Phylogenetics of Perissodactyla and Tests of the Molecular Clock

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Abstract. Two mitochondrial genes, the protein-coding cytochrome *c* oxidase subunit II (COII) gene and a portion of the 12S rRNA gene, were used for phylogenetic investigation of the mammalian order Perissodactyla. The primary objective of the study was to utilize the extensive fossil record of perissodactyls for calibrating molecular clocks and comparing estimates of divergence times using both genes and two fossil calibration points. Secondary objectives included clarification of previously unresolved relationships within Tapiridae and comparison of the results of separate and combined analyses of two genes. Analyses included several perissodactyl lineages representing all three families (Tapiridae, Equidae, and Rhinocerotidae), most extant genera, all four species of tapirs, two to four species of rhinoceros, and two species of *Equus.* The application of a relatively recent fossil calibration point and a relatively ancient calibration point produced greatly different estimates of evolutionary rates and divergence times for both genes, even though a relative rates test did not find significant rate differences among taxa. A likelihood-ratio test, however, rejected a molecular clock for both genes. Neither calibration point produced estimates of divergence times consistent with paleontological evidence over a range of perissodactyl radiations. The combined analysis of both genes produces a well-resolved phylogeny with Perissodactyla that conforms to traditional views of interfamilial relationships and supports monophyly of neotropical tapirs. Combining the data sets increases support for most nodes but decreases the support for a neotropical tapir clade because the COII and 12S rRNA data sets are in conflict for tapir relationships.

Key words: Perissodactyl — Tapirs — Mitochondrial DNA — 12S rRNA — Cytochrome oxidase II — Evolutionary rates — Molecular clock

Introduction

The mammalian order Perissodactyla, the odd-toed ungulates, contains 17 extant species divided into three quite distinct families, Equidae (horses, zebras and asses), Rhinocerotidae (rhinoceros), and Tapiridae (tapirs). Arguments have been made for the inclusion of Hyracoidea (hydraxes) within Perissodactyla and the designation of a group composed of perissodactyls exclusive of hyraxes called Mesaxonia (Schoch 1989). Also, some investigators have placed Hyracoidea as the sister taxon to Perissodactyla (Fischer 1989; Prothero et al. 1988), while others have considered Hyracoidea to belong in the group Paenungulata as the sister taxon to Tethytheria, a clade that consists of proboscideans, sirenians, and several fossil groups (McKenna 1975). Recent molecular studies from a number of different genes have supported the monophyly of Paenungulata (Porter et al. 1996; Stanhope et al. 1996, 1998a, b; Springer et al. 1997a, b, 1999), so a sister-group relationship between hyraxes and perissodactyls seems doubtful. We therefore focus on intraordinal and intrafamilial relationships among traditional perissodactyls. Perissodactyls are strict herbivores (browsers or grazers) adapted for running. The nature of the running adaptation is an important synapomorphic character for the order that involved a

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Table 1. Species, common names, and references used in this study

Taxon	Common name	Reference(s)
Tapirus indicus	Malayan tapir	(COII) Ashley et al. (1996); (12S) this study
Tapirus pinchaque	Mountain tapir	(COII) Ashley et al. (1996); (12S) this study
Tapirus bairdii	Baird's tapir	(COII) Ashley et al. (1996); (12S) this study
Tapirus terrestris	Lowland tapir	(COII) Ashley et al. (1996); (12S) this study
Equus caballus	Horse	Xu and Arnason (1994)
Equus asinus	Donkey	Xu et al. (1996)
Dicerorhinus sumatrensis	Sumatran rhino	(12S) Amato et al. (1993)
Rhinoceros unicornis	Indian rhino	Xu et al. (1996)
Diceros bicornis	Black rhino	(12S) this study
Ceratotherium simum	White rhino	(COII) Xu and Arnason (1997); (12S) Douzery and Catzeflis (1995)
Balaenoptera physalus	Fin whale	Arnason et al. (1991)
Balaenoptera musculus	Blue whale	Arnason and Gullberg (1993)
Sus scrofa	Domestic pig	(COII) Ursing and Arnason (1998)
Tayassu tajacu	Collared peccary	(12S) Douzery and Catzeflis (1995)
Odocoileus virginianus	White-tailed deer	(COII) Honeycutt et al. (1995); (12S) Miyamoto et al. (1990)
Bos taurus	Cow	Anderson et al. (1982)
Gazella thomsoni	Thomson's gazelle	(12S) Allard et al. (1992)
Gazella spekei	Speke's gazelle	(COII) Honeycutt et al. (1995)

remodeling of the ankle joint with restricted lateral movement (Radinsky, 1969). Living perissodactyls represent a small remnant of a diverse, speciose group that arose during the late Paleocene (Radinsky, 1969) or early Eocene (Gingerich 1991) and became widely distributed. Today several members of each family are threatened with extinction.

Perissodactyla has an abundant fossil record compared to other mammals, which has allowed extensive investigation and knowledge of their evolutionary history (e.g., Simpson, 1945b, 1951; Radinsky 1965; Forsten 1989; Prothero and Schoch 1989a and references therein; MacFadden 1992). Traditionally, Perissodactyla has been divided into two suborders: Hippomorpha for the superfamily Equoidae (and two extinct superfamilies) and Ceratomorpha for the modern superfamilies Tapiroidea and Rhinocerotoidae (Wood 1937; Simpson 1945a; Radinsky 1966). The grouping of tapiroids and rhinocerotoids in the suborder Ceratomorpha has been generally well accepted, based primarily on dental characters of fossil and modern taxa. This study examines relationships within Perissodactyla using representatives of all three extant families. We chose to investigate perissodactyl relationships using both separate and combined analyses of the two linked mitochondrial genes in order to compare the single gene phylogenies with each other and with a combined gene phylogeny. Equid relationships have recently been addressed with molecular data (Flint et al. 1990; Ryder and Chemnick 1990; Ishida et al. 1995), as have those among rhinoceros (Amato et al. 1993; Morales and Melnick 1994; Xu and Arnason 1997). Less attention has been paid to Tapiridae. The four extant species of tapirs are generally considered to belong to a single genus, *Tapirus.* They have a strikingly disjunct distribution, with three species being neotropical (*T. bairdii, T. pinchaque,* and *T. terrestris*) and one spe-

cies Asiatic (*T. indicus*). In a previous cytochrome *c* oxidase subunit II (COII) gene study, the position of *T. bairdii* was unresolved (Ashley et al. 1996). To clarify tapir relationships, here we expand our study to include a second mitochondrial gene (12S rRNA) and additional outgroups.

The use of a molecular clock to estimate divergence times has been controversial since it was first suggested (Margoliash 1963; Zuckerkandl and Pauling 1965). It is now well accepted that different genomes (e.g., mitochondrial vs nuclear) within organisms, genes within genomes, and taxa or clades of organisms evolve at different rates (Li 1997 and references therein). Hillis et al. (1996) argue that even for a perfect molecular clock following a Poisson process, stochastic variation causes the clock to be extremely imprecise for estimating divergence dates. Nevertheless, arguments have been made in support of a mammalian molecular clock (Easteal et al. 1995) as well as local molecular clocks within taxonomic groups (e.g., Bailey et al. 1991; O'hUigin and Li 1992; Schneider et al. 1993) and molecular clocks continue to be routinely applied. Although the paleontological record has been used to compare rates of molecular evolution between unrelated taxa (e.g., Martin et al. 1992), rarely has the fossil record been used to examine rates at different times within a single taxonomic group. We investigated the utility and accuracy of a molecular clock using perissodactyl mitochondrial gene sequences together with the multiple, well-supported divergence dates offered by the perissodactyl fossil record. A recent calibration point (divergence of extant equid species at 3 MYA) and a relatively ancient calibration point (divergence of Ceratomorpha and Hippomorpha at 50 MYA) were used to compare independently calibrated rates of molecular evolution and dates of divergence using both the COII and the 12S rRNA genes.

Table 2. Pairwise COII absolute numbers of nucleotide differences (above diagonal) and HKY85-corrected distances (below diagonal)[a]

	R. unicornis	C. simum	E. asinus	E. caballus	T. pinchaque	T. indicus	T. terrestris
R. unicornis	—	72	121	107	101	102	99
C. simum	0.13378	—	106	100	101	97	99
E. asinus	0.25152	0.20582	—	49	115	120	115
E. caballus	0.21022	0.19019	0.0839	—	108	118	108
T. pinchaque	0.21428	0.21687	0.25076	0.23093	—	57	8
T. indicus	0.22261	0.20749	0.27062	0.26559	0.10851	—	53
T. terrestris	0.20546	0.20784	0.24744	0.22797	0.01273	0.09905	—
T. bairdii	0.21392	0.23691	0.24799	0.23199	0.09754	0.09213	0.09198
Suina	0.24707	0.19916	0.24681	0.25161	0.24451	0.24199	0.2444
B. taurus	0.26004	0.21654	0.24888	0.25153	0.29458	0.26741	0.28294
Gazella	0.2713	0.21759	0.22326	0.2561	0.24209	0.2446	0.24157
O. virginianus	0.25281	0.26062	0.28163	0.28791	0.27416	0.27154	0.26306
B. musculus	0.25396	0.21237	0.25019	0.22325	0.23959	0.23056	0.23641
B. physalus	0.26893	0.23763	0.28304	0.28258	0.27247	0.24075	0.2651

[a] See text for parameters used for calculating HKY85-corrected distances.

Table 2. Extended

	T. bairdii	Suina	B. taurus	Gazella	O. virginianus	B. musculus	B. physalus
R. unicornis	102	121	126	129	124	123	128
C. simum	109	104	111	111	126	109	111
E. asinus	116	122	122	113	132	123	134
E. caballus	110	124	123	124	134	114	134
T. pinchaque	52	115	129	114	122	112	122
T. indicus	50	113	121	114	121	108	111
T. terrestris	50	116	127	115	120	112	121
T. bairdii	—	122	133	124	133	110	120
Suina	0.25904	—	122	128	113	120	122
B. taurus	0.30047	0.24463	—	108	104	114	120
Gazella	0.26574	0.26691	0.22415	—	104	121	115
O. virginianus	0.30267	0.22237	0.21084	0.21093	—	117	122
B. musculus	0.22772	0.23836	0.22326	0.2503	0.23537	—	57
B. physalus	0.25862	0.24336	0.23991	0.23081	0.24988	0.10095	—

Materials and Methods

The taxon names and sources of specimens of the newly reported 12S rRNA sequences are given in Table 1 along with previously published COII and 12SrRNA sequences used in our analyses. Total genomic DNA was isolated from white blood cells or frozen tissue samples following standard protocols of proteinase K digestion and phenol/chloroform extraction. A portion of the 12S rRNA gene was amplified using conserved mitochondrial primers that amplify an approximately 490-bp region of the third domain of the 12S rRNA gene (Kocher et al. 1989). Double-stranded PCR amplifications were performed in 50-μl reaction volumes that contained a 100 n*M* concentration of each primer, a 200 μ*M* concentration of dNTPs, 0.25 U of Taq polymerase, 1× reaction buffer, and approximately 50 ng of genomic DNA. Amplification proceeded for 30 cycles of 94°C for 1 min., 50–55°C for 1 min., and 72°C for 2 min. Five microliters of PCR reaction product was electrophoresed in 1% agarose gels and stained with ethidium bromide for visualization. The remaining product from successful PCR reactions was purified by digestion with exonuclease I and shrimp alkaline phosphatase (United States Biochemical). Using the PCR primers, both strands of double-stranded products were sequenced directly using standard dideoxy chain-termination methods with the Sequenase PCR Product Sequencing Kit (United States Biochemical).

In two instances, chimeric sequences were constructed to represent outgroup lineages where data were not available for both genes from a single species. These were both artiodactyl lineages (used for outgroups; below) and consist of *Sus scrofa* COII and *Tayassu tajacu* 12S rRNA to represent the infraorder Suina and *Gazella thompsoni* 12S rRNA and *Gazella spekei* COII to represent the genus *Gazella*. Sequences were entered and aligned using the MacVector program (IBI). The 12S rRNA sequences were further aligned manually using a secondary structure model (Springer and Douzery 1996) for first aligning stem regions and then aligning the loop regions between stems (Kjer 1995). Seventeen bases (positions 329–345) in the 12S rRNA data could not be aligned unambiguously and were deleted from analyses. There were no informative indels in this data set.

Nucleotide compositional bias was calculated by the formula given by Irwin et al. (1991). Heterogeneity of nucleotide base composition between taxa for both genes was assessed using a chi-square test implemented in PAUP* 4.0d58–60, written by David L. Swofford. This test was performed on the data from each gene as well as data partitions within genes. Data partitions tested included stem and loop subsets of 12S rRNA and codon positions for COII. All phylogenetic analyses were performed using PAUP*4.0. To correct for nucleotide compositional differences in the third codon position of the COII gene, the LogDet transformation (Lockhart et al. 1994) was applied and a neighbor-joining analysis (Saitou and Nei 1987) was performed on the COII data. All maximum-parsimony (MP) analyses were performed using the branch-and-bound algorithm. Artiodactyl and cetacean sequences were designated as outgroups in all phylogenetic analyses. Cetacean sequences were included because recent molecular systematic studies

have shown Cetacea to be nested within or as a sister group to Artio-dactyla (Irwin et al. 1991; Graur and Higgins 1994; Gatesy et al. 1996; Montgelard et al. 1997), and we wanted to sample widely within the outgroup in order better to polarize characters within the ingroup. We recognize that the relative positions of Artiodactyla and Perissodactyla, as well as many mammalian interordinal relationships, are poorly re-solved. Although some recent molecular studies support their affinity (Graur et al. 1997; Irwin et al. 1991), we are not assuming a sister-group relationship between these two orders. Usually, the sister taxon to the study group is the best outgroup choice, but since this is unknown for Perissodactyla, we chose a mammalian group that was well repre-sented in the databases for both genes. Sequences of 12S rRNA are available from some paenungulate taxa (African elephant, dugong, and hyrax), and we also performed preliminary MP analyses using these as outgroup taxa. There was no difference in the phylogenies produced with paenungulates versus artiodactyls and cetaceans as outgroups (data not shown).

Maximum-likelihood analyses for 12S rRNA was performed as-suming a gamma distribution with four rate categories and estimating the gamma parameter, base frequencies, transition/transversion ratio, and number of invariable sites. In the case of the COII data, maximum likelihood was performed using estimated parameters as measured from a neighbor-joining tree (Saitou and Nei 1987) constructed with maximum-likelihood distance estimates. Bootstrap analysis of 1000 replications of heuristic MP searches were used to determine support for various nodes. Decay values were generated by the program Au-todecay (T. Eriksson, unpublished) and used in addition to the boot-strap as a measure of support for the various nodes.

Distances used for estimating evolutionary rates and divergence times were corrected using the Hasegawa–Kishino–Yano method (HKY85) assuming a gamma distribution. Gamma parameters and tran-sition:transversion ratios were taken from the maximum-likelihood analyses. The partition homogeneity test [originally called the incon-gruence length difference test (Farris et al. 1994)] was performed on the 12S rRNA and COII data in order to detect conflicting signals using PAUP* 4.0. This test compares the incongruence length difference (ILD), the difference in the number of steps in separate and combined analyses of the original partitions, to a series of randomized partitions generated from the data (Farris et al. 1994).

Differences in relative rates of COII and 12S rRNA evolution among Perissodactyla taxa were tested by the two-dimensional (2D) method of Tajima (1993). The *Bos taurus* sequence was used as the outgroup sequence in the relative rates tests. The results of the 2D test were corrected for multiple comparisons using the sequential Bonfer-roni method (Rice 1989). Likelihood-ratio tests were performed assum-ing three tree topologies for each gene to test for the presence of rate heterogeneity among sequences. These tests can identify significant differences in the likelihood values ($-\ln$ likelihood) of a tree topology with versus without a molecular clock assumption. Yang et al. (1995) conclude that a likelihood-ratio test for a molecular clock can be per-formed even if the true tree is unknown but suggest using several best trees to generate likelihood values for comparison because they can differ dramatically on different topologies. We tested three topologies indicated by the phylogenetic analyses, described below. Next we did likelihood-ratio tests for the most parsimonious trees for each gene after removing taxa suspected of having elevated or slowed rates due to the results of the 2D test. The likelihood values for the trees with and without the assumption of a molecular clock were generated using PAUP* 4.0. Two times the difference of the likelihood values with and without the assumption of a molecular clock is expected to follow a chi-square distribution for the true tree (Yang et al. 1995). We therefore compared the test statistic to a chi-square table for significance.

Evolutionary rates were calculated using two divergence dates from the fossil record. The earlier date is the divergence of Hippomorpha (including equids) and Ceratomorpha (including rhinoceroids and ta-piroids). The fossil record indicates that the radiation of Hippomorpha was under way by the early Eocene, 50 MYA (e.g., Prothero and Schoch 1989b), so the divergence of Ceratomorpha and Hippomorpha

must have preceded this. Ceratomorpha diversification, including the appearance of "tapiroids" (e.g., *Heptodon, Helates, Selenaletes*) and "rhinocerotoids" (*Hyrachyus*), can be traced to the late early Eocene, about 45 MYA (Prothero and Schoch 1989b). There are many fossil specimens of *Equus* and species assignment varies among authors, but there is general agreement that modern species of *Equus* diversified in the late Pliocene, 2–4 MYA (e.g., Bennett 1980; MacFadden 1992; Hulbert 1993, 1996). *E. asinus* is a domesticated offshoot of the Afri-can ass, *Equus africanus,* that belongs to a group (probably monophy-letic) of asinines and hemionines that dates to 2.5 MYA in the form of *E. calobatus* (Skinner and Hibbard 1972). This sets a minimum age for the separation of the asinines and hemionines from the caballine lin-eage (represented here by *E. caballus*). We therefore used a conserva-tive estimate of 3 MYA for the divergence date for our two equid sequences. The rates for each gene were calculated using both fossil divergence dates and HKY85 gamma-corrected distances among taxa. To calculate the rate for the early divergence, the distances between each equid and all four tapirs were averaged and then divided by 50 myr. We used half of this value as the rate of evolutionary change for a gene per million years per lineage. This was done for both genes using both equid species. To calculate the rate for the later divergence, the corrected distance between the two equids was divided by 3 myr, then divided by 2 lineages. Both rate estimates for both genes were then used to calculate divergence times for several taxon pairs.

Results

Newly reported 12S rRNA sequences for *Tapirus pin-chaque, T. indicus, T. bairdii, T. terrestris,* and *Diceros bicornis* have been deposited in the GenBank database (accession Nos. AF191833-AF191837 (AF191833)). The 12S rRNA alignment produced using a model of secondary structure (Springer and Douzery 1996) is available at www.uic.edu/depts/bios/ecoevol/ashley.htm. This approach allowed all but positions 329–345 to be aligned unambiguously. The 17 ambiguous alignment positions were not included in phylogenetic analyses. Our previous tapir COII study (Ashley et al. 1996) in-cluded multiple representatives of each tapir species ex-cept *T. pinchaque,* because only one sample of this highly endangered species was available at that time. The COII sequence of a second *T. pinchaque* sample was obtained for this study and was identical to that reported previously. Pairwise comparisons for COII and 12S rRNA sequences (absolute and HKY85-corrected dis-tances) are shown in Tables 2 and 3, respectively.

We found base compositional bias in both genes, and the different data partitions within genes showed differ-ent biases. Mammalian mtDNA has been reported to have a strand-specific nucleotide compositional bias at third codon positions in protein-coding genes (Adkins and Honeycutt 1994; Honeycutt et al. 1995; Irwin et al., 1991) and in nontranslated rRNA genes (Nedbal et al. 1996). The perissodactyl, artiodactyl, and cetacean mtDNA sequences showed no significant heterogeneity in base compositions among taxa for 12S rRNA or in the first and second codon positions of COII. However, the third codon position of the COII gene has significant base compositional heterogeneity among all taxa ($p = 0.000025$), among perissodactyls ($p = 0.018922$), and between tapirs and equids ($p = 0.009367$). An equid
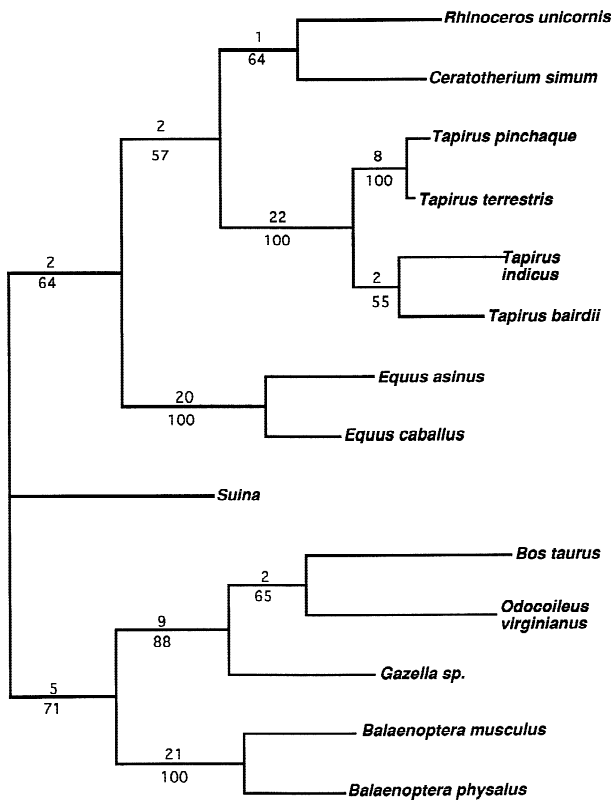
Fig. 1. The most parsimonious tree from a branch-and-bound search of 684 bp of the COII gene. Artiodactylans and cetaceans were designated as the outgroup taxa. Bootstrap values are *below* each node and decay values are *above*. The tree length is 692 steps.

Fig. 2. The most parsimonious tree from a branch-and-bound search of 431 bp of the 12S rRNA gene. Artiodactylans and cetaceans were designated as the outgroup taxa. The bootstrap values are *below* each node and decay values are *above*. The tree length is 259 steps.

(zebra) mitochondrial gene (cytochrome *b*) had been reported previously to have a composition different from that of many other mammals (Irwin et al. 1991).

We first analyzed the two genes separately to determine if phylogenies were congruent between data sets. Unweighted MP analysis of the COII data set grouped *T. pinchaque* with *T. terrestris* and *T. bairdii* with *T. indicus* (Fig. 1). The *bairdii/indicus* node (with 55% bootstrap support) is of primary interest because it aligns the neotropical *T. bairdii* with the Asian species rather than with the other neotropical species. The relative position of *T. bairdii* could not be resolved in a previous COII study (Ashley et al. 1996). Ceratomorpha is recovered in this analysis, but support is weak (bootstrap value is 57% and decay value is two steps). Maximum-likelihood analysis (performed using parameters measured from a neighbor-joining tree constructed with maximum-likelihood distance estimates) produced a phylogeny with the same topology as for unweighted maximum parsimony. Neighbor-joining analysis performed on the COII data after LogDet transformation produced a phylogeny that grouped equids and rhinoceros as sister taxa, a clade that was not suggested by any other analysis. Downweighting third-position transitions by the estimated transitional bias (7:1) resulted in a tree that breaks up the *bairdii/indicus* clade and supports monophyly of neotropical tapirs with a bootstrap value of 70%. The
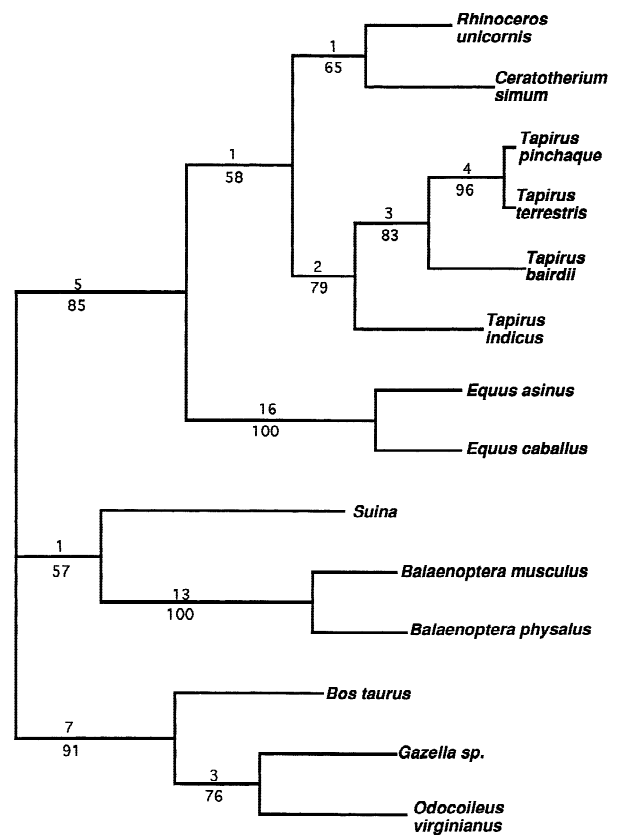
weighted analysis also supports Ceratomorpha with a bootstrap value of 71%. Both weighted and unweighted COII nucleotide analyses produced the same topology for higher-order perissodactyl relationships, recovering the traditional suborder Ceratomorpha and monophyly of Perissodactyla.

With all positions equally weighted, the 12S rRNA analysis produced the same topology as the weighted COII MP analysis, and with better support for the monophyly of neotropical tapirs (83% bootstrap value and decay value of three steps; Fig. 2). The support for Ceratomorpha is weak. The addition of two rhinoceros sequences not available for the COII analysis (*Diceros bicornis* and *Dicerorhinus sumatrensis*) did not alter the topology of the tree but did increase bootstrap support for some relationships (Fig. 3) including the monophyly of rhinoceros and Ceratomorpha. In the case of 12S rRNA, downweighting transitions relative to transversions (7:1) yielded a tree with unconventional relationships among perissodactyl families, with equids as the sister group to tapirs. Maximum-likelihood analysis of the 12S rRNA data also results in a phylogeny that placed Equidae as the sister group to Tapiridae, contradicting the group Cerotomorpha.

The two genes were not significantly incongruent (*p* = 0.4700 when invariant positions are excluded and data
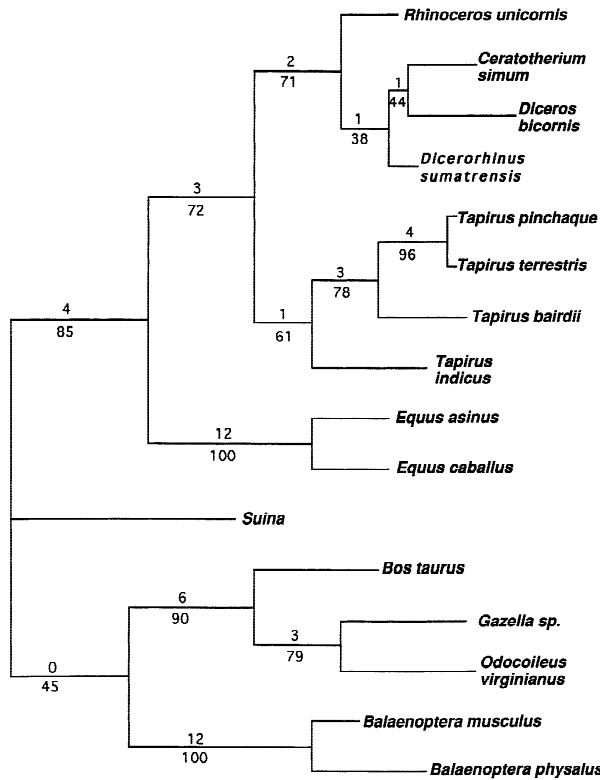
Fig. 3. The most parsimonious tree from a branch-and-bound search of 431 bp of the 12S rRNA gene including two additional rhinoceros taxa. Artiodactylans and cetaceans were designated as the outgroup taxa. The bootstrap values are *below* each node and decay values are *above*. The tree length is 275 steps.

are unweighted) according to the partition heterogeneity test. When both genes were analyzed together using unweighted maximum parsimony, the same perissodactyl phylogeny is recovered as for unweighted 12S rRNA alone or with weighted COII analysis. This tree, shown in Fig. 4, supports monophyly of neotropical tapirs with 61% bootstrap support (decay value of one step) and recovers Ceratomorpha (62% bootstrap support and decay value of three steps).

The application of a molecular clock requires rate constancy across lineages through time. Relative rate tests are designed to assess rate constancy independent of fossil records (Sarich and Wilson 1973). Relative rates of COII and 12S rRNA evolution among perissodactyls were not significantly different by the 2D relative rate test (Tajima 1993) after correction for multiple tests using the sequential Bonferroni method (Rice 1989). Before the correction, there were five taxonomic pairs exhibiting significant rate differences ($p < 0.05$) and the relative influence of these taxa on rate heterogeneity was examined using a likelihood-ratio test.

Likelihood-ratio tests for a molecular clock were first performed on two different but plausible in group tree topologies suggested by the phylogenetic analyses, namely, the topology shown in Fig. 1 (unweighted COII MP) and the topology shown in Figs. 2 and 4 (unweighted 12S rRNA MP and unweighted combined MP,

respectively). For the 12S rRNA, likelihood values were 1109.47 and 1113.74 without the assumption of a molecular clock and 1124.37 and 1130.54 assuming a molecular clock, respectively. Twice the difference between these values is highly significant ($p < 0.005$) with six degrees of freedom, corresponding to the number of taxa minus two (Yang et al. 1995). Similarly, for the COII gene, likelihood values are 2265.05 and 2264.78 without assuming a molecular clock and 2294.84 and 2294.52 assuming a molecular clock (p < 0.005). We then removed those taxa shown in the 2D relative rate tests to be significantly different at the 0.05 level (before correction) and performed a likelihood-ratio test for a molecular clock again to determine whether the 2D test had identified all taxa involved in rate heterogeneity. We could not do this with the 12S rRNA data; only three taxa were left in the data set after removal of those with at least one instance of rate heterogeneity. *Ceratotherium simum, Tapirus indicus, T. pinchaque,* and *T. terrestris* were removed from the COII data set (leaving only four taxa), but the likelihood-ratio test still rejected a molecular clock. We then tested the phylogeny that was recovered from the weighted 12S rRNA data that showed equids and tapirs as sister taxa but still rejected a molecular clock. Therefore the 2D relative rate test was too conservative in this case to identify all lineages exhibiting rate heterogeneity even before correction for multiple tests.

We further evaluated the application of a molecular clock using both genes and the two fossil calibration points described above. Table 4 gives estimates of divergence times for 12S rRNA and COII for selected taxa of interest using both calibration points. Using the early calibration point of 50 MYA for the separation of Ceratomorpha and Hippomorpha and the HKY85-corrected distances among equids and tapirs, the rate of the 12S rRNA evolution is 0.16%/myr (calculated for *E. caballus*) to 0.17%/myr (calculated for *E. asinus*). The rate of COII evolution is 0.24%/myr (calculated for *E. caballus*) to 0.26%/myr (calculated for *E. asinus*). This early divergence calibration provides estimates for the separation of Rhinoceritidae and Tapiridae of approximately 26–32 MYA for 12S rRNA and 42–45 MYA for COII. Various divergence dates within Tapiridae vary substantially between the COII and the 12S rRNA estimates, but neither is consistently higher or lower than the other. Most dramatically, this early calibration rate places the separation of the two equid species at greater than 13 MYA using the average rate from either gene, a date incompatible with fossil evidence suggesting that this event occurred within the last 2.5–3 million years. It is possible to introduce systematic bias into an average rate calculation because the taxa involved share branches on the phylogeny (Hillis et al. 1996). Therefore we estimated the divergence time between the equids using the highest pairwise rate between one ceratomorph and one hippomorpha for both genes. For 12S, the highest rate

Table 3. Pairwise 12S rRNA absolute numbers of nucleotide differences (above diagonal) and HKY85-corrected distances (below diagonal)[a]

	R. unicornis	C. simum	D. bicornis	D. sumatrensis	E. asinus	E. caballus	T. terrestris	T. indicus
R. unicornis	—	20	22	6	46	46	26	30
C. simum	0.05586	—	18	7	44	43	30	30
D. bicornis	0.06512	0.05143	—	11	45	42	29	33
D. sumatrensis	0.02536	0.03052	0.05088	—	19	19	15	16
E. asinus	0.15391	0.1454	0.15831	0.09439	—	16	47	42
E. caballus	0.17081	0.15524	0.14856	0.09274	0.04652	—	40	43
T. terrestris	0.08027	0.09609	0.09219	0.07161	0.17482	0.13809	—	23
T. indicus	0.09583	0.09644	0.10955	0.07828	0.14996	0.15536	0.07035	—
T. pinchaque	0.08821	0.10473	0.10071	0.06585	0.18678	0.14843	0.00527	0.07808
T. bairdii	0.08508	0.10029	0.10983	0.06145	0.16061	0.18259	0.05105	0.0867
Suina	0.16314	0.1311	0.17268	0.13575	0.21876	0.20945	0.17238	0.17178
B. taurus	0.18514	0.17462	0.20287	0.15543	0.22574	0.23206	0.15786	0.16074
Gazella	0.17951	0.15378	0.19042	0.16603	0.188	0.20958	0.16818	0.17392
O. virginianus	0.17737	0.18788	0.17726	0.1324	0.17253	0.19736	0.17379	0.17692
B. musculus	0.2011	0.18487	0.23873	0.14913	0.16566	0.22025	0.20955	0.17889
B. physalus	0.21683	0.19992	0.26587	0.17071	0.17604	0.22054	0.23987	0.19676

[a] See text for parameters used for calculating HKY85-corrected distances.

(calculated from *T. pinchaque* and *E. asinus;* 0.187%/myr) still gave a divergence time of 12.4 MYA, and for COII the highest rate (calculated from either equid vs *T. indicus* at 0.27%/myr) estimated the divergence of equids at 14.8 MYA. These individual calibrations do not recover a divergence time that is very different from the average rates.

Alternatively, using the late divergence of 3 MYA for separation of the equid species *E. caballas* and *E. asinus,* the rate for 12S rRNA evolution is 0.765%/myr and the rate for COII evolution is 1.4%/myr. The late divergence calibrations infer a separation of 8–11 MYA for Ceratomorpha and Hippomorpha and 6–8 MYA for Rhinocerotidae and Tapiridae. These molecular clock dates are obviously too recent, since fossil representatives clearly identifiable to each of three major lineages are abundant in the Eocene (e.g., Prothero and Schoch 1989b). Therefore, neither fossil calibration point gives reasonable estimates of divergence times for the range of perissodactyl diversification, suggesting that changes in rates of evolution (possibly confounded by the stochastic nature of nucleotide substitution) for both genes preclude useful application of a molecular clock.

Discussion

Two goals of this study were to use molecular data to address certain unresolved perissodactyl relationships and to compare the results of separate and combined phylogenetic analyses of two linked but functionally distinct mitochondrial genes, the protein-coding gene, COII, and an untranslated ribosomal gene, 12S rRNA. Phylogenetic analysis (unweighted MP) of the COII alone places the neotropical species *T. bairdii* as the sister taxon to the Asian *T. indicus* but 12S rRNA alone places *T. bairdii* as the sister taxon to the other neotropical

tapirs. MP analysis of the unweighted combined data sets results in the same tapir phylogeny as the 12S rRNA data alone (Fig. 4) but with lower support for the neotropical tapir clade. Signal in the 12S rRNA data was apparently enough to overcome the conflicting signal in the COII data but lowered support for the relationship that was in conflict. Support for Ceratomorpha was higher in the combined analysis than for either gene alone, but the strongest support for this relationship was found in the 12S rRNA data set that included all four rhinoceros sequences (Fig. 3). In this case, additional sampling within the family was more helpful than additional sequence from taxa already represented. Downweighting of third-position transitions in COII resulted in topological congruence with 12S rRNA and combined analysis, but downweighting of transitions in 12S rRNA produced unlikely relationships at the interfamilial level.

All phylogenetic analyses recovered a sister taxon relationship between the two South American tapir species, *T. terrestris* and *T. pinchaque.* Although this conclusion contradicts a recent morphological study of fossil and extant New World tapirs that reports *T. terrestris* and *T. bairdii* to be closest sister taxa (Hulbert 1995), both bootstrap and decay index support for this node is very high in both separate and combined analysis of our genes. The high sequence similarity between the genes of these two species suggests that they had a recent common ancestor and also the possibility that a single tapir lineage migrated to South America following the formation of the Panamanian land bridge approximately 3 MYA and subsequently speciated.

We tested a molecular clock for mitochondrial genes in four ways: (1) by conducting all pairwise relative rates tests using the *Bos taurus* sequence as an outgroup (Tajima 1993), (2) by conducting likelihood-ratio tests for the presence of a molecular clock, (3) by comparing taxonomic divergence estimates from two linked mito-

Table 3. Extended

|	T. pinchaque	T. bairdii	Suina	B. taurus	Gazella	O. virginianus	B. musculus	B. physalus
R. unicornis	28	29	49	53	51	52	57	60
C. simum	32	33	42	51	46	54	54	57
D. bicornis	31	34	49	54	51	50	61	65
D. sumatrensis	14	13	25	27	28	24	27	30
E. asinus	49	47	60	61	54	51	50	52
E. caballus	42	48	55	59	55	53	57	57
T. terrestris	2	17	48	45	46	48	55	60
T. indicus	25	27	48	45	47	48	49	52
T. pinchaque	—	17	50	47	48	48	57	62
T. bairdii	0.05108	—	52	51	49	50	51	53
Suina	0.18356	0.17792	—	50	49	51	50	50
B. taurus	0.16848	0.17532	0.16985	—	33	33	47	53
Gazella	0.1797	0.1692	0.16249	0.10198	—	27	50	53
O. virginianus	0.17388	0.16981	0.17264	0.10049	0.07775	—	45	51
B. musculus	0.22229	0.17086	0.16854	0.15439	0.16664	0.14506	—	17
B. physalus	0.25379	0.18054	0.1694	0.18572	0.18068	0.17285	0.04568	—
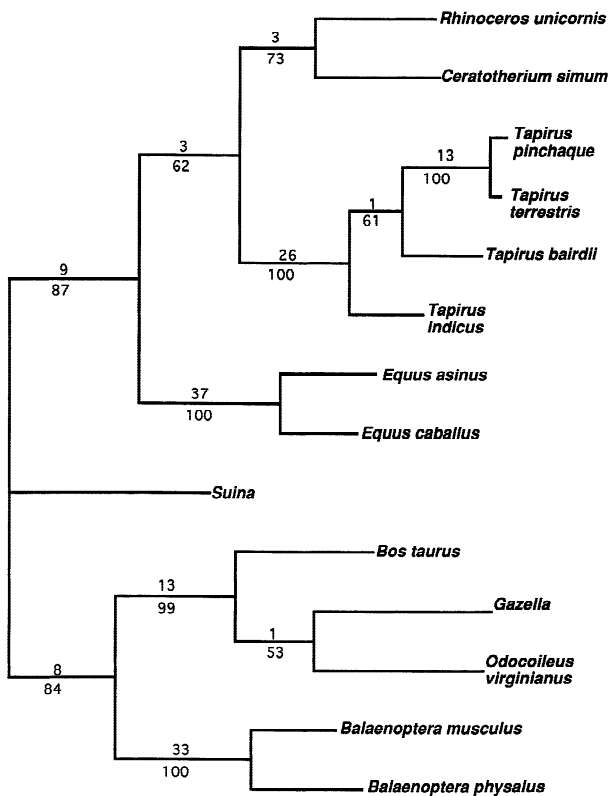
Fig. 4. The most parsimonious tree from a branch-and-bound search of the combined 12S rRNA and COII data. Artiodactylans and cetaceans were designated as the outgroup taxa. The bootstrap values are *below* each branch and decay values are *above*. The tree length is 957 steps.

chondrial gene regions, and (4) by estimating rates and divergence times using two fossil calibration points. After correcting for multiple tests, the relative rate tests failed to reveal significant rate heterogeneity among perissodactyls for either COII or 12S rRNA, but these tests are not designed to detect multiple rate fluctuations within a lineage. The likelihood-ratio tests, however, rejected a molecular clock for both genes. Both fossil based tests suggest that rates of molecular change varied considerably; the divergence time estimates for the two genes did not generally agree and the divergence date estimates produced by the two fossil calibration points were dramatically different (Table 4). For both fossil calibration points, divergence estimates did not agree with paleontological evidence. The use of 50 MYA for the separation of Ceratomorpha and Hippomorpha yields an unlikely estimate for a divergence among the two equid species in our study, on average, more than 13 MYA. Although a 10-million year gap in the fossil record for extant equids is a possibility, we feel that it is rather unlikely given the otherwise dense equid fossil record. This early calibration may also overestimate other perissodactyl divergences, including those among tapirids, although the fossil record for tapirs is not as abundant or as well characterized as for equids. At the other extreme, the late calibration of 3 MYA for the separation of the two equids results in unreasonable estimates for the older divergences among perissodactyls, placing them in the Miocene rather than the Eocene, where the fossil record clearly demonstrates that they occurred. Finally, possible errors in the calibration dates we used would likely inflate the molecular clock discrepancies. The earliest known representative of Hippomorpha at about 50 MYA establishes only a minimum age of divergence; the actual date might be earlier, which would result in an even slower rate for the early calibration. Our recent calibration point, 3 MYA, for divergence of the *Equus* species may well be more recent (fossils clearly belonging to the extant lineages we used do not appear until the Pleistocene), which would result in a higher rate for the late calibration.

Differences in rates of molecular evolution among eutherian taxa have been widely reported, with particular attention given to accelerated rates in rodents (e.g.,

Table 4. Estimated divergence times of various taxa based on a late calibration date of 3 mya for *E. caballus* and *E. asinus* and an early calibration date of 50 mya for Ceratomorpha and Hippomorpha

Taxa compared	Corrected distance (%)		Est. divergence time (mya), COII		Est. divergence time (mya), 12S	
	COII	12S	Late calibration	Early calibration	Late calibration	Early calibration
T. terrestris vs *T. bairdii*	5.1	9.2	3.3	18.0–19.2	3.3	15.0–16.5
T. pinchaque vs *T. bairdii*	5.1	9.8	3.5	19.2–20.4	3.3	15.0–16.5
T. pinchaque vs *T. terrestris*	0.5	1.3	0.5	2.5–2.7	0.3	1.5–1.6
T. indicus vs *T. bairdii*	8.7	9.2	3.3	18.0–19.2	5.7	25.6–28.1
T. indicus vs *T. terrestris*	7.8	9.9	3.5	19.4–20.7	5.1	22.9–25.2
T. indicus vs *T. pinchaque*	7	10.9	3.9	21.4–22.7	4.6	20.6–22.6
R. unicornis vs tapirs (avg)	21.4	8.7	7.6	42.0–44.6	5.7	25.7–28.2
C. simum vs tapirs (avg)	21.6	9.9	7.7	42.4–45.0	6.5	29.2–32.0
E. asinus vs *E. caballus*	8.4	4.7	NA[a]	16.5–17.5	NA[a]	13.7–15.0
Equids vs tapirs (avg)	24.7	16.2	8.8	NA[a]	10.6	NA[a]
Equids vs rhinoceros (avg)	14	21.5	7.7	NA[a]	9.1	NA[a]

[a] A date was not estimated because these taxa were used to calibrate the clock for this divergence.

Catzeflis et al. 1987; Li and Wu 1987; Li et al. 1996) and relatively slow rates in hominoid primates (Kohne et al. 1972; Goodman 1985; Li and Tanimura 1987; Sakoyama et al. 1987; Bailey et al. 1991). Nevertheless, many mammalian molecular systematic studies continue to invoke molecular clocks calibrated by a single (sometimes poorly supported) paleontological date to estimate divergence dates for many other taxon pairs in their study. For example, Arnason and Gulberg (1996) have used the relatively sparse cetacean fossil record to estimate a date of 60 MYA for an Artiodactyla/Cetacea divergence, and the authors and colleagues have applied this single "A/C-60 standard" to date a variety of mammalian divergences, including those with better fossil records such as Perissodactyla (Xu et al. 1996; Xu and Arnason 1997). One defense of a mammalian DNA clock contends that the two widely cited examples of rate differences, among rodents and among hominoid primates, are based largely on poorly established paleontological dates, including the rat–mouse split and the separation of platyrrhine and catarrhine primates (Easteal et al. 1995). We chose therefore to test the utility of a molecular clock in the mammalian order known to have the most complete fossil record—rejection of a molecular clock due to incompatibility with the fossil record may be more convincing in Perissodactyla. Interestingly, unlike the previously reported mammalian rate differences, the discrepancies in DNA versus paleontological dating in Perissodactyla do not appear to be due to an acceleration or slowdown in any one lineage. The equids, for example, exhibit an apparently accelerated rate late in their evolution (as evidenced by the high rate calibrated using two equid species), but early representatives of this lineage are involved in the calibration of the slower rate. We conclude that tests of rate constancy such as relative rates tests may often be too conservative to detect heterogeneity and that extreme caution should be exercised when estimating divergence dates from single calibration points, even if that calibration point is well supported by paleontological or biogeographical evidence.

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