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Genome-wide population affinities and signatures of adaptation in hydruntines, sussemiones and Asian wild asses

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Abstract

The extremely rich palaeontological record of the horse family, also known as equids, has provided many examples of macroevolutionary change over the last ~55 Mya. This family is also one of the most documented at the palaeogenomic level, with hundreds of ancient genomes sequenced. While these data have advanced understanding of the domestication history of horses and donkeys, the palaeogenomic record of other equids remains limited. In this study, we have generated genome-wide data for 25 ancient equid specimens spanning over 44 Ky and spread across Anatolia, the Caucasus, Central Asia and Mongolia. Our dataset includes the genomes from two extinct species, the European wild ass, *Equus hydruntinus*, and the sussemione *Equus ovodovi*. We document, for the first time, the presence of sussemiones in Mongolia and their survival around ~3.9 Kya, a finding that should be considered when discussing the timing of the first arrival of the domestic horse in the region. We also identify strong spatial differentiation within the historical ecological range of Asian wild asses, *Equus*

Jianfei Pan and Xuexue Liu contributed equally to this study.

For affiliations refer to page 11.

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hemionus, and incomplete reproductive isolation in several groups yet considered as different species. Finally, we find common selection signatures at *ANTXR2* gene in European, Asian and African wild asses. This locus, which encodes a receptor for bacterial toxins, shows no selection signal in *E. ovodovi*, but a 5.4-kb deletion within intron 7. Whether such genetic modifications played any role in the sussemione extinction remains unknown.

KEYWORDS
ancient DNA, *ANTXR2*, Asian wild ass, European wild ass, extinction, Sussemione

1 | INTRODUCTION

The equid family emerged from multi-toed dog-sized ancestors ~55 Mya in northern America, and diversified across the world into over a hundred taxa since (MacFadden, 1986). This once flourishing family, which included both grazers and browsers (Owen-Smith, 2013), is, however, only represented by a single genus today, *Equus* (Orlando, 2015). Together with the horse (*Equus caballus*) and the donkey (*Equus asinus*), this genus comprises three African species of zebras (*E. burchelli*, *E. grevyi* and *E. zebra*), as well as two species of Asiatic wild asses (Vilstrup et al., 2013), one (*Equus kiang*) native to Tibetan area, and another (*Equus hemionus*), historically distributed from the Arabian peninsula to the Manchuria (Rosenbom et al., 2015), and now restricted to discontinuous patches in Iran, Turkmenistan, Mongolia and China (Kaczensky et al., 2020) (Figure 1).

The evolutionary history of the *Equus* genus is colourful. It emerged in Northern America ~4–4.5 Mya (Orlando et al., 2013), and dispersed multiple times into the Old World (Jónsson et al., 2014). The ancestors of the so-called stenonine lineage crossed Beringia ~2.6 Mya, and eventually diverged into the zebra and the African ass subgroups ~1.7 Mya, which both reached Africa independently (Jónsson et al., 2014). The ancestors of the horse first crossed Beringia ~1 Mya, extending the geographic span of the so-called caballine lineage into Eurasia (Vershinina et al., 2021). Other caballine dispersals from the New World into the Old World, coincident with global Ice Ages, are documented (e.g. ~120 Kya; Vershinina et al., 2021). However, since the American palaeontological record shows no equid members from after ~12 Kya to the colonization period, the family is generally considered to have become extinct in its original homeland at the end of the Late Pleistocene (or during the first half of the Holocene, according to the persistence of horse-like environmental DNA in sediments dated to ~4–8 Kya; Wang et al., 2021).

The equid family provides many examples of extinctions in the recent past, outside of their American homeland. The quagga zebra (*E. quagga*) from South Africa and the European tarpan (*Equus ferus ferus/sylvaticus*) became, for example, extinct no later than at the late 19th/early 20th century (Olsen, 2006). Palaeoclimatic niche models have also suggested that the European wild ass, *Equus hydruntinus*, survived in patchy isolated regions across their European, Southwest Asian and Caucasian, until they became globally extinct ~2.5 Kya (Crees & Turvey, 2014). Furthermore, ancient DNA work has revealed that the so-called sussemione (*Equus ovodovi*) survived into the Altay region until at least ~32 Kya (Druzhkova et al., 2017; Vilstrup et al., 2013), and in China until ~3.4 Kya (Cai et al., 2022; Catalano et al., 2020), although palaeontological archives previously suggested considerably deeper extinction times into the Middle Pleistocene (Vasiliev, 2013).

In the last decades, ancient DNA has emerged as a powerful tool to study past population dynamics (Orlando & Cooper, 2014). In equids, most of the ancient DNA work has, however, focused on elucidating the domestication history of the horse (reviewed in Orlando et al., 2021), and the donkey (Todd et al., 2022), in which palaeogenomic archives from several hundreds of ancient animals have been characterized (Librado et al., 2021; Librado et al., 2024). While 26 ancient genomes and two almost complete mitochondrial genomes have been sequenced for *E. ovodovi* (Cai et al., 2022; Druzhkova et al., 2017; Vilstrup et al., 2013), the genetic characterization of *E. hydruntinus* has been limited to a handful of partial or pseudo-complete mitochondrial genomes (Bennett et al., 2017; Catalano et al., 2020; Orlando et al., 2006, 2009), and three nuclear genomes (Özkan et al., 2024).Moreover, ancient genome data for their closest phylogenetic relatives, the Asian asses (*E. hemionus*), are still lacking. Therefore, whether these species have evolved in isolation or interbred, and whether their populations were structured in various regional groups, remain unknown.

In this study, we applied ancient DNA shotgun sequencing to characterize the genetic variation in 25 equid archaeological remains at the genome-wide scale. These include the nuclear genome of a >44.1 Ky-old specimen from Emine-Bair-Khosar Cave, Ukraine, morphologically identified as *E. hydruntinus*, as well as four specimens from Mongolia, genetically identified as *E. ovodovi*, and 20 Asian wild asses from western Russia, Georgia, Iran, Kyrgyzstan and Tajikistan (Table S1). Our analyses confirm the close phylogenetic relationship between *E. hydruntinus* and Asian wild asses, but reveal strong spatial differentiation within the historical ecological range of *E. hemionus*, with eastern Iran representing an historical contact zone between a first subgroup spanning Central and East Asia and a second in and around the Caucasian region, Anatolia and Syria. Our work also uncovers the presence of *E. ovodovi* in Mongolia ~3.9 Kya, in stratigraphic layers that were previously considered to be associated with the horse, *E. caballus*, challenging previous

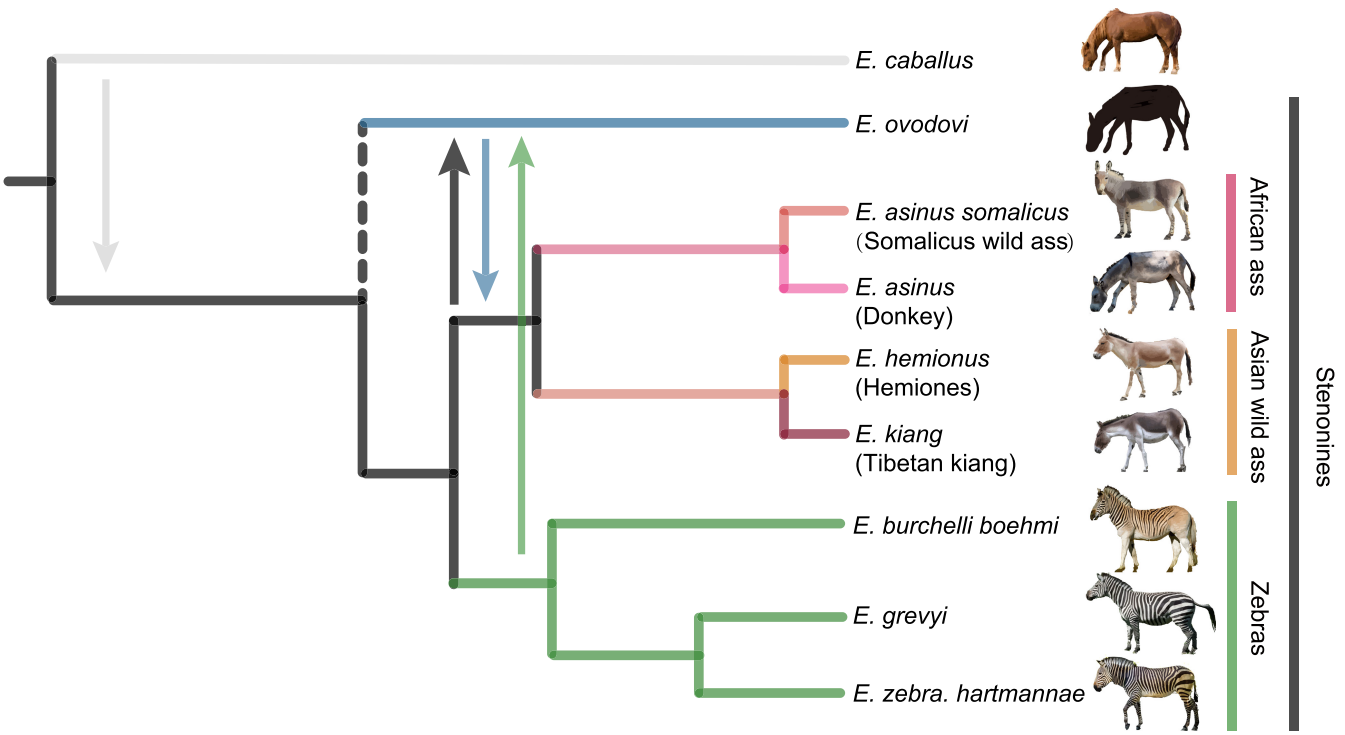

FIGURE 1 Phylogenetic relationships within the *Equus* genus. Phylogenetic relationships are according to Jónsson et al., 2014. The phylogenetic placement of *E. ovodovi* remains ambiguous and difficult to solve, in part due to a history of pervasive gene-flow between various lineages, with different contributions into and from the most recent common ancestors of zebra and assess, respectively (Cai et al., 2022). It is, thus, shown with dashed line.

assumptions about the timing and arrival of the domestic horse in Mongolia. Finally, our work further discloses selection signatures for the *ANTXR2* gene in all stenonine equids investigated except *E. ovodovi*. The role that this cellular receptor for *Bacillus anthracis* may have played in the extinction process of *E. ovodovi* remains to be addressed.

2 | MATERIALS AND METHODS

2.1 | Samples

In this study, a total of 25 equid archaeological remains spread across Anatolia, the Caucasian region, Iran, Central Asia and Mongolia were collected for DNA analysis. These specimens include: four *E. ovodovi* (ZKG, ~3.9 Kya) from Mongolia; one *E. hydruntinus* (Ba2-2343, >44.1 Kya) from the Emine-Bair-Khosar Cave (van Asperen et al., 2012); 20 Asian wild asses from 10 archaeological sites: Godin Tepe (GT, ~3.3–5.2 Kya), Shahr-i Qumis (AM803, ~2.2 Kya) and Yanik Tepe (YTF0148, ~4.8 Kya) from Iran; Kvartshelebi from Georgia (GNM44 and GNM47B, ~4.8 Kya); Oroshaemoei (LR18x57, ~7.6 Kya), Aygurskiy (KAU52, ~5.5 Kya) and Sharakhalsun (BZNKx689, ~4.8Kya) from Russia; Semetey from Kyrgyzstan (SMT1, ~5.4 Kya); Kurteke from Tajikistan (KTK1 and KTK2, ~2.1 and 3.7 Kya, respectively) and Alay from Kyrgyzstan (ALA, ~12.1 Kya) (Figure 2a, Table S1). We also included previously published genome sequences of equid species for comparison, including from three extinct *E. hydruntinus* specimens (Özkan et al., 2024), as detailed in Table S1.

2.2 | Ancient DNA extraction, library construction and sequencing

Ancient DNA analyses were carried out in the clean lab facilities of the Centre for Anthropobiology and Genomics of Toulouse (CAGT), except for specimen Ba2-2343, which was processed in the ancient DNA facilities of the Centre of new Technologies, University of Warsaw (CeNT). At CAGT, DNA extraction was based on 50–630 mg of bone or tooth powder (Table S1). Wet lab procedures at CAGT are fully described in previous work (Librado et al., 2021), and involved the incubation of DNA extracts with the USER™ enzymatic mix to reduce the fraction of nucleotide mis-incorporations in downstream analyses. Wet lab procedures at CeNT are described by Popović et al. (Popović et al., 2021).

2.3 | Read processing, trimming and alignment

Raw paired-end fastq files were demultiplexed, trimmed, and collapsed when showing sufficient sequence overlap using AdapterRemoval v.2.3.0 (Schubert et al., 2016). Reads shorter

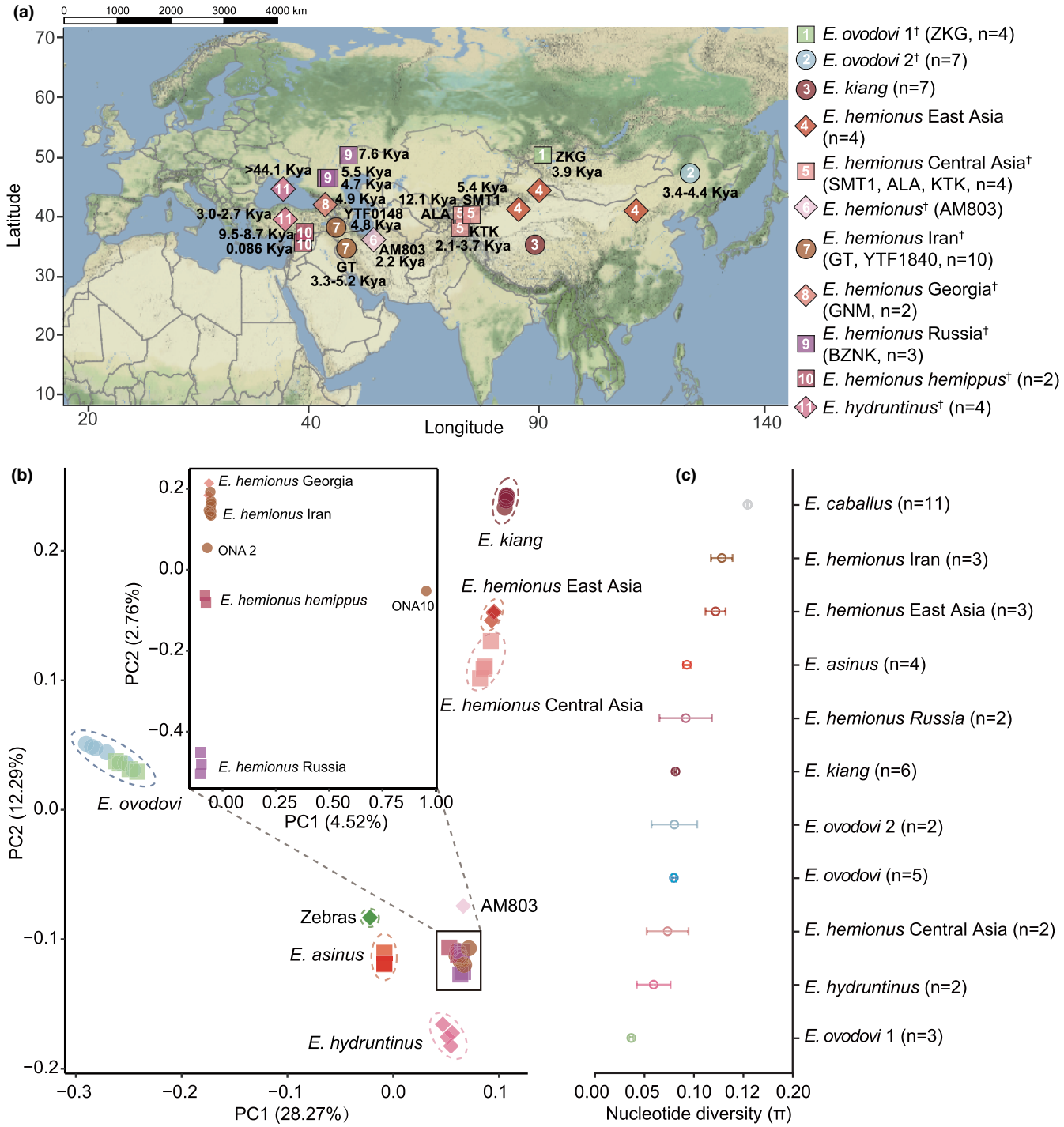

FIGURE 2 Geographical distribution and genetic diversity. (a) Sample map. Archaeological sites, sample names and temporal information are provided (see Table S1 for more details). BP = Before Present; '†' indicates ancient samples. (b) PCA of autosomal variation within stenonine genomes (main plot, $n = 56$) or restricted to *E. hemionus* from Russia, Iran and Georgia, Syrian hemippes, and *E. hydruntinus* (zoomed-in box, $n = 19$). (c) Pairwise nucleotide diversity (π) within each group, based on the distribution of pairwise autosomal differences, limited to samples showing sufficient genome coverage.

than 25 bp were excluded. Sequences were then mapped using Bowtie2 (v2.5.3) against the donkey nuclear reference sequence (GCF_016077325.2), following parameters recommended by (Poullet & Orlando, 2020), and using Paleomix v1.12 (Schubert et al., 2014). Indel realignment was performed using the IndelRealigner from GATK (McKenna et al., 2010), and sequence alignments were filtered to include only those with a minimal mapping quality of 25, while removing PCR duplicates. The average coverage across samples was ~1.10 X (median = 1.03 X; range = 0.11–3.38 X; Table S1). Furthermore, we assessed the

presence of post-mortem DNA damage signatures, including nucleotide mis-incorporation and base compositional patterns, using mapDamage2 v.2.0.8 (Jónsson et al., 2013). Taxonomic and sex identification were carried out using the Zonkey pipeline (Paleomix 1.3.8) on BAM alignments files against the EquCab2 horse nuclear reference (Wade et al., 2009), obtained according to the same procedure (Schubert et al., 2017). The sequence data for the 25 specimens other than those analysed here for the first time were subjected to the same procedure to provide comparative data (Table S1).

2.4 | Mitochondrial sequencing data processing

We used Paleomix v1.12 (Schubert et al., 2014) as described above, to map the sequences against eight distinct mitochondrial reference sequences (*E. asinus* (CM027722.2), *E. burchellii quagga* (NC_044858.1), *E. grevyi* (NC_020432.2), *E. hemionus* (NC_016061.1), *E. hydruntinus* (OP448588.1), *E. kiang* (NC_020433.1), *E. ovodovi* (KY114520.1) and *E. zebra* (NC_018780.1)). To maximize sequence coverage, we selected the alignment maximizing coverage to call the mitochondrial haplotypes using BCFTOOLS v.1.17, ignoring alignments with mapping qualities below 25, individual bases with quality scores lower than 30 and sites showing coverage strictly inferior to 5. A total of 34 additional mitochondrial sequences were obtained from GenBank (see Table S2 for detailed information) for Maximum Likelihood (ML) phylogenetic reconstruction using IQtree v.1.6.12 (Nguyen et al., 2015), assessing node support from 100 bootstrap pseudo-replicates.

2.5 | Genomic variation calling

All samples in our dataset were pseudo-haploidized for their autosomal variation, following the methodology outlined by Librado (Librado et al., 2021), using ANGSD v. 0.933-86-g3fefdc4 (htslib: 1.10.2-106-g9c35744). This procedure returned a total of 2,688,203 nucleotide transversions covered in at least 80% of the total number of samples (average sample missingness = 49.26%).

2.6 | Principal component analysis (PCA)

We constructed PCA analyses using PLINK (v1.90b4.9) (Chang et al., 2015), considering SNPs with a minimum allele frequency (MAF) of 0.05. Analyses were repeated on two data sets. The first included all samples, excepting the 11 *E. caballus* specimens that were included as phylogenetic outgroups (leaving a final set of 1,334,833 transversion SNPs; Figure 2b, main panel). The second analysis was restricted to the samples of *E. hemionus* from Russia, Iran and Georgia, hemippes and *E. hydruntinus* ($N = 19$) to focus on the population structure of the European and Asian wild asses (372,946 transversion SNPs; Figure 2b, zoomed-in panel).

2.7 | Neighbour-joining phylogeny, genetic continuity and population modelling

Phylogenetic relationships were evaluated by reconstructing a BioNJ Neighbour-Joining tree using the set of 1.33 million nucleotide transversions. The matrix of pairwise genetic distances (calculated as 1-ibs, identity-by-state) was generated using PLINK (v1.90b4.9), and served as the input for BioNJ phylogenetic tree reconstruction with FastME v.2.1.4 (Lefort et al., 2015). Node support values were estimated through 100 bootstrap pseudo-replicates, masking for clarity values inferior to 70 (Figure 3b).

2.8 | Ancestry modelling

Genetic ancestry profiles were obtained from ADMIXTURE (v. 1.3.0) (Alexander et al., 2009) ($N = 67$), considering K values from 2 to 10. The optimal number of K ancestry components was determined as $K = 6$ following Cross Validation, with default parameters. A total of 100 bootstrap pseudo-replicates were considered to assess the confidence range of each individual genetic ancestry proportion (Figure 3c and Figures S3,S4).

2.9 | Gene flow and signatures of introgression

We tested the presence of gene flow between the different lineages identified using both phylogenetic reconstruction with migration edges, as implemented by the TreeMix v.1.13 software (Pickrell & Pritchard, 2012), and the Fbranch statistics (f_b), as calculated by Dsuite (Version 0.5 r48) (Malinsky et al., 2021). Both analyses were carried out considering the set of 1,114,884 quality-controlled variants covered in at least 90% of the samples, leaving horse as outgroup ($N = 56$). The optimal number of migration edges for TreeMix ($m = 4$; Figure S6) was determined using the mixed linear model implemented in the optM v.0.1.6 R package (Fitak, 2021). Node support was assessed using modified scripts from BITE (Milanesi et al., 2017), and considering a total of 100 pseudo-replicates (Figures S7,S8). We scanned the genome variation for patterns of introgression, computing f_d statistics (Malinsky et al., 2021) using the Dinvestigate function within Dsuite (Version 0.5 r48), considering sliding windows of 20 SNPs and 5-SNPs increment and the NJ tree topology obtained from FastME v.2.1.4 (Lefort et al., 2015). Genomic regions showing at least three consecutive windows within the top 99.9th f_d percentile provided a list of candidates for introgression. We also assessed the same genomic windows for the Weir and Cockerham F_{ST} fixation index (Leviyang & Hamilton, 2011), considering each population pair in VCFTOOLS (0.1.17) (Danecek et al., 2011). NJ phylogenetic trees were reconstructed within the top 99.9% f_d windows as a further control for gene flow, following previously described methodology (Rocha et al., 2023) (Figure 5a).

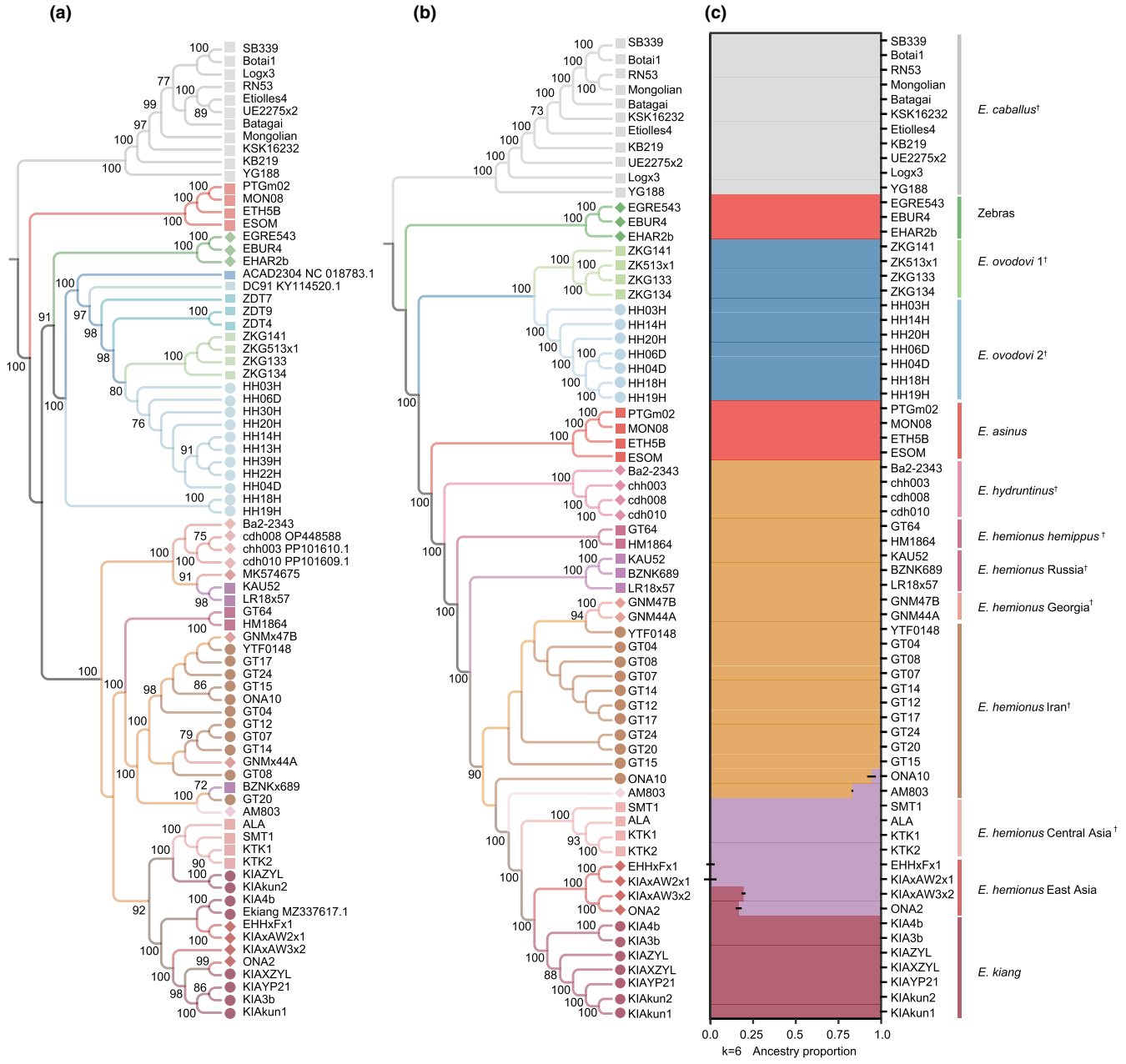

FIGURE 3 Genetic affinities. (a) IQtree Maximum Likelihood phylogenetic tree based on complete mitochondrial DNA sequences. The tree is rooted on horses, and was built using the TVM+F+R3 substitution model. The numbers on each node represent bootstrap supports in percentage (≥70% for clarity). (b) Neighbour-Joining tree constructed using autosomal genome transversions. (c) ADMIXTURE analysis for all samples with the optimal $K=6$ number of ancestry components. The black bars indicate confidence intervals, estimated from bootstrap resampling.

2.10 | Selection signatures

We next used Ohana (Cheng et al., 2022) to look for selection signatures within 50 kb windows (step-size = 5 kb) (Rocha et al., 2023) and across five ancestry components representing the following taxonomic lineages: *E. ovodovi* (Group 1, $n=7$), *E. asinus* (Group 2, $n=4$), *E. kiang* (Group 5, $n=7$), *E. hemionus* from Central and East Asia (Group 4, $n=6$), and the remaining wild asses, including *E. hemionus* from Iran, Russia, Georgia, hemippes and *E. hydruntinus* (Group 3, $n=11$) (Figure 6a,b). For these analyses, positions not covered in at least 90% of the samples were disregarded and MAF was set to 2%. We first obtained the joint distribution of allele frequencies across all ancestral components using Nemeco, and then identified covariance outlier regions using *selescan*, which implements a likelihood model for each individual locus. Functional gene annotation available for protein coding genes within the top 99.5% of each component were obtained from the Ensembl BioMart online genomic database (https://grch37.ensembl.org/info/data/biomart/index.html) and visualized through the OmicShare platform (https://www.omicshare.com/tools) (Figure 6d).

3 | RESULTS

3.1 | Genome sequencing and species identification

We collected one specimen morphologically-identified as *E. hydruntinus* (Figure S1) and associated with a non-finite radiocarbon date (>44,080 Before Present, BP; IntCal20) (Reimer et al., 2020), as well as of archaeological equid remains from across Anatolia, the Russian steppes, Georgia, Iran, Central Asia and Mongolia (Figure 2a; Table S1). These specimens were processed for ancient DNA analysis in the ancient DNA facilities of the Centre for Anthropobiology and Genomics of Toulouse ($N=24$) and those of the Centre of New Technologies University of Warsaw ($N=1$, *E. hydruntinus*) (Supplementary information). DNA preservation levels were compatible with genome characterization through shotgun sequencing at 0.1- to 3.4-fold average depth-of-coverage (median = 1.0-fold), from relatively limited sequencing efforts (37.0–465.5 million read pairs at CAGT and 297.7 single end reads at CeNT; Table S1).

Species identification in Zonkey (Schubert et al., 2017) indicated the clustering of all specimens together within Asian wild asses, except for specimens from Mongolia (ZKG, ~3.9 Kya), not matching the default reference panel of extant *Equus* species. ML phylogenetic reconstruction, including representatives of all ancient and modern *Equus* taxa ever characterized genetically, supported the mitochondrial clustering of ZKG specimens within a monophyletic clade consisting of all other *E. ovodovi* specimens previously sequenced ($n=16$; Figure 3a). The NJ phylogeny reconstructed from autosomal transversion SNPs further confirmed the clustering of ZKG specimens as a sister group to ~3.4–4.4 Ky-old *E. ovodovi* specimens from China (Figure 3b). Moreover, the pairwise genetic distances between ZKG and the other *E. ovodovi* specimens from China were found to be considerably lower than those between any such groups and other Asian wild asses investigated in this study (Figure S2). ADMIXTURE analyses based on the optimal number of genetic components ($K=6$) also indicated that the ZKG specimens exhibited a genetic makeup similar to that of the *E. ovodovi* specimens from China, and maximized a genetic component that was private to this group (Figure 3c and Figure S3,S4). Combined, these analyses indicate that the 4 ZKG specimens belonged to a subpopulation of *E. ovodovi* living ~3.9 Kya in Mongolia, ruling out any possible assignment to the horse species (*E. caballus*).

3.2 | Genetic structure and diversity

We further scrutinized the result of the PCA analysis to assess the genetic affinities amongst different wild asses (sub-) species (Figure 2b). While the first principal component (PC1) mainly separated *E. ovodovi* from the European and Asian wild assess, the second principal component (PC2) provided further resolution into various groups of affinities amongst *E. hydruntinus*, hemippes and *E. hemionus* specimens. In particular, the European wild ass (*E. hydruntinus*) and the Tibetan kiang (*E. kiang*) appeared at the opposite ends of PC2, with various *E. hemionus* occupying intermediate positions (Figure 2b). Those specimens from western Russia (BZNK), Anatolia and Syria (*E. h. hemippus*), and the Caucasus (Godin Tepe, GT; Georgia, GNM) clustered together with two onager specimens (ONA2 and ONA10), relatively close to European wild asses. In contrast, the *E. hemionus* specimens from Central Asia (SMT1, ALA and KTK) placed at the other end of PC2, near Asian wild asses from East Asia and Tibetan kiangs. Such affinities were supported in both NJ and ML phylogenetic reconstructions (Figure 3b and Figure 4c respectively). This indicated a strong phylogeographic structure within Asian wild asses, separating those populations located west of the Caspian Sea from those located in the eastern range of Central Asia (Kyrgyzstan and Tajikistan), China and Tibet. Interestingly, the genomic makeup of the specimen AM803 from Shahr-i-Qumis, Iran shows a mixture of the two genetic components maximized in the two groups of Asian asses west of the Caspian Sea, and in Central Asia and China, both apparent in ADMIXTURE (*E. hemionus* Iran = 83.2 ± 0.6% and *E. hemionus* East Asia = 16.8 ± 0.6%; Figure 3c), TreeMix (Figure 4a), and branch-specific f_b statistics (Figure 4b) analyses. This reveals the region extending from eastern Iran across most of Central Asia represented as a contact zone around ~2.2 Ky BP (i.e. the radiocarbon date of AM803; Table S1).

ADMIXTURE analyses further provided evidence of gene flow between Tibetan kiangs and two *E. hemionus* specimens, including one (KIAxAW3x2) from Xinjiang, North-West China (*E. hemionus* East Asia = 80.4 ± 1.2% and *E. kiang* = 19.6 ± 1.2%; Figure 3c). This confirmed previous phylogenetic findings, reporting mitochondrial haplotype sharing between Tibetan kiangs and so-called Dziggetai hemiones (Bennett et al., 2017). The second such specimen was sampled from a zoo (ONA2) showed a similar genomic composition, consisting of 83.3 ± 1.8% *E. hemionus* East Asia ancestry and 16.7 ± 1.8% of *E. kiang* ancestry. Interestingly, the second zoo specimen (ONA10) also showed evidence of admixture, suggesting a history of pervasive admixture between main population subclusters in captivity (*E. hemionus* Iran = 94.4 ± 2.6% and *E. hemionus* Central Asia = 5.6 ± 2.6%).

ML and NJ phylogenetic reconstructions based on mitochondrial and autosomal DNA variation supported a monophyletic cluster of *E. hydruntinus*, branching off a sister clade of Asian wild asses (Figure 3b). TreeMix ML reconstructions allowing for four migration edges revealed, however, a more complex phylogenetic history, in which the close proximity of *E. hydruntinus* and *E. hemionus* from Russia was strongly supported (Figure 4a). In this analysis, *E. hydruntinus* specimens were modelled to have received a ~ 24% genetic contribution from a lineage basal to all wild asses, which explains their deeper phylogenetic placement in the NJ phylogenetic tree (Figure 3b). *E. hydruntinus* was also modelled to have contributed genetic material (~11%) to Syrian hemippes (*E. h. hemippus*), and branch-specific f_b statistics support significant gene flow between these groups (Figure 4b). Combined, these analyses support a history of contact between *E. hydruntinus* and Asian wild ass populations from Syria and western Russia,

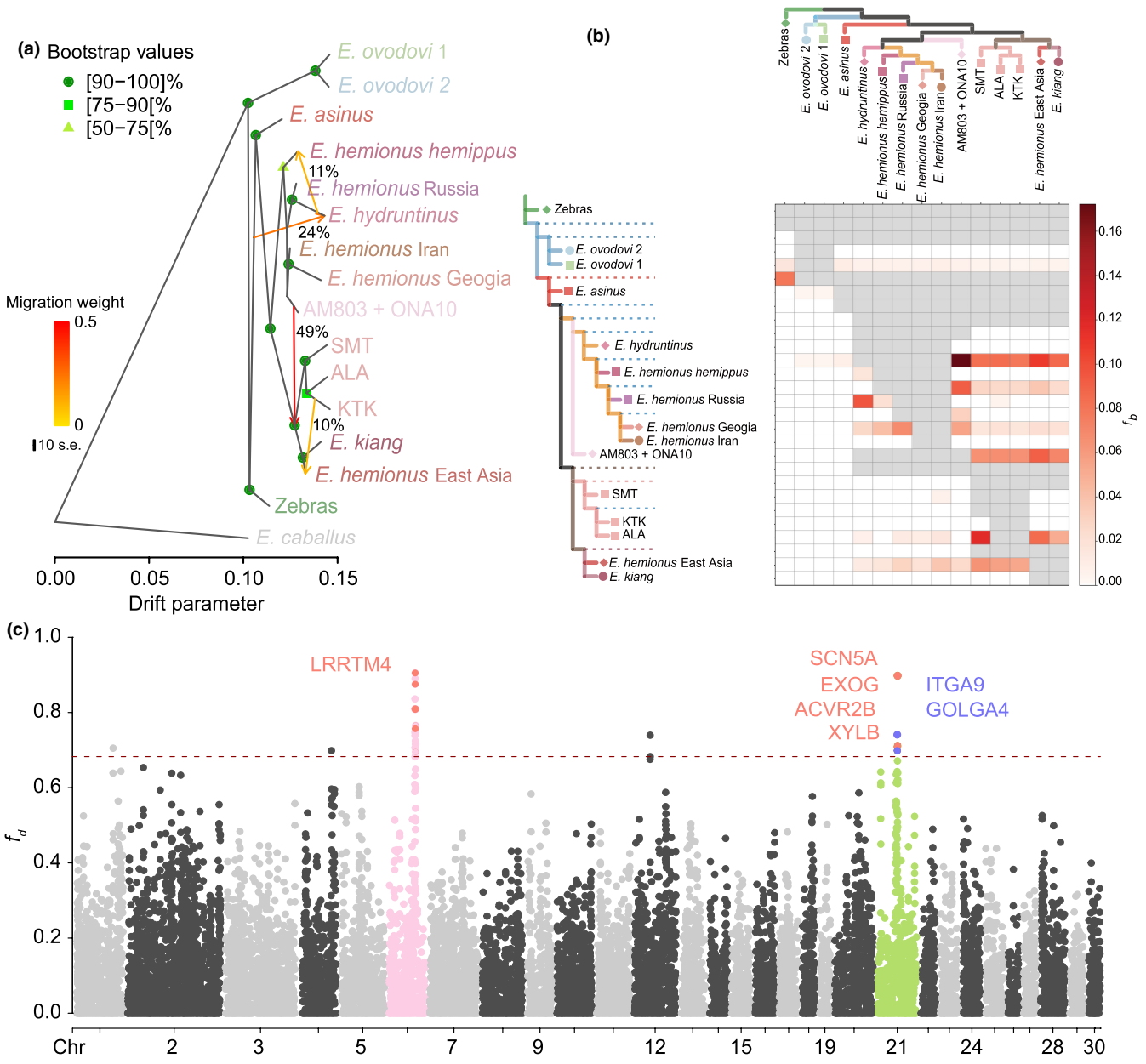

FIGURE 4 Gene flow between various equine groups. (a) TreeMix Maximum Likelihood phylogenetic tree. Coloured arrows represent migration edges, with the colour scaled according to migration weight, indicated in %. The shapes reported on the internal nodes represent increasing bootstrap support. (b) Branch-specific f_b statistics analyses. Significant values of f_b statistics indicate an excessive sharing of derived alleles between the branch of the tree represented above the figure, and the equid species or node presented vertically. The intensity of the red colour reflects statistical support for gene flow, while the grey colour indicates combinations that cannot be tested. (c) Genome scan for gene flow between AM803 and ONA10 and the group of Asian wild asses located East of the Caspian Sea (i.e. Central Asia, East Asia and *E. kiang*), using windows of 20 SNPs (stepsize = 5 SNPs). The red dashed line represents the top 99.9% cutoff. Genes within the top 99.9% f_d and supported by at least three consecutive windows were considered as candidate genes for adaptive introgression.

in line with previous mitochondrial analyses revealing the inclusion of *E. hydruntinus* within a larger, paraphyletic group of Asian wild asses (Bennett et al., 2017). Whether other European wild ass populations located further west of the species' distribution range also showed significant admixture with Asian wild asses remains unknown in the absence of nuclear genome data from *E. hydruntinus* specimens outside of southwest Asia and northwest Russia. However, we noticed that Asian wild asses from Iran and Georgia did not show evidence of gene flow with *E. hydruntinus*. This suggests that the contact zone between *E. hydruntinus* and *E. hemionus* was restricted to the most western range of southwest Asia and the northern range of the Caucasus mountain range, and did not spread further east than eastern Anatolia and the Levant (Figure 2a).

In addition to unveil a strong phylogeographic structure, our analyses also revealed important demographic differences within Asian

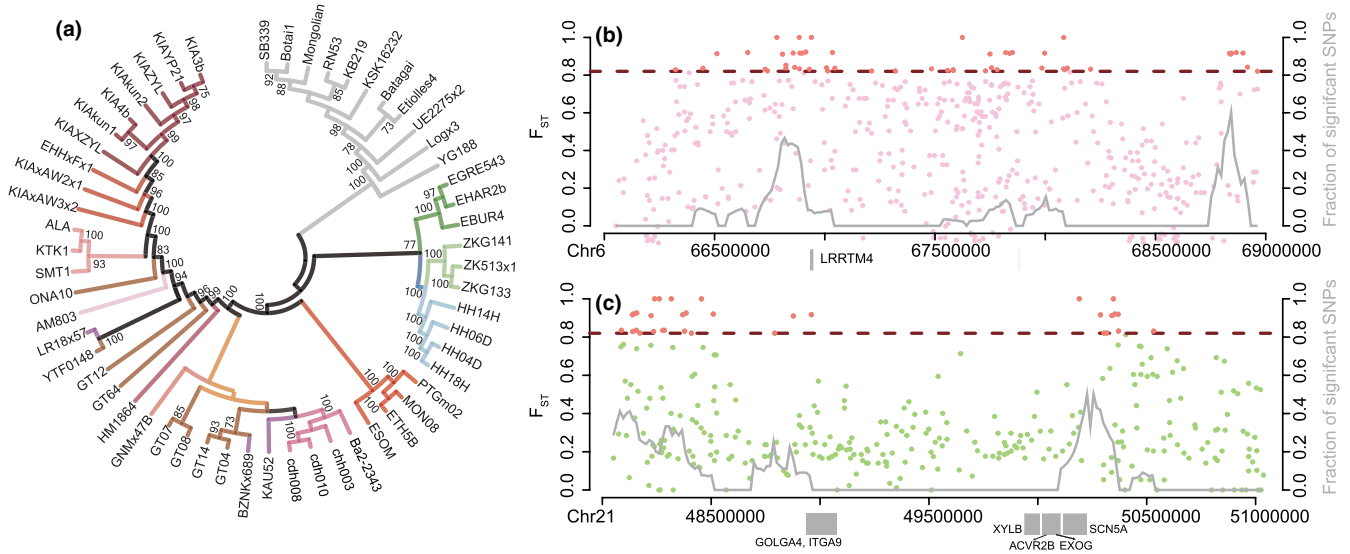

FIGURE 5 Adaptive introgression. (a) Phylogenetic tree for those genomic regions in the genomes of AM803 and ONA10 potentially introgressed from the group of Asian wild asses located East of the Caspian Sea (i.e. Central Asia, East Asia and *E. kiang*). The numbers on each node represent bootstrap supports in percentage (≥70% for clarity). (b, c) F_{ST} fixation indices between Asian wild asses from Eastern Asia plus AM803 and ONA10, and those from west of the Caspian Sea for the candidate introgressive region on chromosomes 6 (b) and 21 (c). Red dots indicate significant SNPs, while pink and green dots refer to chromosomes 6 and 21, respectively (as shown in Figure 4c). The grey line represents the fraction of significant SNPs at the position considered.

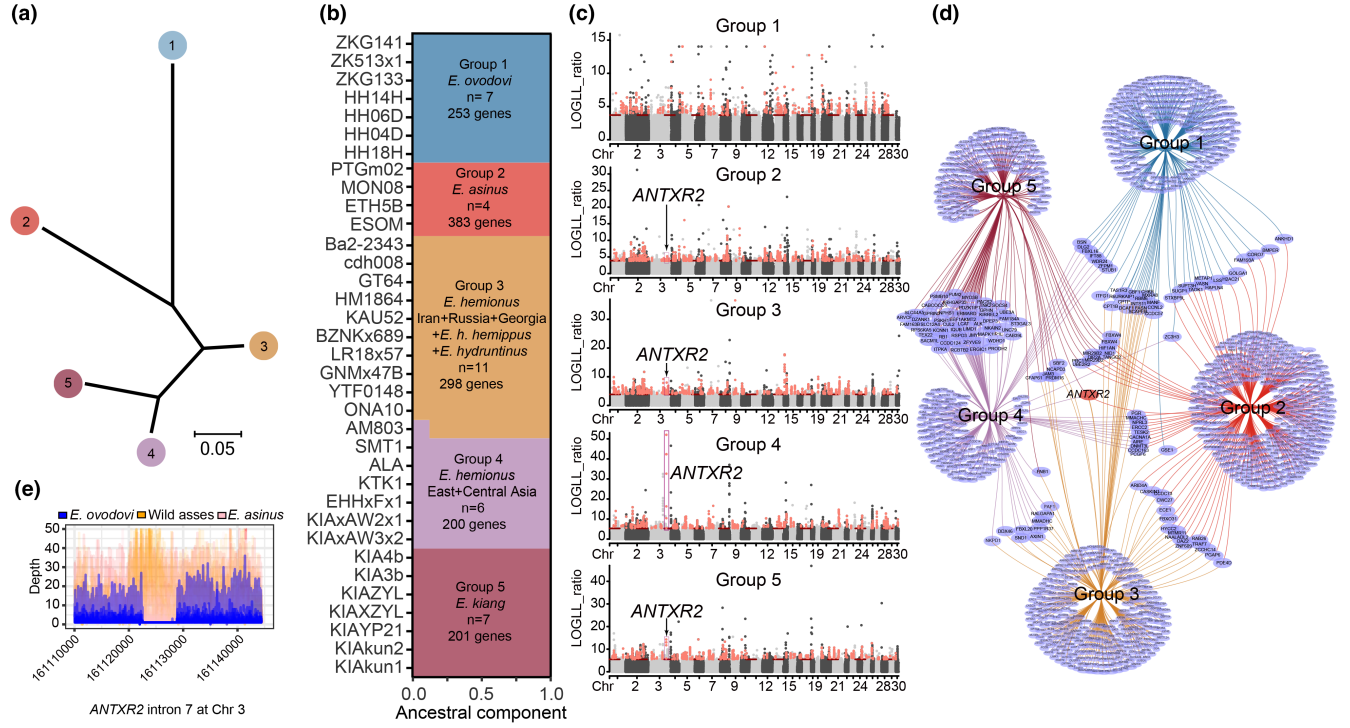

FIGURE 6 Signatures of genetic adaptation in various equine groups. (a) Estimated unrooted tree topologies of ancient ancestry components inferred by Ohana for $K = 5$. (b) Ancestral admixture components and the number of candidate genes for selection in each cluster (cutoff 99.5%). (c) Log-likelihood ratio with the regions comprising at least three consecutive windows within the top 99.5% percentile considered as selection candidates (window size = 50 kb, stepsize = 5 kb). (d) Venn network of annotated protein coding genes located within the candidate regions. (e) Cumulative depth-of-coverage profile within the *ANTXR2* gene.

wild ass populations. The nucleotide diversity of those specimens from Central Asia was more limited than that estimated in northwest Russia, East Asia and Iran (Figure 2c). It was in fact comparable to that measured in *E. hydruntinus* and *E. ovodovi* (Figure 2c), the two extinct species analysed in this study. This indicates populations of limited sizes between 2.0 and 12.1 Ky BP (i.e. the radiocarbon range of

the corresponding specimens; Table S1), in line with the local extinction of this group throughout Central Asia, except in the Badkhyz Reserve from Turkmenistan (Kaczensky et al., 2020).

3.3 | Selection and adaptive introgression

The analyses reported above supported an admixed genomic makeup between the AM803 and ONA10 specimen with Asian wild asses from Eastern Asia (Figure 4a,b and Figure S5). We further scrutinized the AM803 and ONA10 genomes for evidence of adaptive introgression, first identifying those genomic windows of 20 SNPs showing surprisingly-high f_d values in Dsuite (Malinsky et al., 2021), indicative of admixture between AM803 and ONA10 on the one hand, and Asian wild asses from Eastern Asia on the other hand (Figure 5a). Focusing on regions showing at least three such consecutive windows revealed three candidate loci for adaptive introgression on chromosomes 6 and 21 (Figure 4c). We then calculated, within those regions, F_{ST} values between Asian wild asses from Eastern Asia and those from west of the Caspian Sea, but grouping AM803 and ONA10 with the former. Finding high F_{ST} values would depict genomic regions in which the AM803 and ONA10 samples show an Eastern Asian genomic background strongly differentiated from that present west of the Caspian Sea (Figure 5b,c). It would, thus, suggest that the genetic introgression detected may be adaptive.

We found that positions 66,735,924 and 68,324,246 of chromosome 6 depicted a genomic region showing an excess of high F_{ST} values (≥99.9% percentile; Figure 5b). This region only included one protein coding gene, *LRRTM4*, which is a trans-synaptic adhesion protein regulating glutamatergic synapse assembly on the dendrites of central neurons (Table S3) (Sinha et al., 2020). The genomic region between positions 48,910,707 and 50,321,289 of chromosome 21 showed similar f_d and F_{ST} profiles (Figure 5c, Table S3,S4). It encompassed six protein coding genes, with essential roles in the removal of the RNA primer (Karlowicz et al., 2022), Golgi assembly, and spermatogenesis and male fertility (Guo et al., 2020), cell proliferation (Xu et al., 2021), cardiac electrophysiological function (Li et al., 2018), glucose metabolism and lipogenesis (Guo et al., 2022) and insulin sensitivity (Swan et al., 2024) respectively. In light of the relatively limited annotations available in the donkey reference genome, further work is needed before the potentially adaptive introgression signal identified can be confirmed and their functional consequences identified.

To further investigate the adaptive history of European and Asian wild asses as well as *E. ovodovi*, we searched for selection signatures in the equine genome using the ancestry-aware methodology implemented in Ohana (Cheng et al., 2022). More specifically, we supervised the analysis by defining five groups, including *E. ovodovi* (Group 1), donkeys (Group 2) and three groups of Asian wild asses reflecting the population structure uncovered above (*E. hydruntinus* and/or *E. hemionus* from Iran and west of the Caspian Sea, Group 3; *E. hemionus* from Central and East Asia, Group 4; and *E. kiang*, Group 5) (Figure 6a,b). We then calculated the average of the SNP-based LogLikelihood ratio returned by Ohana within 50 kb genomic windows, and considered the top 99.5% ranked values, requiring support from three consecutive windows, as candidates for selection in each ancestry component (Figure 6c,d).

Strikingly, the genetic component characterizing each of the Groups considered returned a set of candidate genomic regions including between 200 and 383 annotated protein coding genes (Figure 6d, Tables S5–S14). The number of shared candidates for selection not only recapitulates the phylogenetic affinities between these groups, but also supports the adaptive nature of the signal identified as Central and East Asian *E. hemionus* (Group 4) share more adaptive signatures with *E. kiang* (Group 5), endemic to East Asia, than with *E. hydruntinus* and/or *E. hemionus* from Iran and west of the Caspian Sea (Group 3). Importantly, one gene, *ANTXR2*, was identified as a selection candidate in all stenonine lineages investigated in this study, except *E. ovodovi* (Figure 6d,e and Table S14). The loss of *ANTXR2* expression in mice is known to enhance resistance to bacterial toxins (Banks et al., 2005), and *ANTXR2* featured amongst selection candidates previously identified while scanning the genomes of Przewalski's horses (Gaunitz et al., 2018), or plains zebras (Dickson, 2021). Combined with our results, the available evidence may indicate that this locus played a central role during equine evolution in response to infectious diseases, especially through its role in the cellular uptake of bacterial toxins.

4 | DISCUSSION

In this study, we have generated genome-wide sequence data for 25 ancient equids, mainly from members of the *E. hemionus* species, but also from two extinct equine species (*E. hydruntinus* and *E. ovodovi*). We produced the first nuclear genome from a specimen morphologically identified as *E. hydruntinus*, which complements the three genome sequences from Özkan et al., 2024 that were assigned to this species based on phylogenetic placement. Clustering of the four specimens within a single monophyletic group confirms this assignment.

The four *E. ovodovi* genomes characterized here also complement those reported Cai et al., 2022, at the archaeological site of Honghe, China, which documents, for the first time, the presence of the species in Mongolia until ~3.9 Kya (Houle et al., 2022) for elements on the stratigraphic context. Interestingly, *E. ovodovi* belongs to a group of equids called Sussemiones, which was once considered extinct since the Middle Pleistocene (Druzhkova et al., 2017). However, palaeogenetic studies have revealed their survival up until the Late Pleistocene in the Altay region (Vilstrup et al., 2013), and at least until ~3.4–4.4 Kya in Inner Mongolia and China (Cai et al., 2022), and now at approximately the same time in Mongolia. Extensive DNA survey of equine remains across East Asia, including after 3.4 Kya, should be carried out to elucidate the exact geographical range and extinction timing of this species. For now, the relatively limited genetic diversity present amongst the specimens sequenced, especially

from Mongolia, may indicate ongoing demographic declines locally (Figure 2c). More globally, the selection scan presented here shows no evidence of positive selection in the region containing *ANTXR2* for this species, in contrast to what is observed for other stenonines. Further work is needed to understand not only the exact role that this gene may have played in equid survival to infectious diseases in equids, but also the functional consequences of the 5.4-Kb deletion specific to *E. ovodovi* (Figure 6e).

Importantly, the four *E. ovodovi* specimens sequenced in this study were thought at the time of their discovery as possibly representing the earliest domestic horses to have spread into Mongolia, as their associated age is coincident with the previously reported expansion of modern domesticated horses outside their original homeland (Librado et al., 2021). The identification reported here rejects this view, and the earliest genetic evidence for the presence of domestic horses in Mongolia available so far remains in the Bronze Age, some ~3.2–3.1 Kya (Fages et al., 2019; Librado et al., 2024), in line with archaeological evidence for hippophagy (Taylor et al., 2018), horse milking (Ventresca Miller et al., 2022) and bridling (Taylor et al., 2017) around ~3.4–3.2 Kya, ~3.2 Kya and ~3.2–3.0 Kya respectively. That we identified other equid taxa than the horse in northwest Russia around ~4.7 and 5.5 Kya is also central to ongoing debates about the timing of horse domestication in the Pontic-Caspian region. The presence of equine beta-lactoglobulin peptides has indeed been reported from dental calculus of two early Bronze age human individuals contemporary to the onset of the Yamnaya cultural horizon (Wilkin et al., 2021). While the primary sequence of such peptides is identical across all species belonging to the *Equus* genus, these data were interpreted as evidence for the consumption of horse milk at the time, due to horses being the only alleged *Equus* species in the region (Wilkin et al., 2021). This interpretation implied that horses were already domesticated around ~4.5–5.3 Kya. More extensive palaeoproteomic surveys of the region, however, failed to replicate these findings, supporting Neolithic and Early Bronze Age dairying practices focused exclusively on sheep in the North Caucasian steppes, with *Equus*-specific peptides appearing no earlier than the early Iron Age (~3 Kya) (Scott et al., 2022). Together with genetic evidence supporting the demographic and geographical expansion of domesticated horses no earlier than ~4.2 Kya (Librado et al., 2021, 2024), our findings caution against previous reports of horse milking and established horse domestication in the region as early as ~4.5–5.3 Kya.

Moreover, our genome analyses revealed that the four *E. hydruntinus* specimens investigated here shared nuclear genetic affinities with Asian wild asses from north-western Russia and with Syrian hemippes. This is consistent with the geographic origins of the *E. hydruntinus* samples (Ukraine and Anatolia), and suggests population groups living at least partly in sympatry and not completely reproductively isolated. The exact genomic makeup of those *E. hydruntinus* groups inhabiting their more western distribution range, which extended as far as western Europe (Strani & Daniel., 2023) until ~2.5 Kya (Crees & Turvey, 2014), remains to be characterized to delineate the geographic extent of such genetic exchanges. More extensive palaeogenomic analyses charting the genetic diversity of hydruntines across space and time are, thus, needed to understand the impact of past glacial climate changes, human hunting, as well as the temporal dynamic and the genomic footprints of extinction, following inspiring studies carried out on woolly mammoths (Rogers & Slatkin, 2017; van der Valk et al., 2022).

Our analyses also support that the Tibetan kiang emerged from *E. hemionus* populations located at the Eastern range of their distribution, and provide additional evidence of gene flow between those two groups beyond mitochondrial haplotype sharing (Bennett et al., 2017). This finding lends support for classifications considering both taxa as *E. hemionus* subspecies, despite both groups carrying different karyotypes (Musilova et al., 2009), a situation reminiscent to what reported for *E. caballus* and *E. przewalski*, which also show different karyotypes (Ahrens & Stranzinger, 2005), but can have viable and fertile hybrid offspring (Der Sarkissian et al., 2015). Rather than reproductive isolation, the strong phylogeographic pattern unveiled amongst *E. hemionus* and *E. kiang* populations is indicative of isolation-by-distance from the Caucasian range to Kyrgyzstan, Tajikistan and East Asia, with eastern Iran representing an important contact zone between two main genetic subgroups. Unravelling the exact boundaries of this contact zone within Central Asia, and their possible shifts in the face of climate change and/or human activities will require further genome sequencing in the region throughout the Late Pleistocene and the Holocene, and until their local extirpation and global decline in the 19th and 20th centuries.

AUTHOR CONTRIBUTIONS

L.O. conceptualized and coordinated the study. P.M. and L.O. provided funding. H.D., A.F.M., B.S., S.S., E.H., A.P., J.-M.A., P.O., P.W., P. K., M.M., D.L., S.R., S.H., T.H., J.-L.H., E.H., R.B., K.S., B.R., S.S., A.A., N.S., A.S.G., G.S., J.L.G., J.B., A.B.B., A.A.K., K.R., C.W., N.V., J.-L.A., E.C. and W.T.T.T. provided samples. E. N. van A. performed the morphological description of *E. hydruntinus* specimen. R.B., J. L.G., S.R., E.H., M.M. and W.T.T.T. described the archaeological contexts. S.S. performed the wet-lab work at CAGT, with input from L.O. M.B. and D. P. performed the wet-lab work and sequencing at CeNT. J.P., X.L. and L.O. performed data analysis. L.O. wrote the manuscript, with input from J.P. and X.L. All authors revised the manuscript and approved the publication. J.P. and X.L. prepared the figures and tables, with input from L.O.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All code related to this analysis is available in open depository of GITHUB: https://github.com/xuefenfei712/aDNA. All collapsed, and paired-end sequence data sequenced in this study are available in compressed fastq format through the European Nucleotide Archive with the deposited code of PRJEB76252.

BENEFIT-SHARING STATEMENT

This research addresses the population genetics of hydruntines, sussemiones and Asian wild asses. We have ensured that all raw data are provided, together with well-documented scripts and instructions for data analyses. All sample providers are included in the Acknowledgements section.

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