

The first complete genome of the extinct European wild ass (*Equus hemionus hydruntinus*)

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Abstract

We present palaeogenomes of three morphologically unidentified Anatolian equids dating to the first millennium BCE, sequenced to a coverage of 0.6–6.4x. Mitochondrial DNA haplotypes of the Anatolian individuals clustered with those of *Equus hydruntinus* (or *Equus hemionus hydruntinus*), the extinct European wild ass, secular name 'hydruntine'. Further, the Anatolian wild ass whole genome profiles fell outside the genomic diversity of other extant and past Asiatic wild ass (*E. hemionus*) lineages. These observations suggest that the three Anatolian wild asses represent hydruntines, making them the latest recorded survivors of this lineage, about a millennium

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later than the latest observations in the zooarchaeological record. Our mitogenomic and genomic analyses indicate that *E. h. hydruntinus* was a clade belonging to ancient and present-day *E. hemionus* lineages that radiated possibly between 0.6 and 0.8 Mya. We also find evidence consistent with recent gene flow between hydruntines and Middle Eastern wild asses. Analyses of genome-wide heterozygosity and runs of homozygosity suggest that the Anatolian wild ass population may have lost genetic diversity by the mid-first millennium BCE, a possible sign of its eventual demise.

KEYWORDS

ancient DNA, Asiatic wild ass, demography, *Equus hemionus hydruntinus*, population genetics, taxonomy

1 | INTRODUCTION

Since its palaeontological description more than a century ago (Regalia, 1907), the 'European wild ass', *Equus hydruntinus* (or *Equus hemionus hydruntinus*), has remained an enigmatic taxon (Bennett et al., 2017; Boulbes & van Asperen, 2019; Burke et al., 2003; Geigl & Grange, 2012; Orlando et al., 2006). This species, which hereinafter we will name the hydruntine, was a gracile non-caballine equid, once roamed open habitats in Europe and Southwest Asia (Figure 1a) and featured in Upper Palaeolithic cave art (Bennett et al., 2017; BernÁldez-Sánchez & García-Viñas, 2019; Cleyet-Merle & Madelaine, 1991) and on Neolithic pottery (Bennett et al., 2017). Its history in the fossil record starts with the Late Middle or Late Pleistocene and ends within the Middle/Late Holocene, when it goes extinct (Boulbes & van Asperen, 2019; Crees & Turvey, 2014; Geigl & Grange, 2012).

Early research in the 20th century comparing *E. hydruntinus* remains with those of other equids identified resemblances to diverse taxa, including the African asses (*E. asinus*) and the zebra (*E. zebra*), the Asiatic asses (*E. hemionus*), or the extinct stonine horses, leaving its phylogenetic position disputed for many decades (Azzaroli, 1991; Davis, 1980; Eisenmann & Baylac, 2000; Eisenmann & Mashkour, 2000; Forsten & Ziegler, 1995; Stehlin & Graziosi, 1935). Within the last two decades, osteological studies on new fossil findings concluded that *E. hydruntinus* was systematically closer to extant Asian asses, that is, hemionines, than to African asses or zebras (Burke et al., 2003; Eisenmann & Mashkour, 1999; Orlando et al., 2006) (Figure 1a). Ancient DNA analyses of mitochondrial DNA (mtDNA) supported this conclusion: partial and complete mtDNA sequences from *E. hydruntinus* and extant hemionines (the kiang of Tibet; the kulan of Mongolia; the kulan of Turkmenistan; the onager of Iran) were found to cluster together to the exclusion of other equids (Bennett et al., 2017; Catalano et al., 2020; Orlando et al., 2006, 2009). Moreover, these mtDNA studies suggested that the hemione – hydruntine division may represent taxonomic over-splitting (Bennett et al., 2017; Orlando et al., 2006, 2009). Bennett et al. (2017) pointed out that in their mtDNA analyses hemionines and hydruntines did not appear reciprocally monophyletic. This could be explained by rapid diversification of the *E. hemionus* lineages, including hydruntines, creating an unresolved radiation node (Model 1 in Figure 1b). Accordingly, hydruntines could also be considered a

subspecies of *E. hemionus*, *E. h. hydruntinus*, similar to the kulan (*E. h. kulan*) and onager (*E. h. onager*), considered *E. hemionus* subspecies by the IUCN (Kaczensky et al., 2015).

However, the phylogenetic patterns described were only based on partial mtDNA sequences. Thus, it is possible that analyses of full mtDNA and of nuclear loci would reveal different patterns, for example, an early hydruntine–hemione split (Model 2 in Figure 1b). In line with the early-split idea, osteological analyses have suggested that *E. hydruntinus* carried a number of unique adaptations distinct from other hemionines, such as a short and wide muzzle adapted to relatively cold environmental conditions (Boulbes & van Asperen, 2019; van Asperen, 2012). Given the equivocal evidence, there have been calls for in-depth morphometric analyses (Twiss et al., 2017) and for the analysis of nuclear genomic data to resolve the issue (Boulbes & van Asperen, 2019; Crees & Turvey, 2014).

Another controversy surrounding the hydruntine involves its extinction dynamics. Crees and Turvey (2014) studied Holocene zooarchaeological records of hydruntines along with palaeovegetation data, suggesting that during the Holocene, the hydruntine range was highly fragmented and restricted to regions with relatively open habitats, such as the Danube basin and the Anatolian steppe. The authors predicted its extinction in the Danube region by the third millennium BCE, and in Iran and South Caucasus possibly within the first millennium BCE. Other scholars have suggested hydruntines in Iran and in Anatolia could have gone extinct during or before the second millennium BCE (Mashkour et al., 1999), attributing its extinction to a combination of factors including increased aridity associated with the 4.2-ka event, competition with livestock for pasture resources and hunting (Guimaraes et al., 2020). Nevertheless, due to the relative rarity of hydruntines in the zooarchaeological record (compared to, e.g. red deer) and also due to difficulties in morphological identification (Geigl & Grange, 2012; Twiss et al., 2017), the timing of the hydruntine extinction remained largely uncertain (Boulbes & van Asperen, 2019; Crees & Turvey, 2014; Nores et al., 2015).

Here we present the first full genomic data genetically attributable to the hydruntine, obtained from three Anatolian equids from first millennium BCE Central Anatolia. The analysis of these palaeogenomes allows us to resolve questions on the phylogenetics, demographic history and extinction dynamics of this taxon.

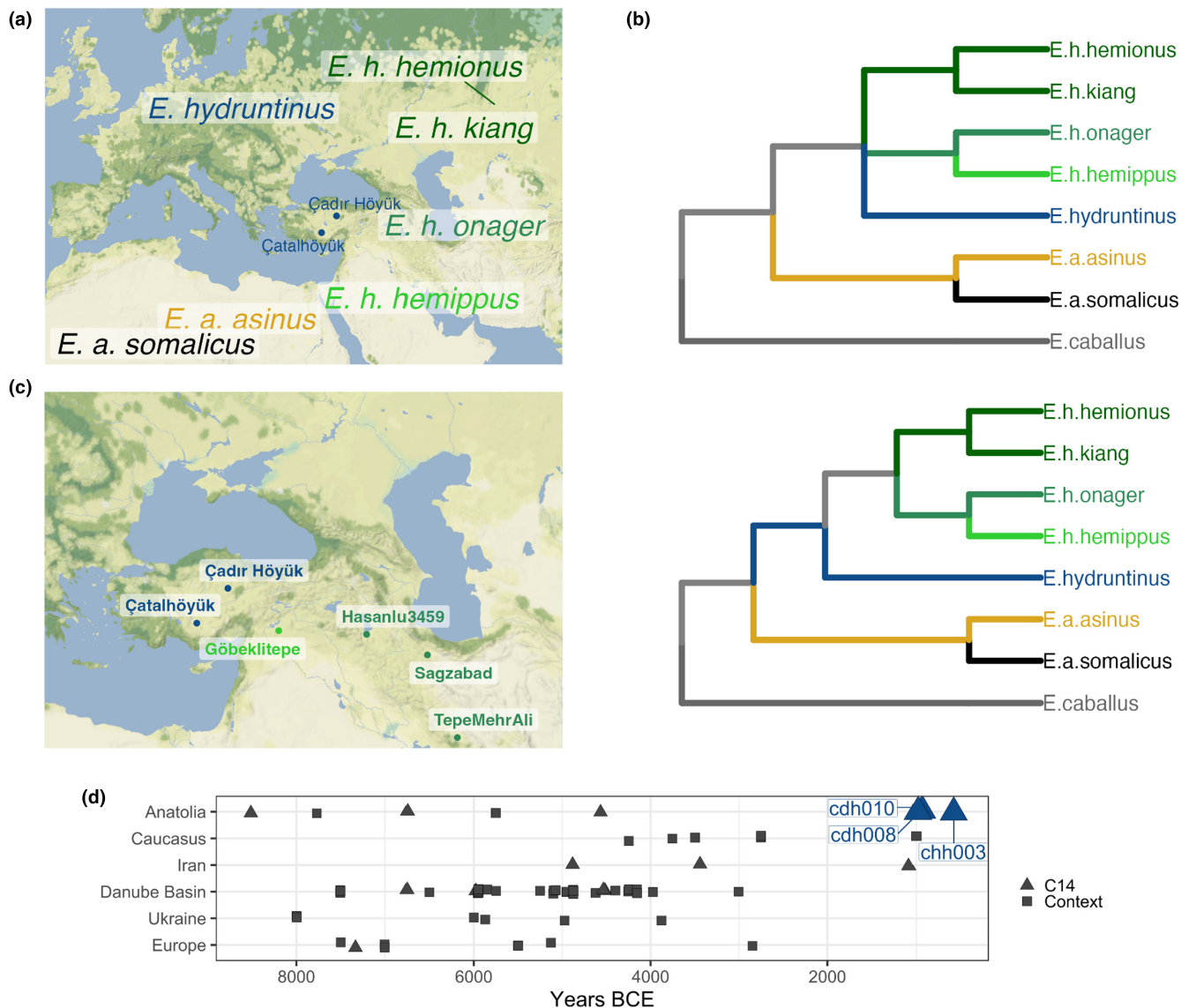


FIGURE 1 (a) The map shows the geographical locations of Çadır Höyük and Çatalhöyük (the excavation sites where the wild ass remains analysed in this study were recovered), as well as the approximate dispersal areas of extant and extinct ass taxa. We avoid showing distinct distribution ranges, especially for *E. h. hydruntinus*, due to scarcity of hydruntine remains in zooarchaeological assemblages and the difficulty of morphological distinction between hydruntines and other Eurasian wild asses (see also Bennett et al., 2017; Crees & Turvey, 2014). (b) Models summarising two hypotheses regarding the taxonomic position of European wild ass relative to other equid lineages. (c) The locations of three ancient hemione individuals and one hemippe individual the partial genomes of which were previously published (Bennett et al., 2022; Fages et al., 2019). (d) Timeline of dated *E. hydruntinus* reportings with samples reported in this study (adapted from Crees & Turvey, 2014).

2 | MATERIALS AND METHODS

2.1 | Archaeological samples

We studied zooarchaeological skeletal samples that were morphologically identified as belonging to equids, excavated in the archaeological sites of Çatalhöyük and Çadır Höyük in modern-day Turkey. Çatalhöyük is a major Ceramic Neolithic period site in Central Anatolia, but its upper layers have also yielded remains dating to the Bronze and Iron Ages, and later periods (Hordecki, 2020; Pawłowska, 2020). Çadır Höyük has demonstrated continuous occupation from the Middle Chalcolithic to the Byzantine Era (early fifth

millennium BCE to 14th century CE) (Ross et al., 2019; Steadman, Hackley, et al., 2019; Steadman, McMahon, et al., 2019). We genetically analysed 11 equid samples from Çatalhöyük and four from Çadır Höyük. See the Appendix S1 for further information on the sites and the archaeological material.

2.2 | Radiocarbon dating

We radiocarbon dated all three equid samples that showed Eurasian wild ass-related genetic profiles (see Section 2.7 below). For each sample, approximately 3 g of bone or tooth material were cut using

2.18 | Masking against reference bias

Ancient genomes and/or genomes mapped to divergent reference genomes can be subject to reference biases (Günther & Nettelblad, 2019), while masking by changing polymorphic sites to N can mitigate this bias (Koptekin et al., 2023). We accordingly masked EquCab2.0 at 2,146,416 polymorphic sites in the main SNP dataset, repeated alignment, and estimated heterozygosity among a selected set of individuals (Sp-5, Kia2, Ona and cdh008). This procedure does not involve masking hydruntine-specific derived positions, but this is not expected to account for the stark heterozygosity differences we observe among lineages.

3 | RESULTS

We extracted ancient DNA from 15 equid tooth and bone samples excavated in two Central Anatolian sites, 11 from Çatalhöyük and four from Çadır Höyük (Figure 1c). We generated genome-wide data from these using shotgun sequencing (Table S1) and obtained 0.02%–11.35% (median=0.07%) of endogenous DNA by mapping to the horse reference genome (EquCab2.0). We chose three samples (cdh008, cdh010 and chh003) with >5% endogenous DNA and authenticity signals (Section 2) for further sequencing (Table 1, Figure S1). We thus produced three genomes with nuclear coverages of 6.38 \times , 0.72 \times and 0.57 \times (Table 1, Table S2). Radiocarbon dating of the skeletal material placed all three individuals within the early/mid-first millennium (Iron Age) in Anatolia (Table 1, Figure 1d). We also constructed mitogenome sequences of these three individuals (Section 2).

3.1 | Genetic analyses assign the iron age Anatolian wild ass individuals to hydruntines

During the Bronze and Iron Ages, horses and the domestic donkey were common in Anatolia, while hydruntines and hemiones were also present, the latter restricted to Southeast Turkey (Table S1 in Appendix S1 see references therein). Due to a lack of sufficient diagnostic characteristics, the three specimens were identified osteologically only to the level of the genus *Equus* (Table S2 in Appendix S1). As a first step towards characterising the three Anatolian individuals, we used the *Zonkey* pipeline (Schubert et al., 2017), which classified all three individuals as 'Asian wild ass-related' (Table S1).

We then compared mtDNA data from these three Anatolian equids with published partial mtDNA sequences from 82 hemiones (Bennett et al., 2017; Huang et al., 2015; Orlando et al., 2009; Wang et al., 2020), 21 morphologically-identified hydruntine individuals (Bennett et al., 2017; Catalano et al., 2020; Orlando et al., 2009; Schubert et al., 2016) and seven other equids (Bennett et al., 2017; Jónsson et al., 2014; Wang et al., 2020). We used a 249-bp-long

fragment shared across all samples to construct a haplogroup network; we also used a 361 bp-long D-loop fragment for *BEAST* analysis (Table S3). In both analyses, mtDNA sequences of the three Anatolian equids clustered with published hemione-hydruntine sequences with 100% support (Figures S3 and S4). Moreover, the three new sequences were a sub-branch of the *H1* haplotype clade (as defined by Bennett et al. (2017)). *H1* is the most prevalent mtDNA haplotype among the 19 hydruntine individuals in this dataset, appears exclusive to hydruntine, and was already detected among Anatolian hydruntines from c.7850–2500 BCE (Bennett et al., 2017; Guimaraes et al., 2020) (Figure S4). Together, these results indicate that the mitochondrial lineage of the three Anatolian equids belonged to the wild asses of Eurasia, and was more closely related to hydruntines than to hemiones.

We investigated this further using complete mtDNA sequences, taking advantage of a recently published near complete mtDNA sequence of a morphologically identified *E.h.hydruntinus/E.h.hydruntinus* specimen from Sicily (Catalano et al., 2020), along with full mtDNA sequences from nine ancient and modern-day equids (Section 2, Table S3). Again, in both the Bayesian and ML trees (Figure 2a, Figure S5) the Anatolian wild asses clustered with the published hydruntine with full support. The mitochondrial phylogenies further suggested that the Eurasian wild asses radiated into four groups within a relatively narrow time range: (a) the hydruntine group encompassing European and Anatolian individuals, (b) the Iranian onagers and the Syrian hemippes, (c) the Gobi kulans and the Tibetan kiangs and (d) the Central Asian kulans represented by a single individual. The order of these radiation events is unclear, however, and is estimated by *BEAST* analyses to have occurred within a relatively short time frame of 300,000 years, between ~0.8 and 0.5 Mya.

The above analyses suggest that the three Anatolian wild asses studied here belonged to the hydruntine lineage. However, mtDNA phylogenies may be incongruent with the overall phylogenetic history of a species due to drift, selection or incomplete lineage sorting (e.g. Toews & Brelsford, 2012). We hence asked whether the whole genome data also support hydruntine ancestry for the three Anatolian wild asses. We compiled a list of ~2 million autosomal transversion SNPs using published asinus and hemionus genomes (Section 2.9). We genotyped the three Anatolian wild ass genomes, 13 published modern-day ass/donkey genomes, as well as three ancient onagers from Iran and three ancient hemippes from the Middle East (Table 1, Figure 1c, Table S3). We then constructed an MDS plot of genomic diversity among these genomes with the outgroup f_3 -statistic (Section 2) (Figure 3, Figure S6, Table S4). This revealed a unique position for the three Anatolian wild asses, distinct from the two other Asiatic wild ass groups included (Gobi kulans/kiangs, and the onagers/hemippes). Because the hydruntine is the only other wild ass lineage identified in the fossil record to have lived in Holocene Anatolia, together with the mtDNA evidence, our results strongly suggest that the three Anatolian wild asses belonged to the extinct hydruntine lineage.

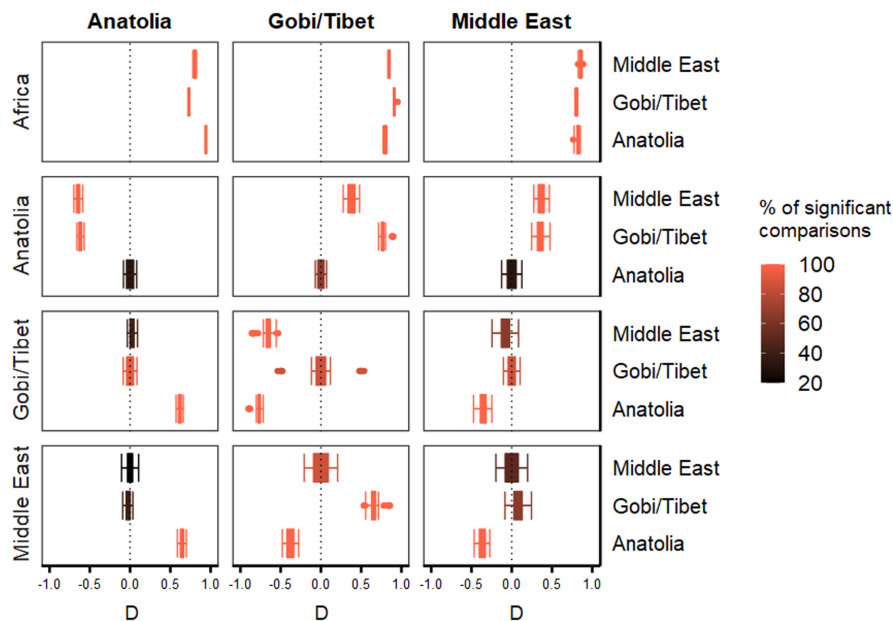


FIGURE 4 Boxplots showing D -statistics calculated between wild ass genomes from different regional groups, using autosomal SNPs from the main dataset. The statistics are calculated as $D(\text{Horse}, \text{Test}; \text{Pop1}, \text{Pop2})$, with *Test* shown on the top, and *Pop1* and *Pop2* on the left and right, respectively. All tests are performed using individual genomes chosen from regional groups. Anatolia: cdh008, cdh010 and chh003; Gobi/Tibet: Aw1, Aw2, Aw3, Kia1 and Kia2; Middle East: GT64 and Hm_1864, Onager, Hasanlu3459, Sagzabad; Africa: Asinus, Somalicus. Each boxplot thus shows a set of D -statistics involving the regional populations shown. The colour gradient from pink to black represents the fraction of D -tests in that comparison that are nominally significant ($Z > 3$). For instance, $D(\text{Horse}, \text{Anatolia}; \text{Middle East}, \text{Middle East})$ involves 30 unique tests, only 20% of which reach $Z > 3$. In comparison, $D(\text{Horse}, \text{Anatolia}; \text{Anatolia}, \text{Middle East})$ also involves 30 unique tests, only 100% of which reach $Z > 3$, marking that the three Anatolians cluster genetically. See Table S5 for the D -test results. See Figure S8 for the same figure drawn using the secondary dataset.

3.4 | Evidence for gene flow between hydruntines and Middle Eastern wild asses

We next asked whether the Anatolian wild asses might be symmetrically related to all Asian wild ass lineages. Following our earlier observations (Figures 2 and 3), here we divided Asiatic wild asses into Gobi/Tibet and Middle East clusters. We then performed D -tests of form $D(\text{Horse}, \text{Anatolia}; \text{MiddleEast}, \text{Gobi/Tibet})$, where *MiddleEast* represents Iranian onager and Syrian hemippe individuals while *Gobi/Tibet* consists of Gobi kulans and Tibetan kiangs. We found affinities of all three Anatolian wild asses towards the modern-day Iranian onager and a museum specimen of hemippe, Hm_1864 over other Asian wild asses, including other onagers and hemippes (27 of 30 comparisons with $Z > 0$; 23 of 30 comparisons with $Z > 3$) (Figure 4, Figure S10, Table S5). This observation is even more pronounced (30 of 30 comparisons with $Z > 3$) in comparisons using the secondary dataset (Figure S11, Table S6). This would be compatible with gene flow between Middle Eastern hemiones and hydruntine populations in Southwest Asia.

We investigated this further by testing $D(\text{Horse}, \text{Anatolia}; \text{MiddleEastX}, \text{MiddleEastY})$ where we compared the genetic affinity of Anatolian hydruntines between the onager and/or hemippe individuals. Using the main variation panel we observed slight affinity towards the 19th century hemippe over the other hemippe and

onagers (12 of 12 with $Z > 0$, 5 of 12 with $Z > 3$; Figure S12). Using the secondary dataset we found a higher affinity to the modern-day onager over all other Middle East wild ass genomes (12 of 12 with $Z > 3$; Figure S13). Although we cannot yet fully determine the exact nature of the possible admixture, we hypothesise that gene flow from hydruntine to the onager and hemippe lineages could explain the observed patterns (see Section 4).

3.5 | The timing of the hydruntine and Asian wild ass split

The above analyses suggest that hydruntines split from other Eurasian wild asses relatively early in their history, although they might have admixed with Middle Eastern wild asses lineages in more recent times as reflected in the autosomal data. Hence we can estimate the hydruntine-Asiatic wild ass split time using mtDNA and also autosomal comparisons between hydruntines and Gobi/Tibet wild asses. As discussed above, the mtDNA data indicated a split involving the various Eurasiatic lineages between 0.8–0.5 Mya. We further used the $F(A|B)$ method for estimating the split time between hydruntines and kiang from the Gobi/Tibet region using autosomal DNA, using both a theoretical estimate and also simulations (Section 2). The intersection of the observed and expected statistics

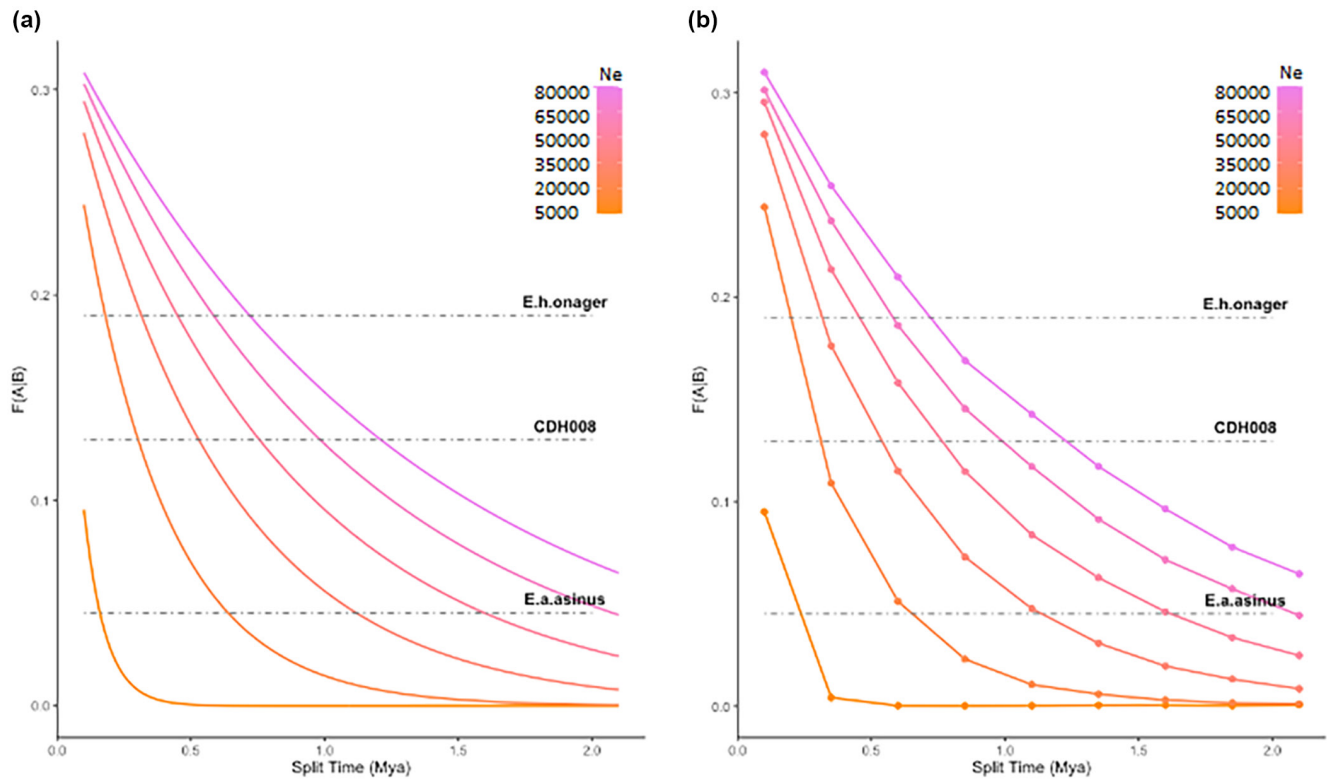


FIGURE 5 Population split time estimation of Anatolian wild ass (cdh008), *E.a.asinus* and *E.h.onager* from Asian wild ass based on the F(A|B) statistic. F(A|B) is the probability of observing a derived allele in the Anatolian wild ass, *E.a.asinus* or *E.h.onager* at the positions where the Asian wild ass genome is heterozygous (Section 2). Observed and expected F(A|B) values, calculated using the formula $e^{-T/(2N)/3}$ in panel (a), and using population genetic simulations in panel (b). We explored the space of divergence times between 100 kya to 2 Mya, and population sizes ranging from 5 k to 80 k.

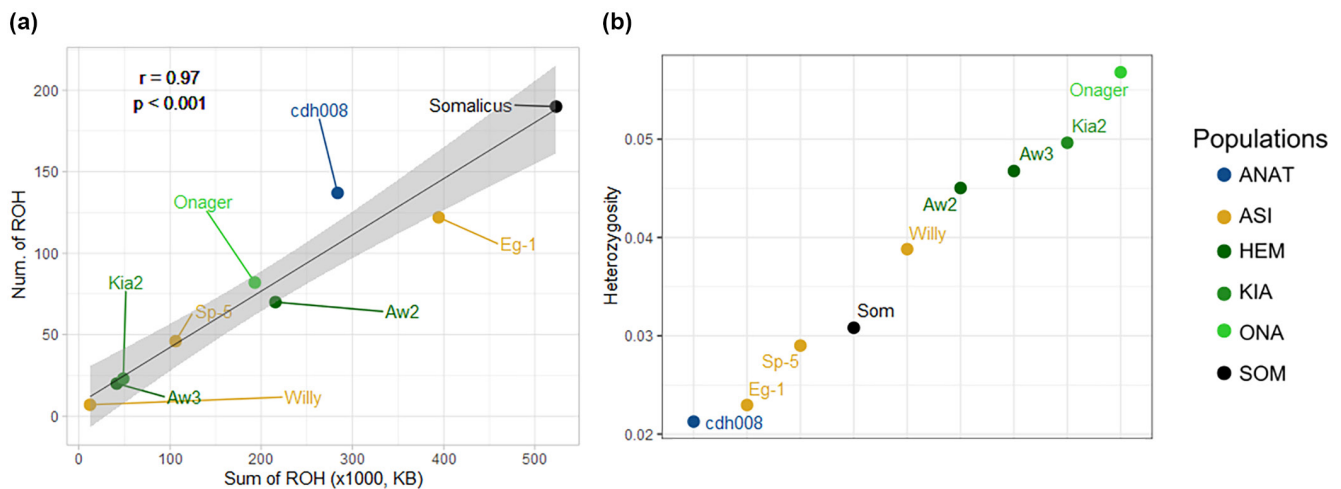


FIGURE 6 (a) Total number versus the total length (sum) of runs of homozygosity (ROH) tracks over 1.5 Mb. The Spearman correlation coefficient and p -value are shown in the inset. (b) Heterozygosity calculated across 2,146,416 transversion sites.

hydruntines were sister taxa to the exclusion of African asses, as suggested earlier based on osteological data (Burke et al., 2003; Eisenmann & Mashkour, 1999; Orlando et al., 2006) and mtDNA analyses (Bennett et al., 2017; Catalano et al., 2020; Orlando et al., 2006, 2009). Thus, we may speak of Late Pleistocene and

Holocene Eurasian wild asses as a broadly coherent evolutionary group.

Our mitogenome phylogeny places hydruntines within Asiatic hemione diversity, splitting after the Central Asian kulan, although with limited statistical support (Figure 1a). In contrast, our nuclear

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SUPPORTING INFORMATION

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