

Revising the recent evolutionary history of equids using ancient DNA

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The rich fossil record of the family Equidae (Mammalia: Perissodactyla) over the past 55 MY has made it an icon for the patterns and processes of macroevolution. Despite this, many aspects of equid phylogenetic relationships and taxonomy remain unresolved. Recent genetic analyses of extinct equids have revealed unexpected evolutionary patterns and a need for major revisions at the generic, subgeneric, and species levels. To investigate this issue we examine 35 ancient equid specimens from four geographic regions (South America, Europe, Southwest Asia, and South Africa), of which 22 delivered 87–688 bp of reproducible aDNA mitochondrial sequence. Phylogenetic analyses support a major revision of the recent evolutionary history of equids and reveal two new species, a South American hippidion and a descendant of a basal lineage potentially related to Middle Pleistocene equids. Sequences from specimens assigned to the giant extinct Cape zebra, *Equus capensis*, formed a separate clade within the modern plain zebra species, a phenotypically plastic group that also included the extinct quagga. In addition, we revise the currently recognized extinction times for two hemione-related equid groups. However, it is apparent that the current dataset cannot solve all of the taxonomic and phylogenetic questions relevant to the evolution of *Equus*. In light of these findings, we propose a rapid DNA barcoding approach to evaluate the taxonomic status of the many Late Pleistocene fossil Equidae species that have been described from purely morphological analyses.

DNA taxonomy | equid evolution | macroevolution | phylogeny | ancient DNA

The original sequence of horse fossils found in the 1870s by paleontologist Othaniel Charles Marsh, and popularized by Thomas Huxley (1), has been enriched by a large fossil record over the years and has now become one of the most widely known examples of macroevolutionary change (2). The original linear model of gradual modification of fox-sized animals (Hyracotheres horses) to the modern forms has been replaced by a more complex tree, showing periods of explosive diversification and branch extinctions over 55 MY (3). The end of the Early Miocene (15–20 MYA) marks a particularly important transition, separating an initial phase of small leafy browsers from a second phase of more

diverse animals, exhibiting tremendous body-size plasticity and modifications in tooth morphology (4). This explosive diversification has been accompanied by several stages of geographic extension from North America to the rest of the New and Old Worlds, so that by the end of the Miocene (5 MYA) more than a dozen distinct genera are represented in the fossil record (4) (*Astrohippus*, *Calippus*, *Cormohipparion*, *Dinohippus*, *Hippidion*, *Nannipus*, *Neohipparion*, *Onohippidium*, *Plesippus*, *Pliohippus*, *Protohippus*, *Pseudhipparion*, *Sinohippus*, Old World *Hipparion*). Nearly all of this diversity is now extinct, with all living members of the Equidae assigned to a single genus *Equus* (4–5), which includes caballines (true horses, *E. caballus* and *E. przewalskii*) and noncaballines: hemionids (*E. hemionus* and *E. kiang* for Asian and Tibetan wild asses, respectively), African wild asses (*E. africanus*, i.e., wild ancestors of the domestic donkeys *E. asinus*), and zebras (*E. quagga*, *E. hartmannae*/*E. zebra*, and *E. grevyi* for plains, mountain, and Grevy's zebras, respectively) (6–9). Plains zebras (*E. burchelli*) have also been considered to be a species distinct from the extinct quaggas (*E. quagga*), both belonging to the plains zebra group (10–11).

Importantly, many of the past equid lineages became extinct as recently as the Late Quaternary (Late Pleistocene and Holocene),

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raising the potential for ancient DNA (aDNA) analyses (12). As a consequence, several aspects of the classical paleontological model of equid evolution can now be tested with molecular data. Indeed, the field of aDNA started with the publication of mitochondrial DNA sequences from a museum specimen of the quagga (13), an equid related to Plains zebras that became extinct in the wild in the late 1870s. Over recent decades, aDNA sequences have been reported from other extinct members of the Equidae family (11, 14–18) and have revealed unexpected patterns in equid evolution with varying degrees of conflict to morphological and paleontological phylogenies. For instance, the morphologically distinct South American hippidions were formerly considered to be descendants of the Pliohippines, a basal North American group that diverged from the lineage that gave rise to the genus *Equus* before 10 MYA (19). Hippidions exhibited a similar distinctive nasal morphology to Pliohippines, and appeared in the South American fossil record \approx 2.5 MYA (20). However, mitochondrial sequences of hippidions from Patagonia and the Buenos Aires Province form a tight clade within a larger paraphyletic group of *Equus*, suggesting either that hippidions and living equids belong to the same genus or that living equids should be split into several genera (15, 16, 18, 21). Similarly, the South American subgenus (*Amerhippus*), which was contemporaneous with hippidion but quite *Equus*-like (22), was shown by aDNA sequences to actually be conspecific with caballines and did not form a separate subgenus within living *Equus* (18). In North America, aDNA revealed that a group of stilt-legged equid species morphologically similar to the hemiones (Asian asses) were in fact New World endemics, and that many putative species of this New World stilt-legged horse (NWSL) group should potentially be synonymized (16). Last, within Europe, aDNA has shown that the extinct European ass (*Equus hydruntinus*) was not related to zebras or primitive Pliocene equids as previously suggested, but was sufficiently closely related to the extant hemiones (Asian wild ass) that separate taxonomic status was questionable (17).

In this paper, we report 29 mitochondrial DNA (mtDNA) sequences (22 control region, HVR-1; and 7 cytochrome *b*, *cyt b*) from 22 fossils of extinct equids from four different geographic regions (South America, Europe, Southwest Asia, and southern Africa). The evolutionary history and phylogeographic structure of hippidiforms was investigated using new specimens from Patagonia and Peru. In Eurasia, the taxonomic relationship of hemionids and *E. hydruntinus* was further analyzed using Siberian hemionid fossils and additional specimens of *E. hydruntinus*, which also permitted the extinction date to be examined. Last, we address the phylogenetic relationship of the Late Pleistocene giant Cape zebra (*Equus capensis*) (23–25) from South Africa to the other zebra species. Phylogenetic analyses and comparison with the genetic diversity found within and between extant equid groups reveal that though two new extinct species (a hippidion and a sussemione) are supported, the temporal and geographic variation in body size of others may have been underestimated, as suggested previously for caballids (16), leading to taxonomic oversplitting.

Results and Discussion

Authenticity of aDNA Sequences. We successfully recovered aDNA sequences from 22 of the 35 specimens (Fig. S1 and Table S1A): one horse (*E. caballus* from the Scladina cave, Belgium); 10 *Hippidion* specimens (five each from Patagonia and Peru); six *E. (cf.) hydruntinus* (two from Europe, three from Russia, and one from the Middle East); one *E. hemionus* (from the Middle East); and four *E. capensis* (from South Africa). Thirteen fossils did not yield any aDNA information despite extensive extraction and PCR attempts (Table S1B), and by default, also acted as cross-contamination controls in different extraction experiments. The poor DNA yields from these fossils are in agreement with their preservation state [high thermal age (26); see *SI Text*]. Thirteen of the samples were successfully amplified and sequenced in two independent laboratories [Lyon and Australian Centre for Ancient DNA (ACAD)],

using different extraction methods, PCR conditions, and sequencing strategies (cloning/sequencing of different amplicons in Lyon versus direct sequencing at ACAD). It is notable that of the 6,569 bp duplicated at ACAD (from 64 amplicons), comparison with the final consensus sequences revealed only minor discrepancies (67 differences; average number of differences per base per fragment = 0.884%). As expected, a large majority of the variants (74.6%) consisted of GC→AT substitutions and are likely to derive from postmortem cytosine deamination characteristic of ancient templates (27, 28). The base recovered from the majority of independent amplicons was accepted as authentic, in agreement with standard recommendations (27, 29), and importantly, the extent of damage-induced errors was well below the intraspecific levels of genetic diversity among equids (Table S3). It is noteworthy that no modern equid DNA was stored in the aDNA laboratories, and though modern Grevy's zebra hairs were analyzed in the Lyon post-PCR laboratory, this haplotype was not found in any ancient sample. Of the new sequences, the mtDNA HVR-I sequences of the Patagonian *Hippidion saldiasi* specimens were highly similar to previously reported *Hippidion* sequences (15–17) (Fig. 1). The mtDNA HVR-I and/or *cyt b* sequences of the *E. (cf.) hydruntinus* from Scladina (Belgium) and Zagheh (Iran) showed high similarity with the two *E. hydruntinus* haplotypes reported in ref. 17 (Fig. 1 and Fig. S2 B and C), as did the new Iranian *E. hemionus* sample analyzed here (CH28). These are consistent with the ancient sequences being authentic.

Phylogenetic Analyses. South America. Both the maximum likelihood and Bayesian phylogenetic analyses (Fig. 1) of the mtDNA HVR sequences identified three monophyletic New World groups: caballine horses (node G); NWSL horses (node F); and hippidiforms (node C2), in agreement with earlier studies (15, 16, 18). The exact branching order of the three groups (relative to the Old World noncaballine horses) could not be resolved with either the HVR-1 dataset alone (node B shows weak supports in Fig. 1; Table S5, topologies L–Q) or various combinations of HVR-1 plus *cyt b*/HVR-2 data (Table S5, topologies R–V). Similarly, although the strongest support was obtained for the placement of hippidiforms within a larger paraphyletic group of *Equus* (with merged datasets, Table S2, and with marginal support from SH tests: *P* values = 0.065–0.083; Table S5, topologies R–V), likelihood-based topological tests cannot significantly reject alternative topologies and the exact position of the root, previously reported between nodes A and H (Fig. 1) (15, 16) should be considered preliminary. The lack of resolution is complicated by the short divergence time among caballines and New World horses (circa 0.5 MY; nodes A and B/B1/B2; Table S4) and the lack of a close outgroup, as has been noted with mammoths (30, 31). When the rhino was used as an outgroup, the data were RY coded to reduce possible mutation saturation artifacts resulting from this deep divergence (55 MYA), but this removed support for most nodes (Table S2).

According to our molecular dating estimates, the different equid lineages (hippidiforms, NWSL, caballines, and noncaballines) originated 3.7–4.3 MYA (95% confidence range: 2.8–6.2 MYA; Table S4). This directly contrasts with classical palaeontological models of hippidiform origins as descendants of the Pliohippines (divergence time with the *Equus* lineage >10 MYA) (19) or as a lineage diverging from a (*Dinohippus*, *Astrohippus*, and *Equus*) clade \approx 7–8 MYA (32), and considerably reduces the time gap between the supposed divergence of the hippidiform lineages and their first appearance in the fossil record 2.5 MYA (20). A deep split within hippidiforms separates one cluster (Fig. 1, node D) from Patagonia and Argentina (*Hippidion saldiasi* and *Hippidion principale*, respectively) from a second (Fig. 1, node E) consisting of specimens from a high-altitude cave in Peru. The genetic differentiation between these clusters is greater than intraspecific levels of genetic diversity in any current equid species (Fig. 2 and Table S3) but comparable to that between indisputable species from the same genus (*Equus*),

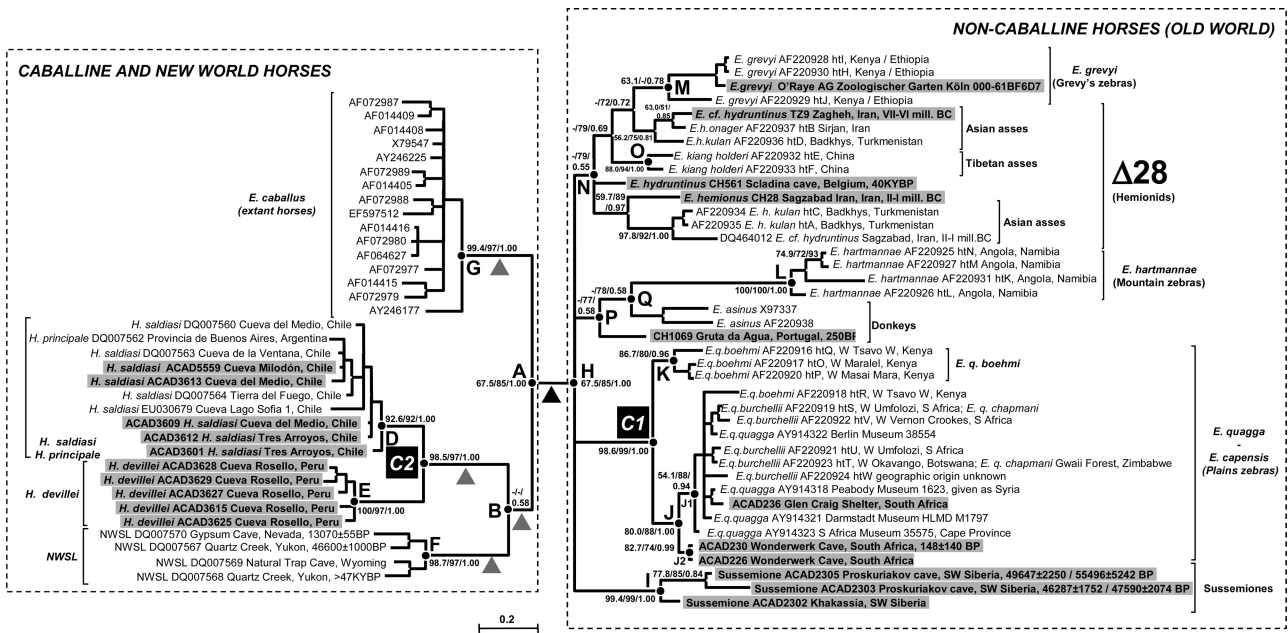


Fig. 1. Phylogenetic relationships among equids. Unrooted phylogenetic tree based on maximum likelihood analysis of the HVR-1 dataset. Sequences generated in this study are highlighted in gray. Numbers above/below branches correspond to node bootstrap values, approximate likelihood ratio test (aLRT SH-like), and Bayesian posterior probabilities. $\Delta 28$: 28-bp deletion in the mitochondrial HVR-1 (positions 15533–15560 according to the complete horse mitochondrial genome; GenBank accession no. X79547) (Fig. 2A). The potential locations of the root are reported with gray (alternative)/black (most likely) triangles according to likelihood-based topological tests (Fig. S4 and Table S5) and previous reports (15, 16). C1 and C2 nodes have been used as calibration points for molecular dating. Two radiocarbon dates were retrieved at the University of Colorado Laboratory for AMS Radiocarbon Preparation and Research (CURL) and the University of California-Irvine Accelerator Mass Spectrometry (UCIAMS) Facility for sussemione samples ACAD2303 and ACAD2305 (see *SI Text*).

such as hemion and donkeys (i.e., two noncaballine Old World equids), and less than that between hippidions and different *Equus* species (such as caballines horses or donkeys). We therefore support the Peruvian specimens as a distinct species within the same genus as hippidiforms from Patagonian/Chile. The extinct Peruvian taxa is referable to *Onohippidium devillei* according to the mean length and width of metatarsals, which are similar to the equids from the type locality of Tarija, Bolivia (33), and should be renamed *Hippidium devillei*. Our analysis therefore shows that *Onohippidium* is a junior synonym genus of *Hippidium* in agreement with Alberdi and Prado (34, 35) and in contrast to a reanalysis of facial characters, dental patterns, and metatarsal proportions (19). The molecular dating estimates (Table S4) suggest that the hippidiforms (from South America) separated from the North American taxa (caballines and NWSL) after 3.2–3.6 MYA, consistent with the final formation of the Panamanian isthmus \approx 3.0–3.7 MYA (36). Though we have only limited sample coverage, especially in Peru, we speculate that after this first vicariant event, the Peruvian *Hippidium* species may have specialized in higher altitude Andean habitats (almost all specimens from *H. devillei* come from high altitude environments) (37), and was genetically isolated from a more generalist species (*H. saldias/principale*) that extended from Brazil to Patagonia and showed large size plasticity (the adult weight of *H. principale* was twice that of *H. saldias*) (see refs. 35–37).

Africa. Four specimens identified as giant Cape zebra (*E. capensis*) were found to have good-quality DNA preservation. Three specimens were from Wonderwerk Cave, south of Kuruman in central southern Africa, and one from Glen Craig Shelter near Port Elizabeth on the southeast coast. Fragments up to 477-bp long could be amplified, and the six target HVR-1 and *cyt b* gene fragments were characterized (687 bp in total) apart from one Wonderwerk specimen (ACAD227), which yielded only the barcode fragment (see below and Table S1A). Maximum likelihood and Bayesian analyses placed the sequences amongst the plains

zebra group (Fig. 1, nodes C1 and J; Fig. S2B and C), which consists of six subspecies that lack population genetic structure (9) and includes the extinct quagga (8) (*E. q. quagga*). The Cape zebra sequences clustered amongst the two southern subspecies (*E. q. quagga*, *E. q. burchellii*), with the Wonderwerk specimens ACAD226 and ACAD230 exhibiting haplotypes outside the previously known variation (node J2), but inside that of the northern subspecies (*E. q. boehmi*, node K, with the exception of specimen AF220918). Although only limited sequence information was retrieved (Table S1A), sample ACAD227 showed minimum genetic distance with node K *E. q. boehmi* specimens (i.e., over 87 nucleotides, one substitution is observed versus four or more for J1/J2 haplotypes). In addition, the Glen Craig specimen (ACAD236) grouped within the southern clade, suggesting limited reproductive isolation (if any) amongst Cape quaggas and plains zebras, or potential issues with the morphological identification of the fossil material. In support of the latter possibility, radiocarbon dating of one Wonderwerk specimen (ACAD230) yielded a recent date (cal. 148 ± 120 BP) in contrast to an expected Pleistocene age. Though further specimens and dates are obviously required, all specimens morphologically identified as Cape zebras yielded sequences that grouped phylogenetically within plains zebras. The genetic distance between the giant Cape zebra and extant plains zebras is within the range of the intraspecific diversity found within other zebra species (11) (Fig. 2 and Table S3), and so we do not consider separate species (or subspecies) status is warranted (until independent information from nuclear loci is available). In contrast, Churcher (24) used the large size of giant Cape zebra fossils (estimated 400 kg and 150 cm height at the withers) to support conspecific status with Grevy's zebra, the largest extant wild equid. However, skulls are considered to be the best taxonomic indicators for equids at the morphological level (38), and the skull proportions of a giant Cape zebra from Elandsfontein (South Africa) showed a close affinity with true Cape quaggas (23), in accordance with our genetic findings. Together, these findings suggest that the giant Cape zebra

convergence observed within Late Pleistocene and Holocene equids. Of course, an mtDNA HVR genetic survey would be only a first step in identifying major taxonomic groups, which would need to be examined with morphological and other molecular studies. Indeed, the relatively short sequences we have generated cannot clearly resolve the exact branching order of major taxonomic groups and the bootstrap values, posterior probabilities, and approximate likelihood ratio tests (aLRT, SH-like) for these nodes were generally not strong. Recently, the use of whole mitochondrial genome sequences from Pleistocene fossils has helped resolve the rapid radiation events within elephantids (31) and ursids (47). Such a mitogenomic approach is likely to uncover the phylogenetic relationships within caballines and New World horses (Fig. 1, nodes A–G), and potentially within extant noncaballine horses as well, which are still poorly understood at the molecular level (Fig. 1, node H). For example, in Fig. 1, Grevy's zebras nest within paraphyletic hemionids, and mountain zebras do not cluster with plains zebras but with donkeys, in striking contrast with traditionally accepted taxonomy based on coat patterns, behavior, morphology, geographic separation (6, 7, 10, 48), and the 28-bp deletion (Fig. 1) (17). However, current datasets provide only moderate (if any) bootstrap and aLRT supports and posterior probabilities (Table S2) for these branches, and they should be regarded with caution. Besides, likelihood-based topological tests show significant support for the standard topology (in accordance with the current taxonomy) (KH and SH tests P values ≤ 0.003 for model E7, and KH test P value = 0.008 for model E7 vs. E6; Table S5). It seems likely that a (mito)genomic approach will be needed to solve major phylogenetic splits within equids and rapid radiation events (Table S4).

In this study, two species of extinct equids have been identified by mtDNA. The first is related to hippidiforms and corresponds to the paleontological genus *Onohippidium*, which should be reclassified within the *Hippidion* genus as *Hippidion devillei*. The second is a unique basal lineage of Old World equid, which appears morphologically related to the Middle Pleistocene *Sussemiones*, yet survived in southwestern Siberia at least until circa 45–50 KYBP. In addition, we propose to synonymize a variety of other species [Cape zebras, quaggas, and plains zebras (11, 13) and potentially *E. hydruntinus* and *E. hemionus*]. This reinforces similar suggestions for hippidiforms, NWSL, and caballids (15, 16, 18) and *E. hydruntinus* (17). This pattern of taxonomic oversplitting does not appear to be restricted to equids but is widespread amongst other Quaternary megafauna [e.g., Late Pleistocene bison (49); Holarctic cave lions (50); New World brown bears (51), and ratite moas (52, 53)]. Together, these findings suggest that the morphological plasticity of large terrestrial vertebrates across space and time has generally been underestimated, opening the way to detailed studies of the environmental, ecological, and epigenetic factors involved. Interestingly, in this regard the human lineage shows a rich fossil record over the last 6 MY, spreading over seven possible genera and 22 species (54). The exact number of taxonomic groups that should be recognized is still debated, even within our own genus (55), and in this context it is pertinent to consider the degree of taxonomic oversplitting, from species to generic levels, that aDNA has revealed amongst Late Pleistocene equids and other megafauna. A further important implication of this finding is that the number of megafaunal extinctions and loss of taxonomic diversity from the Pleistocene to modern day may not have been nearly as large as previously thought, at least at the species or subspecies level. Conversely, at the molecular level, aDNA studies on a wide range of large mammal taxa (49, 50, 56, 57) have revealed that the loss of genetic diversity over this time period has been much larger than previously recognized with major implications for the conservation biology of surviving populations (58).

Materials and Methods

aDNA Extraction, Amplification, and Sequencing. The samples were extracted, amplified, and sequenced in specialist aDNA laboratories in Lyon ($n = 27$) and Adelaide ($n = 24$) (Table S1 A and B) according to appropriate techniques and controls (29). In Lyon, a maximum of three different samples were coextracted at any time to limit the potential for cross-contamination. Mock extractions and the three different amplification controls were included in each experiment to monitor contamination. DNA was extracted using either a phenol/chloroform method (59, 60) or a silica-based method adapted from Rohland et al. (61). The latter method is designed to maximize recovery of PCR-amplifiable DNA from ancient bone and tooth specimens while minimizing coextraction of PCR inhibitors. A 183-bp fragment of the *cyt b* gene and six short overlapping fragments encompassing 546 bp of the horse mtDNA HVR-I (positions 15518–16063 from the complete horse mitochondrial genome; GenBank accession no. X79547) were targeted by PCR, as described in Orlando et al. (15, 18). All PCR products were cloned and sequenced, and a total of 330 PCR products and 2,230 clones were analyzed at Lyon (Table S1 A). In addition, 121 PCR amplicons (16 samples) were independently analyzed by direct sequencing at ACAD (two additional amplicons were cloned and sequenced for ACAD227; Table S1 A). For most samples, each sequence position was determined from at least two independent amplifications to avoid possible artefactual substitutions induced by DNA damage (27). However, for four samples (reported in italics in Table S1 A), it was only possible to amplify a certain fragment once. No individual site with a significant posterior probability of DNA damage could be detected in four independent BYPASS-degr analyses (62).

Sequence Analyses. The sequences reported here have been deposited in GenBank under accession nos. GQ324584–GQ324612 and GU062887. Available sequences from equid species were extracted from GenBank using the first 10,000 BLAST hits recovered from *Equus (Amerhippus) neogaeus* sequences (EU030680 and EU030681). Both gene fragments were aligned manually using the SeaView software (63). A final HVR-1 dataset was constructed to maximize the length of included sequences and to balance taxonomic sampling (SI Text). Unrooted and rooted phylogenies (GenBank accession nos. X97336 and Y07726 for *Rhinoceros unicornis* and *Ceratotherium simum*, respectively) were constructed using maximum likelihood and Bayesian Markov chain Monte Carlo methods (SI Text). A RY-recoded rooted dataset was used for further phylogenetic analyses to limit possible substitution saturation due to the deep divergence of rhinos and equids ≈ 55 MYA (Table S2). Another dataset consisting of all of the available equid sequences for the targeted 143-bp *cyt b* fragment was also generated and analyzed (Fig. S2D). Finally, HVR-1, HVR-2, and *cyt b* datasets were merged [see “merged consensus” described in Orlando et al. (17)]. All datasets were used for phylogenetic inference (Fig. S2 D–F, Table S2, and SI Text), and the monophyly of the principal nodes was assessed using likelihood-based statistical tests implemented in PAUP*4.0 [Kishino-Hasegawa (KH) and Shimodeira-Hasegawa (SH) tests; Fig. S4 and Table S5] (64).

To more accurately estimate the distributions of pairwise genetic distances within and between different taxonomic groups, two other HVR-1 datasets were generated of intermediate sequence length (243 and 301 bp, respectively, according to the reference sequence X79547), but with larger numbers of haplotypes (1,866 and 1,544, respectively). Uncorrected and corrected (GTR+ Γ +I) pairwise distance matrices were recovered from PAUP* 4.0b10 (64), and genetic diversity estimates were calculated and plotted using the R package (65). All datasets were automatically filtered for numts, and levels of missing data greater than 5% using a Perl script. Median-joining network were reconstructed for both datasets using Network (66), weighting all sites equally (Fig. S2 B and C). Bayesian analyses were applied to the unrooted HVR-1 dataset to assess divergence times, using BEAST (67) and assuming a HKY+ Γ +I model of molecular evolution. The age of specimens was recovered from either radiocarbon dating or the literature (SI Text, dating; Table S1 A). A calibration point was established by assuming the emergence of the hippidiform lineage occurred within a normal distribution centered at 2.5 ± 0.1 MYA (Fig. 1, node C1), based on fossil evidence (20). A second calibration point (Fig. 1, node C2) was placed at 0.7 ± 0.1 MYA for the emergence of plains zebras, documented by the fossil *E. mauritanus*, which has been unambiguously placed on the plain zebra lineage (10, 68, 69).

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