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Molecular phylogeny and evolution of the Perissodactyla

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The evolution of perissodactyls (rhinoceroses, tapirs, and horses) has been well studied primarily because of their extensive fossil record. Nevertheless, controversy persists regarding relationships of some of the extant taxa, reflecting inconsistencies between molecular and morphological studies. Here we examine the phylogenetic relationships of 16 living perissodactyl species by concatenating two mitochondrial and nine nuclear genes, and we estimate their divergence times using a relaxed Bayesian molecular clock approach. Our analyses recovered the monophyly of the suborders Ceratomorpha and Hippomorpha, and the families Rhinoceratidae, Tapiridae, and Equidae. We supported the early divergence of the Indian rhinoceros in the late Oligocene (26 Mya) relative to the Sumatran and African rhinoceroses, and the split of caballine (domestic horse and Przewalski's wild horse) and noncaballine equids (zebras and African and Asiatic asses) in the Pliocene (4 Mya). An important implication of this study is that *Equus asinus*, the African wild ass was found to be the sister taxon of Asiatic asses and zebras, diverging from the common ancestor with caballine horses 2 Mya. Rates of chromosome rearrangements were also evaluated in perissodactyls, placing a notably high rate of variation amongst equids, particularly within the zebra clade. The robust phylogenetic results presented here are relevant in terms of understanding the evolutionary history of this highly threatened group of mammals.

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ADDITIONAL KEYWORDS: chromosome evolution – horses – mitochondrial genes – nuclear genes – rhinoceroses – species tree – tapirs.

INTRODUCTION

The Perissodactyla comprises the odd-toed ungulates, a group of strict herbivores (browsers and grazers) adapted for running and dietary specialization. This order includes the family Equidae with eight extant species of horses, asses, and zebras in the genus *Equus*; the monogeneric family Tapiridae with four living species of tapirs; and the Rhinocerotidae with five surviving species of rhinoceroses in four genera (Table 1; Wilson & Reeder, 2005). Living perissodactyls represent a small remnant of a diverse group of mammals that arose during the late Palaeocene in North American and Asia, and became widely distributed except in Australia and Antarctica (Radinsky, 1966; McKenna & Bell, 1997).

The perissodactyls have evolved unique morphological adaptations in relation to other ungulates such as the mesaxonic foot, whereby the symmetry of extremities passes through the third digit (Hulbert, 1996). Another interesting characteristic is their rapid rate of chromosome evolution (Table 1; Wichman *et al.*, 1991; Trifonov *et al.*, 2008). In particular, equid chromosome numbers vary from $2n = 32$ in the mountain zebra to $2n = 66$ in the Przewalski's wild horse (Ryder, Epel & Benirschke, 1978). In tapirs, dramatic chromosomal differences occur between Old World ($2n = 50$) and New World species ($2n = 76$–80; Houck, Kingswood & Kumamoto, 2000).

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Table 1. Species diversity of extant perissodactyls. Scientific and common names, chromosome numbers, and conservation status are shown

Taxonomy	Common name	Chromosome number	Conservation status*
Perissodactyla			
Ceratomorpha			
Rhinocerotidae			
Diceros bicornis	Black rhinoceros	84	Critically Endangered
Ceratotherium simum	White rhinoceros	82	Near Threatened
Rhinoceros unicornis	Indian rhinoceros	82	Vulnerable
Dicerorhinus sumatrensis	Sumatran rhinoceros	82	Critically Endangered
Rhinoceros sondaicus†	Javanese rhinoceros		Critically Endangered
Tapiridae			
Tapirus indicus	Malayan tapir	52	Endangered
Tapirus terrestris	Lowland tapir	80	Vulnerable
Tapirus pinchaque	Mountain tapir	76	Endangered
Tapirus bairdii	Baird's tapir	80	Endangered
Hippomorpha			
Equidae			
Equus caballus	Domestic horse	64	
Equus przewalskii	Przewalski's wild horse	66	Critically Endangered
Equus asinus	African wild ass	62	Critically Endangered
Equus kiang	Kiang	51–52	Least Concern
Equus hemionus	Asiatic wild ass	54–56	Endangered
Equus zebra	Mountain zebra	32	Vulnerable
Equus burchelli	Burchell's zebra	44	Least Concern
Equus grevyi	Grevy's zebra	46	Endangered

*Conservation status obtained from the IUCN Red List of Threatened Species version 3.1 (http://www.iucnredlist.org).
†Species not included in the present survey.

Traditionally, the Perissodactyla have been divided in two suborders: Hippomorpha containing the family Equidae, and Ceratomorpha comprising the modern families Tapiridae and Rhinocerotidae (Radinsky, 1966; Prothero & Schoch, 1989). This classification was initially based on dental and osteological morphology, and soft anatomy (Prothero, Manning & Hanson, 1986; Prothero & Schoch, 1989), finding later support in molecular (Norman & Ashley, 2000; Murphy *et al*., 2001; Amrine-Madsen *et al*., 2003; Agnarsson & May-Collado, 2008; Arnason *et al*., 2008; Raterman & Springer, 2008) and cytogenetic (Trifonov *et al*., 2008) studies, with a few exceptions (Flint, Ryder & Clegg, 1990; Henry, Lance & Conlon, 1993; Madsen *et al*., 2002).

Only a limited number of molecular studies have addressed the phylogenetic relationships of all extant perissodactyl species. In general, surveys have primarily considered mitochondrial genetic markers for intrafamilial analyses (George & Ryder, 1986; Ashley, Norman & Stross, 1996; Norman & Ashley, 2000; Oakenfull, Lim & Ryder, 2000; Pitra & Veits, 2000; Tougard *et al*., 2001), deriving phylogenies with rather poorly supported nodes and divergence time estimates constrained by the molecular clock hypothesis. For instance in these studies, the phylogenetic position of Asian rhinoceroses (*Dicerorhinus sumatrensis* and *Rhinoceros unicornis*) within the Rhinocerotidae remained contentious (Morales & Melnick, 1994; Tougard *et al*., 2001; Fernando *et al*., 2006; Willerslev *et al*., 2009). Three hypotheses have been proposed regarding the phylogenetic relationships of extant rhinoceroses: (1) the Sumatran rhinoceros is closely related to the African rhinoceroses evidenced by these species having two horns instead of one (Loose, 1975; Morales & Melnick, 1994); (2) the Sumatran rhinoceros is the sister taxon of the Indian and Javanese rhinoceroses because of their overlapping geographical ranges (Groves, 1983); (3) the two African rhinoceroses, two Asian rhinoceroses and the Sumatran rhinoceros represent three different lineages (Prothero & Schoch, 1989; Cerdeno, 1995).

In extant tapirs, the relationship amongst New World tapirs remained unresolved with the Baird's tapir from Central America being associated with either South American tapirs or the Malayan tapir (Hulbert, 1995; Ashley *et al*., 1996; Norman & Ashley, 2000). In living equids, the phylogenetic positions of noncaballines (asses and zebras) have been controversial despite a large number of studies that include palaeogenetics

(Orlando *et al*., 2006, 2009), cytogenetics (Ryder & Chemnick, 1990), mitochondrial and nuclear DNA restriction fragment length polymorphisms (RFLPs; George & Ryder, 1986; Flint *et al*., 1990), and more recently the use of microsatellites (Krüger *et al*., 2005), nuclear (Oakenfull & Clegg, 1998) and mitochondrial sequencing data (Oakenfull & Ryder, 1998; Oakenfull *et al*., 2000). In general, genetic studies agree that caballines (domestic horse and Przewalski's wild horse) diverged early from noncaballines, but the relationships such as between the African wild ass and mountain zebra are inconclusively resolved (Oakenfull *et al*., 2000; Wallner *et al*., 2003).

The present work aims to examine the phylogenetic relationships of 16 perissodactyl species representing most of the extant taxa, by concatenating 11 mitochondrial and nuclear genes, and to estimate their divergence times using a relaxed Bayesian molecular clock method. Phylogenetic information is also used to evaluate rates of chromosome number variation amongst perissodactyl families. The understanding of the evolution and taxonomy of perissodactyls is not only of academic interest, but is essential for the appropriate design of conservation plans, as most of the extant species are currently threatened in the wild.

MATERIAL AND METHODS

Sampling

We examined a total of 16 extant species of perissodactyls (see Table 1), including two subspecies of *Equus hemionus*: *E. h. kulan* (kulan) and *E. h. onager* (onager). DNA samples were obtained from the DNA collection of the San Diego Zoo Institute for Conservation Research. Nucleotide sequences for the outgroups *Canis lupus* and *Bos taurus* were obtained from the Ensembl Genome Browser.

Mitochondrial and nuclear markers

We amplified partial sequences of ten genes from exonic and intronic regions, one mitochondrial [Cytochrome *b*, Cyt*b* (1140 bp)] and nine nuclear [*Breast cancer 1, Brca1* (994 bp); *Endothelin receptor type B, Ednrb* (533 bp); *V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog, Kit* (273 bp; 1215 bp); *Melanocortin 1 receptor, Mc1r* (954 bp); *Microphthalmia-associated transcription factor, Mitf* (402 bp); *Snail homolog 2, Snai2* (547 bp); *Sex determining region Y-box 10, Sox10* (505 bp); *T-box 15, Tbx15* (767 bp); *Tyrosinase, Tyr* (576 bp)]. New sequences have been deposited in GenBank (accession numbers JF718840–JF719026). Sequences from another mitochondrial gene, *12S rRNA* [365 bp] were obtained for all taxa from GenBank (accession numbers AF221583, AF221586, AF221588–AF221590, AF221592, AF221593, EU851885, NC012682, NC012684, Y07726, AY739618, AF038012, AF191834, AJ428947, AY012148). PCRs were performed in a 20 μL volume using Eppendorf Mastercycler Gradient thermal cyclers. Each reaction included 30 ng of template DNA, 10 μL of Taq buffer with 1.5 mM $MgCl_2$ (Applied Biosystems), 0.3 μL of 10 mM deoxynucleoside triphosphates, 0.6 μL lM of each primer, and 0.15 units AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR forward and reverse primers were designed in conserved exonic regions by using alignments containing Laurasiatheria mammalian sequences (Appendix S1). The PCR cycling conditions were 95 °C for 6 min, followed by 34 cycles of denaturation at 94 °C for 1 min, 50 °C annealing for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. We used the same PCR primers in the cycle sequencing reactions.

DNA sequences were edited and aligned using SEQUENCHER 3.1.1 (Gene Codes, Ann Arbor, MI) and CLUSTAL W2 (Labarga *et al*., 2007), and then adjusted by eye. Sequences were combined in a single data set using GENEIOUS v. 1.2.1 (Drummond *et al*., 2006) for a total of 8271 bp.

Phylogenetic analyses

MODELTEST 3.7 (Posada & Crandall, 1998) was used to select the model of molecular evolution of all genes according to the Akaike information criterion. We defined three genetic partitions based on the marker's mode of inheritance and rate of evolution corresponding to mitochondrial, nuclear intronic, and nuclear coding partitions. The model of molecular evolution identified for all three partitions was the general time reversible + gamma (G) + proportion invariant (I). Phylogenetic analyses were performed using MrBayes v. 3.1.1 (Ronquist & Huelsenbeck, 2003) and PAUP*v. 4.0b10 (Swofford, 2002). The Bayesian analysis was partitioned, the model parameters unlinked across partitions, and the amongst-partition rate variation accommodated using rate multipliers (Marshall, Simon & Buckley, 2006). Two concurrent runs consisted of four Markov chains (one cold and three heated chains with a temperature of 0.2), ten million generations (sampled every 1000 generations), and a 50% burn-in. We considered runs to have converged on stationarity when there were no trends in generation versus logL plots, potential scale reduction factors were near 1.0 for all parameters, and the average standard deviation of split frequencies was below 0.01. Posterior probabilities were calculated for each node.

Maximum likelihood (ML) trees were obtained with PAUP* using heuristic searches with 100 random addition sequences of taxa and the tree bisection-reconnection (TBR) branch swapping option. Under ML, estimated optimal parameters were used with

neighbour-joining starting trees and TBR branch swapping. For the combined data, we also used the site-specific rate option. Support for internal nodes was assessed by bootstrap analyses (Felsenstein, 1985), with 1000 replicates.

In order to assess compatibility amongst genetic partitions, we performed an incongruence length test (ILD; Farris *et al*., 1994) using PAUP* with 1000 replicates, implementing a significant threshold of $P < 0.01$. In addition, we employed a Shimodaira–Hasegawa (SH) test using 10 000 resampling estimated log-likelihood (RELL) bootstrap replicates ($P < 0.01$; Shimodaira & Hasegawa, 1999).

Molecular dating

We employed a likelihood ratio statistic in PAUP* for testing the molecular clock hypothesis in all genes independently and combined. The molecular clock hypothesis was rejected when $P < 0.001$. Multidivtime (Thorne & Kishino, 2002), the Bayesian relaxed molecular clock approach, was used to estimate divergence times by calculating branch lengths and the variance-covariance matrix from the mitochondrial and nuclear datasets using the Felsenstein (F84) + G model, with ML parameters obtained from PAML 4 v. 3.1 (Yang, 2007). Mean ages of divergence amongst taxa, with the associated standard deviation and 95% confidence intervals were then estimated by running five million generations, with burn-in set to 50%. Chains were sampled every 1000 generations. The following prior gamma distributions were adopted: a mean of 60 Mya for the expected time between tips and root under the absence of constraints on node times [and standard deviation (SD) of 30 Mya], that corresponds to the oldest perissodactyl represented by a fragmentary tooth of the brontotheriid *Lambdotherium* from the Palaeocene of China (Benton, Donoghue & Asher, 2009). The highest possible number of time units between tips and root was 100 Mya, which corresponds to an approximation of the divergence time proposed for the Laurasiatheria mammals (Murphy & Eizirik, 2009). Two calibration points were incorporated from the fossil record: the divergence between the Hippomorpha and Ceratomorpha suborders by early Eocene, lower 52–upper 58 Mya (Prothero & Schoch, 1989), and the modern diversification of the genus *Equus* in the late Pliocene, lower 2–upper 4 Mya (Hulbert, 1996; MacFadden, 2005).

Chromosome number variation

The evolution of chromosome number in perissodactyls was studied by examining data from Trifonov *et al*. (2008); their study reported the first genome-wide comparative chromosome maps of perissodactyls by multidirectional chromosome painting using paint probes derived from human, horse, Grevy's zebra, Malayan tapir, and white rhinoceros. Their data constitute 75 binary characters that correspond to all segmental associations or syntenic disruptions identified amongst perissodactyl chromosomes (fusion, fission, and inversion events).

We mapped chromosome characters on the Bayesian tree generated from the combined data set that includes perissodactyl species considered in the Trifonov *et al*. (2008) survey using MacClade 4.08 (Maddison & Maddison, 2005). We assumed all characters to be unordered and equally weighted, and we estimated the maximum number of changes. Ancestral chromosome number states from Trifonov *et al*. (2008) and the divergence times estimated in this study were then used to calculate rates of chromosome rearrangements per million years (R/Myr) in internal and terminal branches of the tree.

RESULTS

Phylogeny of perissodactyls

The relationships amongst perissodactyl species were initially assessed by comparing the phylogenetic signal of the two coding genetic partitions: mitochondrial (mt), containing two genes (Cyt*b* and *12S rRNA*; 1.5 kb total) and nuclear, including partial exonic sequences of nine genes (*Brca1*, *Ednrb*, *Kit*, *Mc1r*, *Mift*, *Snai2*, *Sox10*, *Tbx15*, and *Tyr*; 5.5 kb total). Data from the nuclear intronic partition alone were not compared because of its unresolved topology (multiple polytomies; data not shown). Within coding partitions, similar topologies were obtained from different approaches: maximum parsimony (MP; mt: Tree length, $T_L = 1483$, nuclear: $T_L = 1455$), ML (mt: -ln $L = 8295.93$, nuclear: -ln $L = 15275.77$), and Bayesian inference (BI; mt: -ln $L = 8309.75$, nuclear: -ln $L = 15295.11$). Bayesian topologies were compared between data sets, both showing the presence of three major clades corresponding to the perissodactyl families Equidae, Rhinocerotidae, and Tapiridae (Fig. 1). Inconsistencies between trees were observed in that equids and tapirs clustered together in the mitochondrial tree (Fig. 1A), in contrast to the grouping of Ceratomorpha (rhinoceroses plus tapirs) resulting from the nuclear coding data (Fig. 1B). In addition, some species switched phylogenetic positions within the Rhinocerotidae and Equidae clades (e.g. *D. sumatrensis*, *Equus kiang*). Results from the ILD and SH tests suggested that the mitochondrial and nuclear coding topologies are significantly different (ILD: $P = 0.43$; SH tests: $P < 0.01$). The mitochondrial partition seemed to provide stronger phylogenetic support for terminal nodes (e.g. *Equus burchelli–Equus grevyi* group), whereas the nuclear

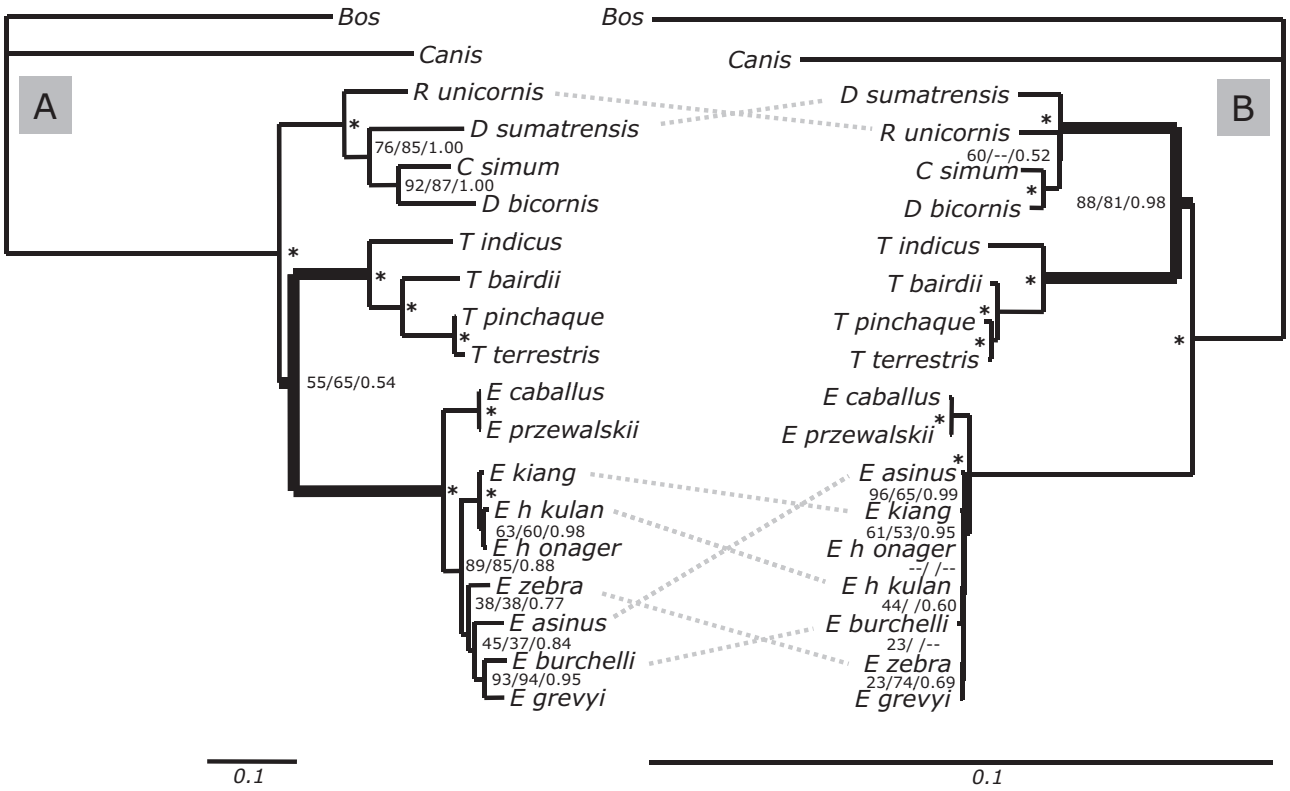

Figure 1. Comparison of Bayesian topologies obtained from (A) mitochondrial (-ln L = 8322.90) and (B) nuclear coding (-ln L = 15275.77) genetic partitions of perissodactyls. Maximum parsimony (MP) and maximum likelihood (ML) bootstrap (BP) and Bayesian posterior probabilities (PP) values are indicated for all nodes. An asterisk indicates the highest support for all three approaches [$BP_{MP} = BP_{ML} = 100\%$, PP = 1.00]. Dotted grey lines indicate species that have switched phylogenetic positions and bold lines incongruent clusters amongst topologies.

data better sustained internal nodes such as the one grouping all ceratomorphs [bootstrap, $BP_{MP} = 88$, $BP_{ML} = 81$, posterior probability (PP) = 0.98].

Phylogenetic analyses were also conducted for the complete data set of all genes utilizing three partitions: mitochondrial, nuclear coding, and nuclear intronic [intron 3, *Kit* gene (*KitI3*); Fig. 2]. Similar topologies were obtained for the combined data set from different approaches (MP: $T_L = 3586$; ML: -ln L = 29768.46; BI: -ln L = 28352.36). The combined Bayesian tree was similar to that obtained previously from the nuclear coding partition, suggesting a major contribution of the nuclear exonic genes in the phylogenetic signal. This is unexpected given that a higher number of substitutions per site were estimated in mitochondrial relative to nuclear genes (Fig. S1). Support by bootstrap and posterior probability values was relatively high for most of the nodes, with the exception of some relationships amongst equid taxa and the placement of the Sumatran rhinoceros (*D. sumatrensis*) amongst rhinoceroses.

The monophyly of the suborders Ceratomorpha and Hippomorpha, and the families Rhinoceratidae, Tapiridae, and Equidae was recovered in the combined analyses. Within the Rhinocerotidae, Asian rhinoceroses (*R. unicornis* and *D. sumatrensis*) appeared to diverge prior to the African rhinoceroses (*Ceratotherium simum* and *Diceros bicornis*), with the Indian rhinoceros as the most basal lineage. In the Tapiridae family, the Malayan tapir, *Tapirus indicus* was placed as the sister taxon of Central (*Tapirus bairdii*) and South American tapirs (*Tapirus pinchaque* and *Tapirus terrestris*), with both South American species forming a monophyletic group. In equids, the deepest divergence corresponded to the split between caballine (domestic horse and Przewalski's wild horse) and noncaballine equids (zebras and African and Asiatic asses). Within noncaballines, the African wild ass was identified as the sister taxon of two monophyletic clades composed of the Asiatic asses (*E. kiang* and *E. hemionus*) and zebras, with the mountain zebra (*Equus zebra*) having diverged first relative to Burchell's (*E. burchelli*) and Grevy's (*E. grevyi*) zebras.

Rare genomic changes or indels (insertions/deletions) were identified in intron 3 of the *Kit* gene of

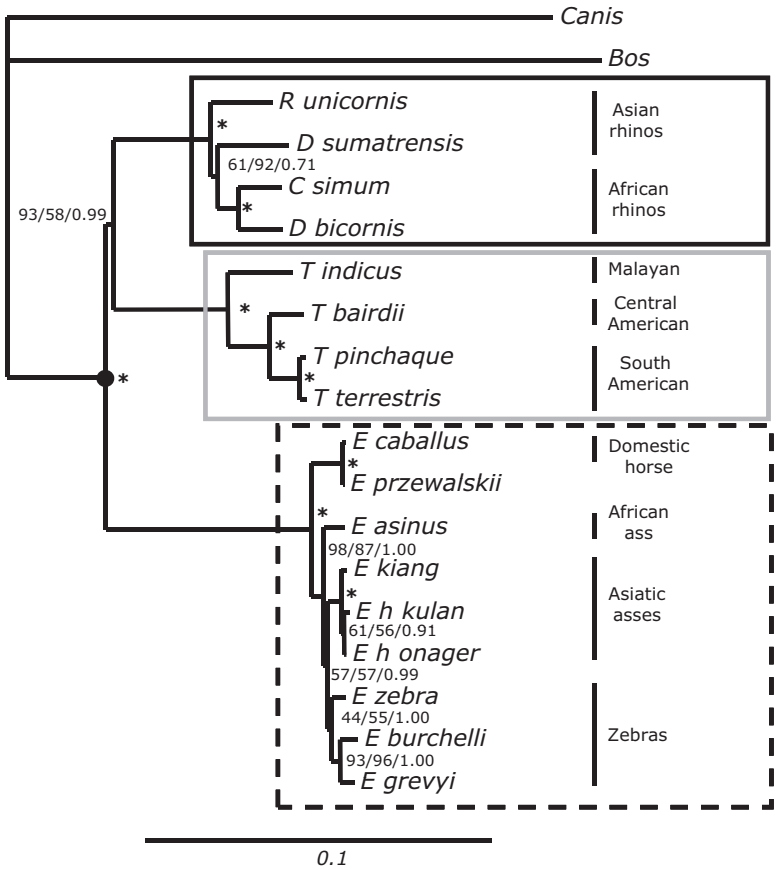

Figure 2. Bayesian tree (-ln $L = 29351.97$) of perissodactyls for the combined data. Maximum parsimony (MP) and maximum likelihood (ML) bootstrap (BP) and Bayesian posterior probability (PP) values are indicated for all nodes. An asterisk indicates the highest support for all three approaches ($BP_{MP} = BP_{ML} = 100\%$; $PP = 1.00$). Open boxes on the phylogram refer to Figure 3, which represents the *Kit* gene (intron 3) synapomorphic indels for the three families of perissodactyls: black for the Rhinocerotidae, grey for the Tapiridae, and black dashed lines for the Equidae.

perissodactyls (Fig. 3). Six unique deletions were observed supporting the Tapiridae family (8 bp deletion), Rhinocerotidae (3 bp), Equidae (4 bp), the suborder Ceratomorpha (tapirs plus rhinoceroses; 8 bp), and subclusters within the Equidae. These indels provide an independent and less homoplasic source of phylogenetic information, in particular for those nodes showing limited bootstrap support, such as the clade composed of zebras and Asiatic asses (6 bp deletion).

Divergence times

The molecular clock hypothesis was tested for all genes independently and for the combined data set. *Brca1*, *Mift*, *Tyr*, *KitI3*, Cyt*b*, and *12S rRNA*, and the combined data rejected the null hypothesis of equal rates of evolution in all lineages ($P < 0.001$; see Table S1). Accordingly, the combined data were analysed under a Bayesian relaxed molecular clock model using two calibration points (see Material and methods). The diversification of living perissodactyls was estimated to occur in the early Eocene, approximately 54 Mya (SD = 1.4; Fig. 4), followed by a rapid radiation of extant tapirs and rhinoceroses about 51 Mya (SD = 1.7). Asian rhinoceroses evolved in the late Oligocene, with the Indian rhinoceros diverging around 26 Mya (SD = 3.1), and the Sumatran rhinoceros at 25 Mya (SD = 3.4). African rhinoceroses evolved later in the middle Miocene around 17 Mya (SD = 3.1). In tapirs, the Malayan tapir diverged early in the late Oligocene about 25 Mya (SD = 3.0), simultaneously with Asian rhinoceroses, in contrast to a more recent diversification of American tapirs in the Miocene of Central America (11 Mya, SD = 2.7) and late Pliocene of South America (2.0 Mya, SD = 1.3).

Although a remarkable radiation of hippomorphs occurred during the Miocene according to the fossil record, the only extant genus of hippomorphs, *Equus*, was estimated to have a relatively recent origin in the

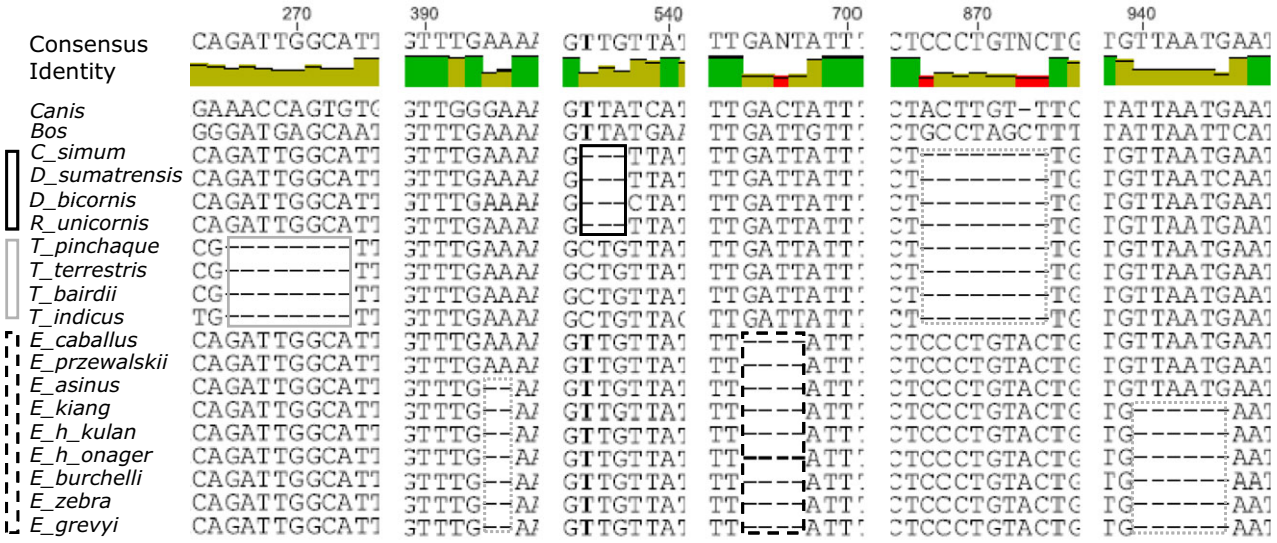

Figure 3. Synapomorphic deletions found in intron 3 of the *Kit* gene: 8 bp supporting the Tapiridae family (grey), 2 bp the noncaballines group (grey dotted lines), 3 bp the Rhinocerotidae (black), 4 bp the Equidae (black dashed lines), 8 bp the Ceratomorpha clade (grey dotted lines), and 6 bp the Asiatic asses and zebras group (grey dotted lines). Location of the deletions in the consensus sequence and the sequences identities are indicated at the top of the alignments.

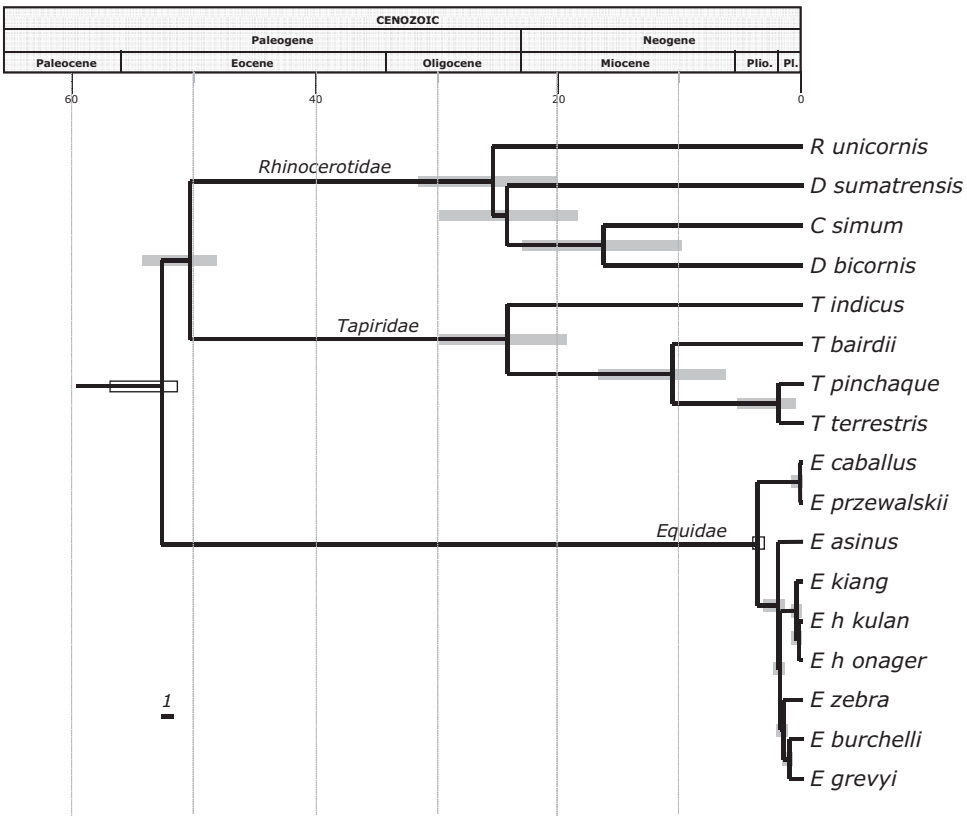

Figure 4. Divergence times estimated for perissodactyl species using the combined data set. Grey bars represent the 95% confidence interval obtained from Multidivtime. White bars represent the two calibration points: 52–58 Mya for the split between the Hippomorpha and Ceratomorpha suborders, and 2–4 Mya for the origin of modern horses (genus *Equus*).

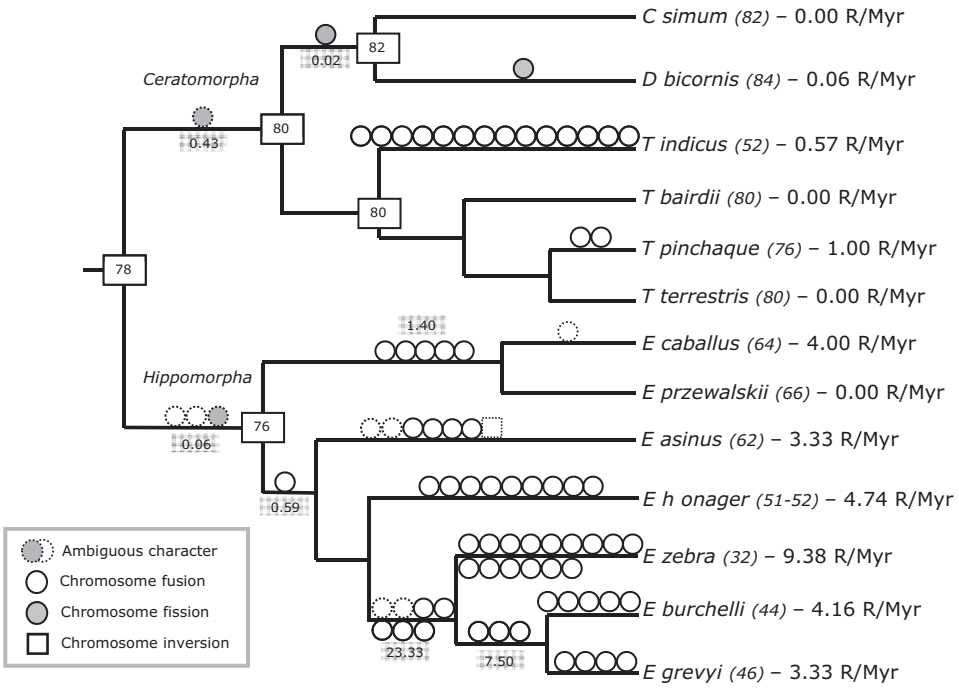

Figure 5. Mapping of chromosome rearrangements on the Bayesian tree (-ln $L = -26549.49$) of perissodactyl species included in the Trifonov *et al*. (2008) survey. Open circles represent chromosome fusions, grey circles fissions, open squares inversions, and dotted symbols ambiguous characters. Rates of chromosome evolution are shown for terminal and internal branches (small grey boxes). For some nodes, chromosome number ancestral states are indicated in open boxes. R/Myr, rate of chromosome rearrangements per million years.

Pliocene (3.8 Mya, SD = 0.1). Diversification within caballines was estimated to occur recently, around 250 000 years ago (SD = 0.2). The African wild ass diverged from its common ancestor with caballine horses at approximately 2.1 Mya (SD = 0.4), which is slightly prior to the diversification of Asiatic asses and zebras at about 1.9 Mya (SD = 0.4). Zebras diverged around 1.6 Mya (SD = 0.3), with the mountain zebra divergence being basal to the split between the Burchell's and Grevy's zebras about 1.2 Mya (SD = 0.3). Asiatic asses were estimated to diversify more recently in comparison to extant zebras, with the kiang around 500 000 years ago and Asiatic wild asses (onager and kulan) about 300 000 years ago.

Rates of chromosome evolution

Chromosomal rearrangements identified in Trifonov *et al*. (2008) were mapped onto a combined Bayesian tree that includes the perissodactyl species analysed in that study (-ln $L = -26549.49$; Fig. 5). Rates of chromosome evolution (rearrangements per million years) were estimated by incorporating the divergence times from the relaxed molecular clock method. Different evolutionary rates of chromosome rearrangement were observed amongst ceratomorphs and hippomorphs, with rhinoceroses and tapirs showing the lowest rates of chromosomal change ranging from zero to 1 R/Myr. In the Rhinocerotidae, only the black rhinoceros showed variation of the diploid number in relation to the ancestral state, represented by one fission event (0.06 R/Myr). In the Tapiridae, the Malayan tapir showed a high number of unique chromosomal rearrangements (14 fusions), but because of its ancient origin, the rate estimated was relatively low (0.57 R/Myr). Higher rates of chromosome rearrangements were calculated in the Equidae family ranging from zero (Przewalski's wild horse) to 9.38 R/Myr (mountain zebra). Amongst equids, lower rates of chromosome evolution were observed on the caballines' and noncaballines' internal branches, corresponding to 1.40 and 0.59 R/Myr, respectively. In contrast, higher rates of chromosome rearrangements represented primarily by chromosome fusions were mapped on the internal branch of the clade comprised of extant zebras (23.33 R/Myr) and on the terminal branches of the zebra clade. For instance, the species showing the highest rate of chromosome evolution amongst equids was the mountain zebra (9.38 R/Myr), with 15 fusion events. These calculations assume a uniform probability per unit time for the identified rearrangements, so that, for karyotypic changes that

have occurred more recently, higher average rates of chromosomal rearrangement are derived, as opposed to rates determined for longer branches (e.g. the Malayan tapir branch). We cannot eliminate the possibility that karyotypic changes occur discontinuously or sporadically within branch nodes or between a node and the tip of the tree.

DISCUSSION

Phylogenetic relationships of the Perissodactyla

The phylogenetic analyses performed in this study, combining nuclear and mitochondrial genetic markers, resolved the relationships amongst extant species of perissodactyls with high levels of confidence. Discrepancies observed between genetic partitions are likely to be the result of differences in gene properties, including base composition and substitution rates (as seen in Table S2; Brown, George & Wilson, 1979; Galtier & Gouy, 1995; Perna & Kocher, 1995), or to genetic/biological processes that occurred during the perissodactyls' speciation events, such as incomplete lineage sorting, genetic introgression or hybridization amongst species (Rokas & Carroll, 2006; Hallstrom & Janke, 2008).

The phylogenetic tree obtained from the combined analysis strongly supports the taxonomic status of the suborders Ceratomorpha and Hippomorpha, and of all three perissodactyl families (Rhinocerotidae, Tapiridae, and Equidae), as initially proposed by morphological studies (Prothero *et al*., 1986; Prothero & Schoch, 1989) and later confirmed by nuclear (Murphy *et al*., 2001; Amrine-Madsen *et al*., 2003; Raterman & Springer, 2008) and mitochondrial genetic data (Norman & Ashley, 2000; Agnarsson & May-Collado, 2008; Arnason *et al*., 2008).

The phylogenetic relationships found in the Rhinocerotidae support the monophyletic origin of two-horned rhinoceroses, the Sumatran and African species, and identified the one-horned condition present in the Indian rhinoceros as the ancestral state of extant rhinoceroses. The early divergence of the Indian rhinoceros has previously been documented by Norman & Ashley (2000) using mitochondrial DNA (*12S rRNA*). However, other molecular studies have proposed different phylogenetic inferences. For instance, Fernando *et al*. (2006) suggested that the Sumatran rhinoceros diverged first amongst Asian rhinoceroses, in contrast to Amato, Ashley & Gatesy (1993), and Tougard *et al*. (2001) who proposed the divergence of two monophyletic groups of rhinoceroses consisting of the Asian and African species. We have confirmed the phylogenetic separation of extant rhinoceroses into three different lineages: the two African species (white and black rhinoceroses), with the Sumatran rhinoceros as its sister taxon and the Indian rhinoceros as the most basally diverged lineage. The three-lineage hypothesis and the affinity of the Sumatran rhinoceros to African species have also been supported by the fossil record and morphological studies (Prothero & Schoch, 1989; Cerdeno, 1995). Our survey highlights the importance of including nuclear genetic markers for resolving the extant rhinoceroses' phylogeny, also proposed by other studies exclusively analysing mitochondrial genetic variation (Willerslev *et al*., 2009).

Within the Tapiridae, our estimates of an early divergence of the Malayan tapir and a more recent diversification of Central and South American tapirs are supported by previous mitochondrial DNA studies (Ashley *et al*., 1996; Norman & Ashley, 2000). However, the same authors found other most parsimonious trees that clustered together the Baird's tapir from Central America with the Malayan tapir. In addition, the fossil record and morphological data (Hulbert, 1995) initially grouped the Baird's tapir with the South American lowland tapir, inconsistent with a monophyletic origin of South American tapirs. The recent discovery and incorporation of new tapir fossils in morphological analyses (Ferrero & Noriega, 2007; Hulbert, 2010) provides support to the molecular inferences on the American tapir relationships that we describe here.

Amongst extant equids, the most controversial aspects of their evolution have been the phylogenetic positions of asses and zebras and the validation of an early diversification of caballines relative to noncaballines (Harris & Porter, 1980; Lowenstein & Ryder, 1985; George & Ryder, 1986; Oakenfull & Clegg, 1998). Our results support the taxonomic division of caballines and noncaballines initially proposed by morphological data (Forsten, 1992) and other molecular studies (Lowenstein & Ryder, 1985; George & Ryder, 1986; Oakenfull & Clegg, 1998; Krüger *et al*., 2005). Different scenarios have been proposed in the literature regarding the evolution of African and Asiatic asses. For instance, studies using postcranial morphology, RFLPs, protein electrophoresis, and mitochondrial and nuclear sequences have positioned the African wild ass as the sister taxon of zebras (Kaminski, 1979; Harris & Porter, 1980; George & Ryder, 1986; Oakenfull & Clegg, 1998; Oakenfull *et al*., 2000). In contrast, analyses of cranial and dental morphology (Eisenmann, 1979; Bennett, 1980) grouped together the onager and African wild ass as the sister group of zebras. However, these previous surveys obtained relatively poor levels of node support. The phylogenetic tree obtained in this study strongly supports most of the nodes within the Equidae, positioning the African wild ass (*E. asinus*) as the first diverged taxon amongst noncaballines.

This result has only been suggested by one previous study using nuclear DNA RFLPs (Flint *et al*., 1990).

The three extant zebra species represented a monophyletic group, with the mountain zebra placed as the sister taxon of Burchell's and Grevy's zebras. Most molecular studies support our phylogenetic results (Kaminski, 1979; George & Ryder, 1986; Ishida *et al*., 1995; Oakenfull *et al*., 2000), with exceptions primarily coming from the postcranial (Harris & Porter, 1980) and dental morphological data (Churcher & Richardson, 1978), which have failed to support the basal divergence of the mountain zebra. A paraphyletic origin of the extant zebra species has also been proposed in the literature using other approaches such as cranial morphology (Bennett, 1980), palaeogenetics (Orlando *et al*., 2006, 2008, 2009), and nuclear data (Flint *et al*., 1990; Wallner *et al*., 2003), but these have shown low levels of support.

Divergence times

The fossil record of perissodactyls is considered one of the most extensive and complete of all mammals, which provides a comparative framework for molecular dating estimations. Our study has estimated the divergence of rhinoceroses and tapirs around 51 Mya, which agrees with previous reports documenting the emergence of the first ceratomorph-like taxon (*Hyrachyus*) in the middle Eocene about 50 Mya (Prothero *et al*., 1986). Similar ages have also been suggested by mitochondrial DNA studies (Pitra & Veits, 2000), but with slightly lower estimations (Tougard *et al*., 2001; Arnason *et al*., 2008).

The diversification of extant rhinoceroses in the late Oligocene (~26 Mya) matches the age proposed by other surveys using mitochondrial (Xu & Arnason, 1997; Tougard *et al*., 2001) and allozyme data (Merenlender *et al*., 1989). However, other molecular studies have also suggested slightly older (34 Mya) or more recent (22 Mya) divergence times (Morales & Melnick, 1994; Pitra & Veits, 2000; Willerslev *et al*., 2009). Our study is consistent with the fossil record, which identifies the first rhinocerotids in the late Eocene of Eurasia (Lacombat, 2005). Other fossils have also been reported around 10 and 15 Mya, representing the origins of the Indian and Sumatran rhinoceroses, respectively (Carroll, 1988; Lacombat, 2005). The origin and evolution of African rhinoceroses at about 17 Mya is supported by mitochondrial DNA data (Tougard *et al*., 2001) and the fossil record. In particular, the divergence of the genus *Diceros* is known to have occurred in the late Miocene of Europe and North Africa (Hillman-Smith & Groves, 1994), resulting in the coexistence of the two genera of African rhinoceroses (*Ceratotherium* and *Diceros*) during the early Pliocene about 5 Mya (Groves, 1972).

The estimated divergence time of the extant Tapiridae in the late Oligocene agrees with dates of between 20 and 30 Mya previously proposed by mitochondrial surveys (Ashley *et al*., 1996; Norman & Ashley, 2000; Pitra & Veits, 2000). This interval also overlaps with palaeontological records that have documented the first representatives of true tapirs (Tapiridae) from the Oligocene of North America and Europe (Schoch, 1989). The genus *Tapirus* extends from late Miocene in North America and Europe (Padilla & Dowler, 1994; Hulbert, 2005), whereas in South America the earliest known record is from the early Pleistocene of Argentina (Tonni, 1992). Our data suggest a scenario of an early divergence in Asia of the Malayan tapir and migration from North to Central America of the ancestor of the extant Central and South American tapirs during the late Miocene. The last diversification event of extant tapirs then took place in South America after the emergence of the Isthmus of Panama about 3 Mya, as proposed by Ashley *et al*. (1996).

In the Equidae, the early split of caballines relative to the other equids about 4 Mya does not seem to be supported by palaeontological data as their presence in North America has been reported later, at approximately 2 Mya (Eisenmann, 1992; Forsten, 1992), and because *Equus ferus*, the precursor of the domestic horse, first appeared in the late Pleistocene of North America (1.1–1.2 Mya; Azzaroli & Voorhies, 1993) and middle Pleistocene of Eurasia (1.0–0.4 Mya; Azzaroli, 1983). Within caballines, the divergence of the domestic horse and Przewalski's wild horse around 250 000 years old overlaps with estimates from previous studies on horse evolution using mitochondrial (Oakenfull *et al*., 2000), nuclear (Wallner *et al*., 2003) and microsatellite data (Krüger *et al*., 2005) that have suggested their split between 80 000–380 000 years ago.

The divergence of the African wild ass, estimated from our data at around 2 Mya has conflicting support. For example, the mitochondrial DNA RFLP study by George & Ryder (1986) suggested a more ancient divergence of the African wild ass about 3.9–3.4 Mya, whereas Krüger *et al*. (2005) calculated a more recent origin by coalescent analyses of nuclear data that ranges from 0.49–1.5 Mya. In addition, our results do not seem to be supported by the palaeontological record, as no African ass fossils have been found in eastern and central Asia from that time. Fossil evidence for extant asses in Asia first appeared during the middle or late Pleistocene about a million years ago, and in Africa between 0.5–1.7 Mya (Forsten, 1992; Bernor *et al*., 2010). However, the genus *Equus* has been reported in eastern Africa around 2.3 Mya (Bernor *et al*., 2010), suggesting that ancestral noncaballine horses prob-

ably appeared first in Africa. Thus our data support that the origin of the African wild ass came after the first dispersal event of the genus *Equus* from North America to Eurasia, proposed to have occurred at about 2.6 Mya (Lindsay, Opdyke & Johnson, 1980).

The phylogenetic split of zebras and Asiatic asses estimated about 1.9 Mya appears more ancient than dates estimated in a previous analysis using nuclear markers (~0.9 Mya; Oakenfull & Clegg, 1998). However, it matches palaeontological records that have documented the first dispersion of noncaballines from Eurasia to Africa around 2 Mya (Forsten, 1992). The diversification of extant zebras occurred more recently (~1.6 Mya), as supported by the first reliable fossils of *E. burchelli* in Africa around 0.7 Mya (Eisenmann, 1992) and Grevy-type zebra fossils known from the early Pleistocene in Africa (Churcher, 1993). The fossils of the mountain zebra are rather sparse, with the oldest reported record from southern Africa at about 0.6 Mya (Forsten, 1992).

Finally, the diversification of Asiatic asses about 500 000 years ago is consistent with mitochondrial DNA data (Ryder & Chemnick, 1990). This age indicates that the Asiatic asses diversified after the migration of the African wild ass ancestor into Africa around 1.6 Mya (Lindsay *et al*., 1980). An alternative and more likely hypothesis, according to the ancient fossil record of *Equus* in Africa (Bernor *et al*., 2010) is that Asiatic asses occurred after an African-like ass emigrated from Africa into central Asia. The fossil record for Asiatic asses includes an extinct species of Pleistocene equid, *Equus conversidens*, a species similar to *E. kiang* (St-Louis & Cote, 2009). The divergence of *E. hemionus* around 300 000 years ago is supported by palaeontological records that report Asiatic wild asses in Asia during the middle and late Pleistocene (Grinder, Krausman & Hoffmann, 2006).

Chromosome evolution

Well-supported phylogenies improve the understanding of the evolution of phenotypic traits by allowing the reconstruction of ancestral states and character mapping across lineages. Chromosome number variation in perissodactyls has been the subject of great interest because of their rapid rate of chromosome evolution (Wichman *et al*., 1991; Trifonov *et al*., 2008), especially in horses (Ryder *et al*., 1978). Numerous studies have documented chromosome variation and synteny amongst perissodactyl species (Ryder *et al*., 1978; Ryder & Chemnick, 1990; Houck *et al*., 1994; Houck *et al*., 2000; Trifonov *et al*., 2003; Yang *et al*., 2003). However, only two major studies have focused on incorporating phylogenetic information and divergence times into efforts to deduce accurate rates of chromosome evolution in perissodactyls (Bush *et al*., 1977; Trifonov *et al*., 2008). In our study, using phylogenetic and dating information, we estimated new rates of chromosome rearrangements in perissodactyls in comparison to results obtained by Trifonov *et al*. (2008). Similar rates of chromosome evolution were found in the Rhinocerotidae and Tapiridae families, but variation was observed amongst equids. For instance, we estimated lower rates of chromosome rearrangements in all terminal branches of the zebra species (e.g. *E. zebra*: 9.38 versus 20 R/Myr), but higher rates in the internal branches (e.g. 23.3 R/Myr). Discrepancies with previous reports are likely to be because of differences in the phylogenetic relationships and divergence times used to map chromosome traits. The rapid rate of chromosome evolution in zebras seems to be concomitant with speciation and dispersion events of the genus *Equus* occurring in Africa in the late Pliocene and early Pleistocene (Bernor *et al*., 2010). Comparative chromosome maps of the kiang and other *E. hemionus* subspecies are needed to interpret better the rates of chromosome evolution in the recently diverged Asiatic asses.

Comments on perissodactyl conservation

The role of molecular genetics and phylogenetics in species conservation appears crucial not only for clarifying species relationships and boundaries, but in facilitating the understanding of species' evolutionary history and processes contributing to speciation (Ryder, 2005). For instance, the identification of ancestral lineages such as the Indian rhinoceros (Rhinocerotidae) and the Malayan tapir (Tapiridae) that potentially preserve 'ancient genes' shared by other more recently diverged species is fundamental for undertaking biodiversity conservation strategies. Furthermore, the recognition of recently diversified species such as zebras and Asiatic asses opens new opportunities to address questions about potential speciation processes and hybridization events currently happening in nature (Cordingley *et al*., 2009; Ryder & Steiner, 2009), and important for defining species boundaries. In conclusion, the development of a robust phylogenetic framework of perissodactyl evolution is critical for future species' taxonomic revisions and the potential readjustment of global conservation priorities (Moehlman, 2001).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Number of expected substitutions per site estimated in all perissodactyl families for the three genetic partitions: mitochondrial (black), nuclear coding (grey), and nuclear intronic (white). Values were obtained using PAUP* from patristic distances, considering three subsets of data that correspond to each perissodactyl family. A star on the phylogenetic tree indicates the highest support for all three approaches [bootstrap $_{\text{maximum parsimony}}$ (BP_{MP}) = BP $_{\text{maximum likelihood}}$ = 100%, posterior probability = 1.00].

Table S1. Likelihood ratio test performed on independent and concatenated genes of perissodactyls. Likelihood values for the best tree and the alternative tree (under the molecular clock assumption) are shown with the *P*-values. Significant differences are highlighted in bold.

Table S2. Genetic characterization of mitochondrial and nuclear genes. Parameters considered are: total sequence length, number and proportion of parsimony-informative characters (No. of sites, Var.), nucleotide frequencies (% A, C, G, T), relative nucleotide rate substitution (AG, TC, CA, AT, CG, GT), amongst-site rate heterogeneity (alpha parameter), proportion of invariable sites (Inv. sites), transition/transversion ratio (TI/TV), consistency index (CI), and saturation (S).

Appendix S1. List of primers used to amplify partial sequences of mitochondrial and nuclear genes.