target SNPs (tables S1 to S3). Several modifications of these primers were designed to increase sensitivity of qPCRs by minimizing the likelihood of primer dimers and artifacts and increasing primer efficiency. A list of primers used and product sizes is given in table S3. qPCR was performed using LightCycler 1.5 or LightCycler 2 (Roche Applied Science, Mannheim, Germany). qPCR programs varied depending on primer requirements and product length, but a typical program involved UNG incubation

at 37°C for 15 min, followed by polymerase activation at 95°C for 5 min, then two-step cycles of denaturation at 95°C for 10 s, then primer annealing and extension at 62°C for 40 s, and finally a temperature increase of 0.1°C/1 s from 62° to 95°C with continuous fluorescence measurement to generate melt curves of the products. Products were purified with a QIAquick PCR Purification kit (Qiagen, Hilden, Germany), and both strands were sequenced by capillary electrophoresis at Eurofins/MWG Operon (Ebersberg, Germany) using the ABI 3730xl DNA Analyzer (Life Technologies). Samples that yielded sequence results for the Y-chromosome are shown in table S1. An average of one nontemplate control (NTC) was run for every 6.6 samples (including mocks). No DNA was amplified in either NTCs or mocks, demonstrating that no detectable equid DNA was introduced during sample preparation or was present in reagents.

Samples from the two individuals from Umm el-Marra with the best preserved DNA identified via qPCR (UMM4 and UMM9) were selected for shotgun sequencing, and 24 double-stranded libraries using dual barcodes were prepared from DNA purified from three or four different areas of each bone using the protocol described by Massilani *et al.* (*44*). Seven of these libraries were treated with UNG to reduce the presence of cytosine deamination damage in the resulting sequences.

Double-stranded libraries of the two *E. h. hemippus* (hemippe) museum specimens were constructed using the NxSeq ampFREE Low DNA Library Kit (Lucigen, Middleton, WI, USA) following the protocol and the modifications described by Bennett *et al.* (*45*). Barcodes were added during an amplification reaction using dual-barcoded single-stranded library adapters (*46*) as primers, rather than those in the kit, where 20 µl of eluted library was added to 25 µl of OneTaq 2× Master Mix (Roche) and 0.6 µM of each adapter for 50-µl total volume, and amplified with the following protocol: 5 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 60°C, and 45 s at 68°C, followed by a 5-min cycle at 68°C. A library for a hair and skin sample belonging to specimen NMW5493/B 3625 was constructed using the NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA).

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

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

All libraries were quantified with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific), with Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA), and by qPCR. Screening by shotgun sequencing of Umm el-Marra samples and of the two hemippe samples was performed on an Illumina MiSeq system using a v3 reagent kit for 2 × 75 cycles. The libraries constructed from sample SP345, which came from a molar belonging to specimen NMW6048/ST345 (1864 hemippe) and a hair and skin sample belonging to specimen NMW1308/B4690 (1892 hemippe), and the two libraries from the UMM9 sample purified using the 5M40 and 2M70 buffers were selected for deep genomic sequencing.

Sequencing of the 1864 hemippe genome was performed on Illumina NextSeq using NextSeq 500/550 High Output Kit v2 (2 × 75 cycles). The custom sequencing primer CL72 (*46*) was substituted for the read 1 primer sequencing steps, which is compatible with the single-stranded adapters used for these samples. Sequencing of the 1892 hemippe and of two of the UMM9 libraries was performed first on Illumina MiSeq using a v3 reagent kit for 2 × 75 cycles and then on Illumina NovaSeq 6000 using an S2 flow cell for 2 × 50 cycles. Sequencing of the GT64 libraries was performed on NovaSeq 6000 using an S4 flow cell for 2 × 75 cycles.

Paleogenetic data analyses

Sequences from PCRs were manually curated, assembled, and aligned using the Geneious software suite (*47*). Median-joining network analysis (*24*) was performed on mitochondrial sequences covering 357 bp of the hypervariable region generated in this study by PCR combined with those previously reported (*23*) (accession numbers given in table S2) and Y-chromosome sequences generated in this study combined with those publicly available (samples and sources shown in table S1). Maximum likelihood (ML) analyses of the complete mitochondria of the two hemippes combined with donkey and hemione complete mitochondria after deletion of the 11-bp tandem repeat in the HVR were computed using RAxML (*48*) with a generalised time reversible (GTR) nucleotide substitution model, a gamma-distributed rate of variation among sites with four rate categories, and invariant sites (i.e., GTR-GAMMA-I) (fig. S6). We used 100 bootstraps to estimate node robustness.

Genomic analyses

Fastq reads from six modern horse genomes (*24*), six domestic donkey genomes (*25*), three Mongolian khulan genomes (an *E. hemionus* subspecies from the Gobi) (*25*, *26*), two kiang genomes (*E. h. kiang* or *E. kiang*) (*25*, *27*), and one Persian onager genome (an *E. hemionus* subspecies from Iran) (*27*) (table S4) were trimmed with cutadapt (v1.18) (*49*) and aligned to the *E. caballus* reference genome (eqCab2.0) using the BWA (v0.7.17) (*50*) mem program. PCR duplicates were removed using Picard MarkDuplicates (v2.20.0) (*51*), and reads aligning to the reference genome with mapping quality score below 30 were removed using samtools 1.9 (*52*).

We curated the 36 million biallelic variant list used to differentiate equids in the Zonkey workflow (*53*) to filter out variants found in repeated sequences using an EqCab2 genome repeat mask downloaded from the UCSC browser (https://genome.ucsc.edu/cgi-bin/hgTables). The rationale for this filtration was that these variants would be less reliably called using the short, damaged reads typical of ancient DNA libraries, in particular, when mapping reads from a noncaballine equid to the horse reference genome because these genomes are expected to differ markedly in repeat location and sequence variability. This filtration reduced the variant list to 22 million. We then called variants from this list on the modern equid genomes using bcftools (v1.9) (*54*) mpileup -B -q30 -Q30 and bcftools call -m. The vcf file was imported in plink (v1.9) (*55*) and filtered to include only SNPs, removing invariant and multiallelic positions. The final curated list contains 15.5 million SNPs.

Shotgun reads for the Umm el-Marra (UMM9) and Göbekli Tepe (GT64) samples were merged with leeHom (*56*) using the ancientdna option, while the historical hemippe reads were trimmed with cutadapt (v1.18) (*49*). Fragments smaller than 28 bp were discarded, and the remaining reads were aligned to the *E. caballus* reference genome (eqCab2.0) using the BWA (v0.7.17) (*50*) aln program with parameters "-n 0.01 -l 0" followed by samse (UMM9 and GT64) or sampe (hemippe). PCR duplicates were removed

using Picard MarkDuplicates (v2.20.0) (*51*), and reads aligning to the reference genome below a mapping quality score of 20 and a length of 28 bp were removed. To reduce the increase in spurious alignments from shorter reads described in (*57*), mapped reads less than 35 bp containing indels were also removed using an awk script. The ancient nature of the UMM9 and GT64 sequences was confirmed by analyzing the damage profile using mapDamage2 (*58*) of libraries generated from extracts not treated with USER-enzyme (fig. S3). To remove the C->T mutations at the end of the molecules that escaped the USER treatment (fig. S3), the base quality was rescaled at the last two bases using mapDamage2 (*58*). Since all Umm el-Marra samples had very low levels of endogenous equid DNA (0 to 0.18% of reads), they were additionally aligned to the human and bovine genome reference sequence (GRCh37 and ARS_UCD1.2, respectively). Only libraries that had at least fivefold more reads mapping to the horse than to the cow or human genome when a seed length of 18 was used during bwa aln mapping were kept. Summaries of the sequencing results are given in table S5. Hemippe reads were additionally aligned to the kiang mitochondrial genome (NC_016061.1) (*59*) using bwa aln and bwa mem. The resulting *E. h. hemippus* mitochondrial genomes had a mean coverage of 52× (1864 hemippe), 40× (1892 hemippe), and 51× (Göbekli Tepe GT64). Complete mitogenome sequences were generated by consensus calling of the bases using Geneious (*47*). To obtain a full-length mitogenome, gaps were filled using both targeted PCR data of the HVR (*23*) and by analyzing, at the boundary of the gaps, the soft clipped reads resulting from mapping with bwa mem rather than bwa aln.

Nuclear SNPs were called from the Umm el-Marra and hemippe bam files using the samtools (*54*) mpileup command with the following parameters: -B -A -Q20 and specifying only the 15.5 million SNP positions described above. Calling and selection of a single allele for all heterozygous sites were performed using pileupCaller (*60*). This resulted in 6.8 million shared positions between extant equids and the 1864 hemippe, 2.2 million shared with the 1892 hemippe, 10.9 million with the Göbekli Tepe GT64 sample, and 45,604 with the UMM9 sample (table S5).

PCA was performed using EIGENSOFT SmartPCA (v16000) (*61*, *62*) by projecting the samples with partial coverage onto eigenvectors calculated from all shared positions of well-covered equids (projectlsq: YES). For the PCA represented in Fig. 4A and fig. S4 (D and E), we used the 15.2K SNPs shared between UMM9 and both the GT64 and the 1864 hemippe, and only the 1892 hemippe was projected. For the PCA represented in fig. S4 (A to C), we used the 4.1M SNPs shared between the extant equids used and both the GT64 and 1864 hemippe, with both the UMM9 and 1892 hemippe being projected. Admixture (v1.3.0) (*30*) was used to estimate ancestry of the six horses, six donkeys, three Mongolian khulans, two kiangs, the onager, the 1864 and 11,000-year-old GT64 hemippe, and UMM9 using the 15.2K SNPs shared between UMM9 and both the GT64 and 1864 hemippe and a four-population model ($K = 4$). Figure 4B represents the admixture bar graph obtained in 70% of the 30 iterations (90% showed the UMM9 sample as a 1:1 admixture of onager/hemippe and donkey). The bifurcating tree with gene flow was performed using treemix (*31*) with the 40.4K SNPs shared between UMM9 and either of the three hemippes, and considering the following equid groups (number of individuals): horse (six), donkey (six), hemippe (three), UMM9 (one), onager (one), Mongolian khulan (three), and kiang (two). Horses were used as the outgroup, and sample size correction was disabled. The tree obtained with one migration/admixture event is represented in Fig. 4D, and the residuals are plotted in fig. S5. From these 40.4K SNPs, we identified those where all donkeys are identical and all hemippes that have the corresponding positions covered are identical and distinct from the donkeys (4738 SNPs) and counted the SNPs where the UMM9 SNPs are identical to either the donkey- or hemippe-specific SNPs. These counts are represented in Fig. 4C.

The genome phylogeny shown in fig. S7 was obtained using the 738.5K SNPs shared between all three hemippes and equids, after calculation of the pairwise distance matrix between all equids using plink (*55*) and construction of the phylogenetic tree using fastme with nni optimization (*63*).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abm0218

View/request a protocol for this paper from *Bio-protocol*.

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