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Phylogenetic Relationships of the Five Extant Rhinoceros Species (Rhinocerotidae, Perissodactyla) Based on Mitochondrial Cytochrome *b* and 12S rRNA Genes

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A major question in rhinocerotid phylogenetics concerns the position of the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) with regard to the other extant Asian (*Rhinoceros unicornis* and *R. sondaicus*) and African (*Diceros bicornis* and *Ceratotherium simum*) species. We have examined this particular question through the phylogenetic analysis of the complete sequences of the mitochondrial 12S rRNA and cytochrome *b* genes. Three additional perissodactyls (one tapir and two equids) plus several outgroup cetartiodactyls were included in the analysis. The analysis identified a basal rhinocerotid divergence between the African and the Asian species, with the Sumatran rhinoceros forming the sister group of the genus *Rhinoceros*. We estimate the Asian and African lineages to have diverged at about 26 million years before present.

Key Words: **Rhinocerotidae; Perissodactyla; 12S rRNA; cytochrome *b;* morphological features; divergence dates.**

INTRODUCTION

Rhinocerotidae are included within the order Perissodactyla, together with Tapiridae and Equidae. This family comprises five living species: the white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceroses in Africa and the Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*), and Sumatran (*Dicerorhinus sumatrensis*) rhinoceroses in Asia. Tapiridae are currently represented by one genus (*Tapirus*) with four species found in South America and in southeast Asia, whereas Equidae include one genus (*Equus*) with six species widespread throughout the world (Wilson and Reeder, 1993).

The family Rhinocerotidae, much more diversified and widespread in the past, is morphologically well defined (Prothero and Schoch, 1989; Cerdeño, 1995) but the taxonomy and the phylogenetic relationships among the five surviving species remain debated. Most authors agree that the two African genera should be considered as sister taxa but relationships within and between the Asian forms are not resolved. Three hypotheses, based upon morphological features (number of horns) and/or geographic distribution (Africa or Asia), are commonly proposed. According to Simpson (1945) and Loose (1975), the two-horned African and Sumatran rhinoceroses are closely related and distinct from the one-horned Indian and Javan rhinoceroses (Fig. 1A). Pocock (1945) and Groves (1967, 1983) regarded the three Asian forms as sister taxa, notably because of their geographic closeness (Fig. 1B). Finally, Guérin (1982), Prothero and Schoch (1989), and Cerdeño (1995) considered the two African rhinos, the two Asian *Rhinoceros,* and *Dicerorhinus* as three separate lineages (Fig. 1C). As a result, numerous classifications were proposed which differ by the taxonomic rank (tribal or subtribal level; see Table 5) of the different clades. The main question concerns the phylogenetic position of *Dicerorhinus* with regard to the African (*Ceratotherium* and *Diceros*) and the other Asian (*Rhinoceros*) genera. At first, we adopted the classification of Cerdeño (1995), which leaves *Dicerorhinus* as *incertae sedis* (see Table 1).

The molecular investigation of Morales and Melnick (1994) is the only study sufficient in species sampling to test the three hypotheses (Fig. 1). That study was based on restriction site mapping of an mtDNA ribosomal region for the four living genera (*Ceratotherium, Diceros, Dicerorhinus,* and *Rhinoceros*) and supported the morphological hypothesis (Fig. 1A) of Simpson (1945) and Loose (1975). Xu and Arnason (1997) used complete mitochondrial genomes but included only the white and Indian rhinoceroses. Other studies have dealt with the conservation genetics of the Sumatran (Amato *et al.,* 1995; Morales *et al.,* 1997) and the black

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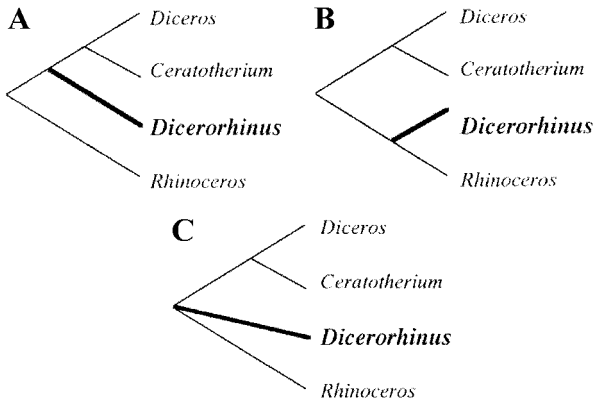

FIG. 1. Systematic relationships among the living rhinoceros genera: three hypotheses (as illustrated in Fig. 1 of Morales and Melnick, 1994) based on (A) the number of horns (Simpson, 1945; Loose, 1975), (B) the geographic distribution (Pocock, 1945; Groves, 1983), and (C) and both morphological and geographic features (Guérin, 1982; Prothero and Schoch, 1989; Cerdeño, 1995).

(O'Ryan *et al.*, 1994) rhinoceroses using mitochondrial DNA markers.

In contrast, our molecular analysis, based on complete cytochrome *b* (Cytb) and 12S rRNA (12S) mitochondrial sequences, includes all five living species of Rhinocerotidae, one species of Tapiridae, and two of Equidae. Xu *et al.* (1996; complete mitochondrial DNA) and Porter *et al.* (1996; exon 28 of the vWF factor gene) recognized a sister group relationship between Perissodactyla and Carnivora, to the exclusion of Cetartiodactyla. In our study dealing with mitochondrial genes, we follow Xu *et al.* (1996) by accepting Carnivora as the sister group of Perissodactyla; therefore, Cetartiodactyla were considered as outgroup.

The purpose of this study was to test the three hypotheses mentioned (Fig. 1) to clarify the phylogenetic relationships within the living rhinoceroses, particularly with respect to the systematic position of the Sumatran rhinoceros. Divergence dates were estimated for each perissodactyl split, and intra-Rhinocerotidae and intra-Perissodactyla phylogenetic relationships are discussed in the light of morphological and paleontological data.

MATERIALS AND METHODS

Species Sampling

We sequenced the cytochrome *b* and 12S rRNA genes from the Sumatran (*D. sumatrensis*) and Javan (*R. sondaicus*) rhinoceroses and the 12S rRNA from the black rhinoceros (*D. bicornis*). DNA samples from the Sumatran and black rhinoceroses were provided by Dr. Oliver Ryder and Leona Chemnick (Zoological Society of San Diego, Center for Reproduction of Endangered Species, San Diego, CA) and by Professor Terence J. Robinson (Department of Zoology, University of Stellenbosch, South Africa), respectively. In the case of the endangered Javan rhinoceros, a rib fragment was provided by Daniel Robineau (Laboratoire d'Anatomie Comparée, Museum National d'Histoire Naturelle, France).

Our dataset includes 16 taxa (Table 1), among which 8 are in the Perissodactyla: 5 Rhinocerotidae, 2 Equidae, and 1 Tapiridae. Carnivora were represented by 2 Caniformia and 2 Feliformia, whereas 4 Cetartiodactyla were used as outgroup taxa.

DNA Sequencing

From DNA samples of *Diceros* and *Dicerorhinus*, complete 12S and/or Cytb genes were PCR-amplified using conserved primers located in the flanking tRNAs of each gene: primers R1 and S2 were used for the 12S (Douzery and Catzeflis, 1995) and primers L6 and H7 for the Cytb (Table 2).

DNA extractions from the rib fragment of *R. sondaicus* were performed using ancient DNA methods, as described in Hänni *et al.* (1994), slightly modified. DNA amplification was performed in a 100-μl reaction containing 2 μl of DNA, 200 ng/μl of bovine serum albumin (Sigma), and 1 μl of *Taq* polymerase Gold Perkin. A set of primers was defined for each gene (Table 2). Five overlapping fragments of about 150 to 470 bp were amplified, covering the complete Cytb and 12S genes.

The 12S of *Diceros* and *R. sondaicus* were directly sequenced, whereas the 12S and Cytb from *Dicerorhinus* and the Cytb from *R. sondaicus* were cloned in the pGEM-T vector system 1 (Progema) or in the original TA cloning kit vector using INVαF′ one-shot bacteria (Invitrogen). Sequencing (three different clones in the case of cloning) was performed either manually, using the thermosequenase kit (Amersham) with [^{33}P]dNTP, or automatically on a 377A Perkin–Elmer sequencer. All sequences were obtained on both strands using PCR and additional internal primers. The five new sequences are deposited in the EMBL database under Accession Nos. AJ245721–AJ245725.

Sequence Alignment and Saturation Analysis

Sequences were aligned by hand using ED editor (MUST package; Philippe, 1993). The alignment of the 12S rRNA sequences was made using a previous alignment of mammalian sequences (Douzery and Catzeflis, 1995; Dubois *et al.*, 1996) as reference, in which indels are preferentially introduced in loops. One hypervariable region, corresponding to the region between stem 39 and its complement 39′ in the study of Springer and Douzery (1996), was removed from analyses because of alignment ambiguities. For cytochrome *b* gene, no indel was detected.

Saturation was evaluated as described by Philippe *et al.* (1994) and Hassanin *et al.* (1998). Using the adjusted and patristic distance matrices calculated by PAUP 3.1.1 (Swofford, 1993), the observed differences

TABLE 1

Systematic Arrangement of the Considered Taxa According to Simpson (1945), Wilson and Reeder (1993), and Cerdeño (1995) for the Rhinocerotidae

	12S rRNA	Cytochrome *b*
Order Perissodactyla		
Suborder Ceratomorpha		
Family Rhinocerotidae		
Subfamily Rhinocerotinae		
Tribe Rhinocerotini		
Dicerorhinus sumatrensis	This study, AJ245722	This study, AJ245723
Subtribe Rhinocerotina		
Rhinoceros sondaicus	This study, AJ245724	This study, AJ245725
Rhinoceros unicornis	Xu *et al.*, 1996 (X97336)	Xu *et al.*, 1996 (X97336)
Subtribe Dicerotina		
Ceratotherium simum	Xu and Arnason, 1997 (Y07726)	Xu and Arnason, 1997 (Y07726)
Diceros bicornis	This study, AJ245721	Irwin *et al.*, 1991 (X56283)
Family Tapiridae		
*Tapirus pinchaque**	Springer, 1998 (AF038012)	
*Tapirus terrestris**		Veits, D. and Pitra, C., unpublished (AF056030)
Suborder Hippomorpha		
Family Equidae		
Equus caballus	Xu and Arnason, 1994 (X79547)	Chikuni, K., unpublished (D32190)
Equus grevyi	Douzery and Catzeflis, 1995 (X86943)	Irwin *et al.*, 1991 (X56282)
Order Carnivora		
Suborder Feliformia		
Family Felidae		
Panthera tigris	Ledje and Arnason, 1996a (Y08504)	Arnason *et al.*, 1995 (X82301)
Family Herpestidae		
Herpestes auropunctatus	Ledje and Arnason, 1996a (Y08506)	Ledje and Arnason, 1996b (X94926)
Suborder Caniformia		
Family Phocidae		
Phoca vitulina	Arnason and Johnsson, 1992 (X63726)	Arnason and Johnsson, 1992 (X63726)
Family Ursidae		
Ursus arctos	Ledje and Arnason, 1996a (Y08519)	Arnason *et al.*, 1995 (X82308)
Order Artiodactyla		
Infraorder Suina		
Family Suidae		
Sus scrofa	Ursing and Arnason, 1998 (AJ002189)	Ursing and Arnason, 1998 (AJ002189)
Family Tayassuidae		
Tayassu tajacu	Douzery and Catzeflis, 1995 (X86944)	Irwin *et al.*, 1991 (X56296)
Suborder Ruminantia		
Family Bovidae		
Bos taurus	Sanger and Young, 1982 (V00654)	Sanger and Young, 1982 (V00654)
Order Cetacea		
Family Balaenopteridae		
Balaenoptera physalus	Arnason *et al.*, 1991 (X61145)	Arnason *et al.*, 1991 (X61145)

Note. Author references and accession numbers are indicated for each sequence. Species involved in a chimera are noted by an asterisk (*).

from pairwise comparisons are plotted against the corresponding number of inferred substitutions (Philippe *et al.*, 1994). The slope of the linear regression (S) equals one when no saturation is observed, whereas the slope tends toward zero as the level of saturation increases (Hassanin *et al.*, 1998).

Phylogenetic Reconstructions

Maximum-parsimony (MP) analyses were performed with PAUP 3.1.1 using a heuristic search with random stepwise addition of taxa (10 replicates), TBR branch-swapping, and MULPARS options. Only informative sites equally weighted were considered. Robustness was assessed with bootstrap resampling (Felsenstein, 1985) after 1000 replicates with one random addition of taxa. The decay index (DI; Bremer, 1988), which gives the number of extra steps necessary to break or to build a clade, was calculated with the option "topological constraints enforced."

Maximum-likelihood (ML) methods were conducted using the program PUZZLE (version 4; Strimmer and von Haeseler, 1996) based on the quartet puzzling approach with the Tamura–Nei model and a gamma distribution (with eight categories and a fraction of invariable sites) for substitution rates. Robustness was estimated through reliability percentages (RP) representing, as a percentage, how often a group appears

TABLE 2

Primers Used for PCR and Sequencing of the 12S rRNA and Cytochrome *b* Genes

12S rRNA gene primers		Cytochrome *b* gene primers	
R1	5′-AAAGCAAGGCACTGAAAATGCCTAGA-3′	L7	5′-ACCAATGACATGAAAAATCATCGTT-3′
S2	5′-TCTTCTGGGTGTAGGCCAGATGCTTT-3′	H6	5′-TCTCCATTTCTGGTTTACAAGAC-3′
12S6	5′-GTGACTTTAATACATTCGCCC-3′	C3	5′-ATCTCAGCCCTAGCAATCAC-3′
12S7	5′-TATGGAACAGGCTCCTCTAGG-3′	C7	5′-ATCACTCTGGTTTGATAGT-3′
12S9	5′-CTAAAGTAAGCACAAGTATAA-3′	C10	5′-GGCAGATAAAAAATATGGAT-3′
12S10	5′-CCATTTCTCTCCATCCCATAA-3′	C11	5′-CACCAGACACAACAACTGCC-3′
12S11	5′-CGGGCGGTGTGTGCGTGCTTT-3′	C16	5′-GGATTCCTGATGGGTTGTTG-3′
12S14	5′-TTTGACTAAGTTATACTAAACAGA-3′	C17	5′-ATCTAGGAGACCCTGACAAC-3′
12S15′	5′-TTGACACGCTTTACGCCGAGGTTC-3′	C18	5′-CTCCACACATCCAAACAACG-3′
12S17	5′-TAAGAATAGAGAGCTTAATTGAAC-3′	C20	5′-ATAGGATTGATGCTAGTTGG-3′

after 1000 puzzling steps. The Intree option was used to evaluate alternative topologies with the test of Kishino and Hasegawa (1989), which compared log-likelihood difference (ΔLnL) with regard to the standard error (SE) of this difference.

The level of incongruence between the two genes was tested with the ARNIE program (Random Cladistics package; Siddall, 1996), which used the incongruence length difference test with the parsimony approach (Farris *et al.,* 1995); 1000 randomizations were performed on variable sites only (Cunningham, 1997).

RESULTS

12S rRNA Sequence Analysis

The alignment of 12S rRNA sequences of 16 taxa was 1006 nucleotides long, among which 885 were conserved after elimination of the indels and the hypervariable terminal region (representing 55 positions in our alignment). A graph illustrating the level of saturation displayed by the 12S is presented in Fig. 2A. Intra-Perissodactyla pairwise comparisons appear moderately subject to saturation (slope of the regression analysis S = 0.67) as compared to the whole dataset (S = 0.52). Since our interest bears on intra-Perissodactyla relationships, all substitutions were therefore retained for phylogenetic reconstructions.

Phylogenetic analyses were performed on 885 sites (343 variable and 249 informative sites). Only one most-parsimonious tree was obtained (L = 890, CI = 0.544, RI = 0.499). ML puzzling quartet and MP bootstrap analyses yielded the same topology, similar to Fig. 3. Monophyly of Perissodactyla is supported by 100% RP in ML and 98% BP (DI = +11) in MP. Among the Perissodactyla clade, at least 95% BP is provided in MP analyses to Rhinocerotidae (99%, DI = +5), Equidae (100%, DI = +24), Rhinocerotina (*R. sondaicus* and *R. unicornis;* 100%, DI = +14), and Dicerotina (*C. simum* and *D. bicornis;* 95%, DI = +7). Support for these nodes is 99, 98, 87, and 97% in ML, respectively. Ceratomorpha (Tapiridae + Rhinocerotidae; 83% RP, 79% BP, DI = +5) and *Dicerorhinus*–Rhinocerotina (71% RP, 76% BP, DI = +3) appear less supported.

Cytochrome b Sequence Analysis

The alignment of the cytochrome *b* sequences of 16 taxa is 1140 nucleotides long. Saturation analysis was conducted on positions 1 and 2 (Figs. 2B and 2C, respectively), whereas transversions (Tv; Fig. 2D) and transitions (Ts; Fig. 2E) were analyzed separately at the third codon position. Considering the whole dataset, position 2 appears slightly more affected by homoplasy than position 1 (S = 0.51 and S = 0.65, respectively). In intra-Perissodactyla comparisons, saturation is much reduced and to the same level (S = 0.75 for both positions). At the third codon position, a low level of saturation is detected for Tv in Perissodactyla (S = 0.81) as compared to the complete dataset (S = 0.51). On the other hand, Ts are equally saturated in Perissodactyla (S = 0.37) as in the whole dataset (S = 0.30). Further analyses were then performed with all positions, excluding Ts in the third codon position.

Phylogenetic analyses were conducted on 1140 positions representing 378 variable and 290 informative sites when Ts in position 3 are excluded (522 variable and 412 informative sites for the whole dataset). Only one most-parsimonious tree was obtained (L = 832, CI = 0.406, RI = 0.490). The topology is the same as that in Fig. 3 except for the African clade, which is not recovered, because *Diceros* constitutes the first emergence among Rhinocerotidae. In the quartet puzzling and bootstrap trees, Perissodactyla (RP = 100%, BP = 93%, DI = +6), Rhinocerotidae (RP = 99%, BP = 99%, DI = +14), Equidae (RP = 80%, BP = 100%, DI = +26), Rhinocerotina (RP = 100%, BP = 99%, DI = +9), and the clade Rhinoceros + *Dicerorhinus* (RP = 93%, BP = 65%, DI = +2) are monophyletic with high support. However, Dicerotina are supported in likelihood only (RP = 86%, BP = 38%, DI = −2), whereas Cera-

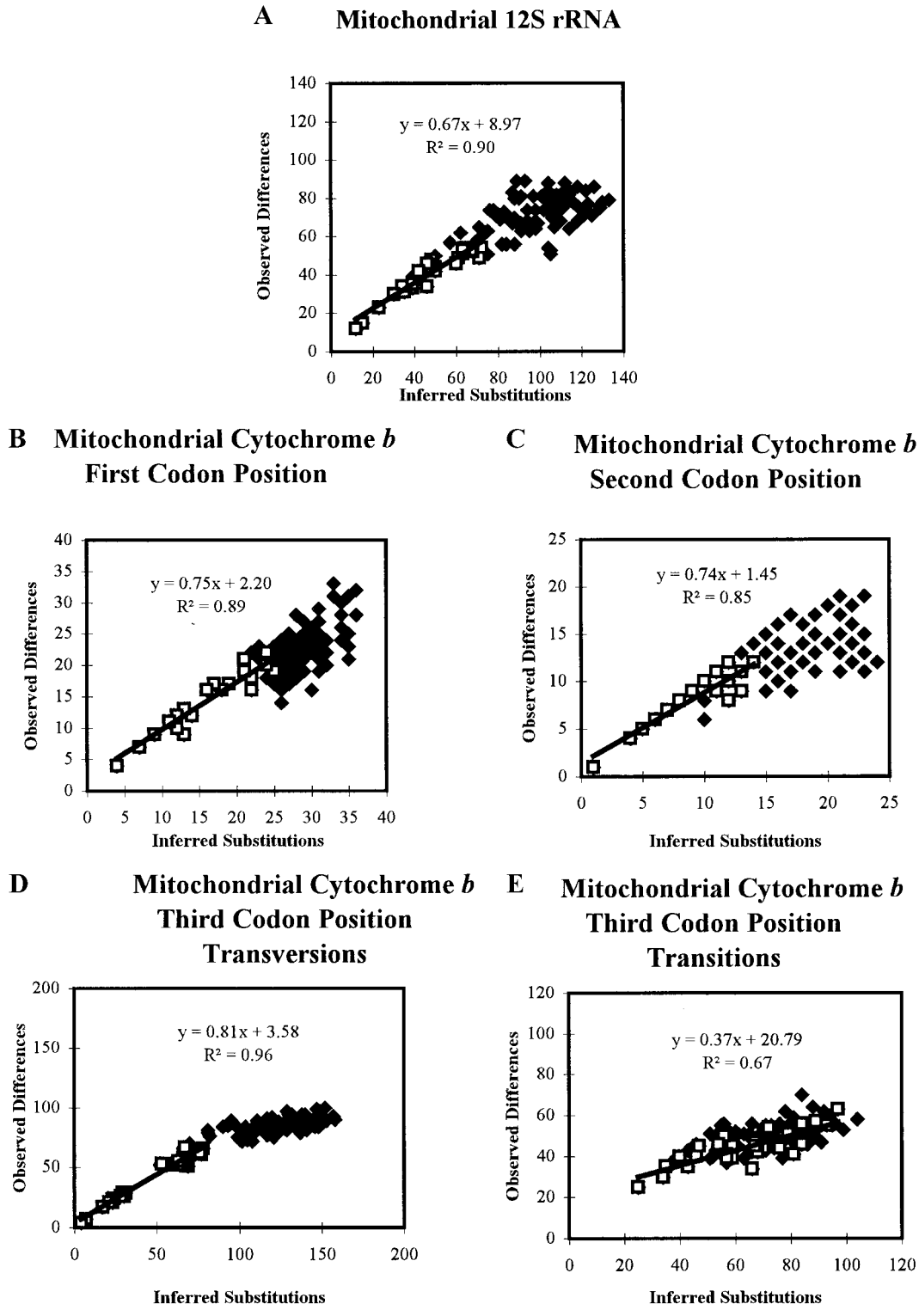

FIG. 2. Graphic estimation of saturation: the pairwise numbers of observed differences are plotted against the corresponding numbers of inferred substitutions for the most-parsimonious tree. Saturation analysis was conducted on 249 informative sites (I) for the 12S rRNA, whereas 86 I and 26 I were analyzed, respectively, for the first and second positions of cytochrome *b*. At the third codon position, transversions and transitions (114 I and 178 I, respectively) were considered separately. White squares represent intra-Perissodactyla pairwise comparisons and black diamonds correspond to comparisons between other taxa. Equations of the linear regression (straight lines) and correlation coefficients are given for intra-Perissodactyla comparisons (regression slopes for the whole dataset are given in the text).

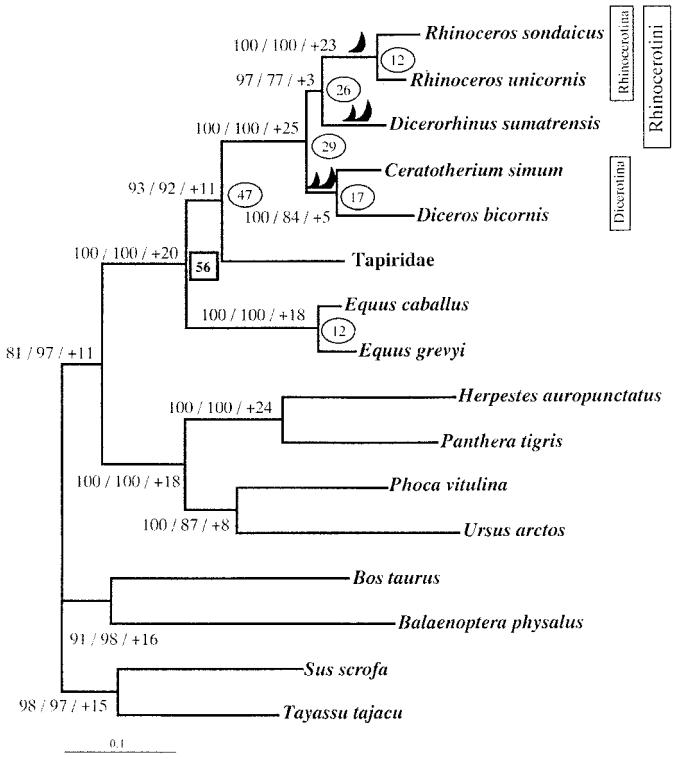

FIG. 3. Maximum-likelihood puzzling tree from the combined 12S rRNA (excluding indels and the terminal hypervariable region) and cytochrome *b* (excluding transitions in third codon position) genes. The numbers at nodes refer, respectively, to (from left to right) reliability percentage in maximum-likelihood (with PUZZLE), bootstrap percentage in parsimony (with PAUP), and the decay index (number of extrasteps to remove a grouping). The encircled numbers represent divergence dates estimated for the different perissodactyl splits, calibrated on the equid–ceratomorph divergence time at 56 Myr (bold-framed number). Tapiridae indicates that a chimera was built for the combined analysis (see Table 1). The number of horns of each rhinocerotid clade is drawn on the branches.

tomorpha are supported in parsimony (RP = 48%, BP = 72%, DI = +4).

Amino acid sequences were analyzed on 379 sites representing 110 variable and 67 informative positions (data not shown). Perissodactyla are weakly supported (RP = 79%, BP = 63%) but monophyly of Rhinocerotidae (RP = 97%, BP = 85%), Rhinocerotina (RP = 76%, BP = 73%), and Equidae (RP = 100%, BP = 99%) is still recovered. The association *Rhinoceros* + *Dicerorhinus* is supported in ML (RP = 88%) but not in MP (BP = 31%). In contrast, Dicerotina are not monophyletic (RP = 6%, BP = 44%) because *Ceratotherium* was the sister group of the clade *Rhinoceros* + *Dicerorhinus* (RP = 92%, BP = 40%). More surprising is the clustering of Tapiridae with Equidae with a high support (RP = 98%, BP = 84%), as already noted by Xu (1996) with the amino acid analysis of 12 mitochondrial protein-coding genes.

Combined Analysis of the 12S rRNA and Cytochrome b Genes

Despite some discrepancies between the branching patterns given by the 12S and Cytb genes, the test performed with the ARNIE program did not reveal significant incongruence between the two markers ($0.14 > P > 0.05$). The two datasets were therefore concatenated.

The combined analysis, performed on 2025 nucleotides (865 variable and 661 informative sites), yielded one most-parsimonious tree (L = 1617, CI = 0.441, RI = 0.492) identical to the quartet puzzling and bootstrap trees (Fig. 3). Four clades are evidenced by 100% in both methods, Perissodactyla (DI = +20), Equidae (DI = +52), Rhinocerotidae (DI = +25), and Rhinocerotina (DI = +23), whereas Dicerotina are supported by 100% RP and 84% BP (DI = +5). Lower supports are observed for Ceratomorpha (RP = 93%, BP = 92%, DI = +11) and for the cluster *Dicerorhinus*–Rhinocerotina (RP = 97%, BP = 77%, DI = +3).

Alternative hypotheses of intra-Perissodactyla relationships were investigated using the test of Kishino–Hasegawa. We tested all 105 possible trees among the five most supported clades in Perissodactyla, that is Equidae, Tapiridae, Rhinocerotina, Dicerotina, and *Dicerorhinus*. Over these 105 topologies, the highest likelihood tree differs from the puzzling tree by placing *Dicerorhinus* as the sister group to the two African rhinos (tree 1 in Table 3). At the 5% confidence level (1.96 SE of log-likelihood difference), 98 topologies (93%) are significantly less likely than the best ML tree and 6 trees are not significantly different (Table 3). Trees 1 and 2 differ by only 0.027 SE ($P = 0.98$), which means that the two topologies are equivalent in their likelihood. These two trees cluster *Dicerorhinus* either with Dicerotina (tree 1) or with Rhinocerotina (tree 2). The topology in which *Dicerorhinus* is external to the

TABLE 3

Seven ML Topologies Nonsignificantly Different (at the 5% Confidence Level) Including *Dicerorhinus sumatrensis* (DS), Dicerotina (DIC), Rhinocerotina (RHI), Tapiridae (TAP), and Equidae (EQU)

Tree	ΔLnL[a]	SE[b]	ΔLnL/SE
1: ((((DIC, DS), RHI), TAP), EQU)	[−11357.20][c]		Best tree
2: (((DS, RHI), DIC), TAP), (EQU)	0.19	6.91	0.027
3: (((RHI, DS), DIC), (EQU, TAP))	11.54	9.84	1.17
4: (((((RHI, DS), DIC), EQU), TAP)	12.75	9.75	1.31
5: ((((RHI, DIC), DS), TAP), EQU)	6.38	4.69	1.36
6: ((((DIC, DS), RHI), EQU), TAP)	10.48	7.07	1.48
7: (((DIC, DS), RHI), (EQU, TAP))	10.76	6.99	1.56

[a] Log-likelihood difference between the best ML tree and the evaluated topology.
[b] Standard error of log-likelihood difference.
[c] Log-likelihood value of the best ML tree.

TABLE 4

Molecular and Paleontological Estimations of Divergence Dates among Perissodactyla

Clade	Molecular estimation[a] (in MYBP)	Paleontological estimation[b] (in MYBP)
Perissodactyla	56	56 (Upper Paleocene)
Hippomorpha (Equidae)	12.2 ± 2.2	5–3 (Lower Pliocene)
Ceratomorpha (Tapiridae + Rhinocerotidae)	46.7 ± 3.7	50 (Middle Eocene)
Rhinocerotidae	29.3 ± 1.8	49–37 (Middle Eocene)
Dicerotina	17.1 ± 2.5	13 (Middle Miocene)
Rhinocerotina	11.7 ± 1.9	3.3–1.6 (Upper Pliocene)
Dicerorhinus–Rhinocerotina	25.9 ± 1.9	23–16 (Lower Miocene)

[a] Calibrated on the date of 56 MYBP for the equid–ceratomorph split (Garland *et al.*, 1993). Standard error on dates was estimated from standard error on branch length.
[b] See text for references.

remaining rhinoceroses (tree 5) is worse than the best tree by the criterion of 1.4 SE ($P = 0.16$). In the four remaining trees, statistically less likely at least by the criterion of 1.2 SE ($P = 0.23$), Tapiridae either clusters with Equidae (trees 3 and 7) or is the first branch among Perissodactyla (trees 4 and 6).

Divergence Dates among Perissodactyla

To estimate the divergence dates among Perissodactyla, the hypothesis of a molecular clock was tested for cytochrome *b* (without transitions in the third position) and for the 12S rRNA (without indels) based on a likelihood-ratio test (option z in PUZZLE). In both cases, the clocklike hypothesis is rejected for the whole dataset ($\delta = 76.8$, $P < 0.001$ for Cytb; $\delta = 60.13$, $P < 0.001$ for 12S). Therefore, we checked for a molecular clock by resampling a subset of taxa among the Carnivora and the Cetartiodactyla. We found that the 12S data fit the clock hypothesis ($\delta = 19.4$, $0.01 < P < 0.05$) when Carnivora are represented by *Panthera* and *Phoca* and Cetartiodactyla by *Sus* and *Tayassu*. Divergence dates were then calculated from branch length estimated in ML under the molecular clock model. Standard errors on branch length were used to calculate standard error on dates.

Because of the uncertain phylogenetic relationships among Perissodactyla, Carnivora, and Cetartiodactyla, divergence dates among Perissodactyla were estimated using as calibration point the date of 56 Myr for the split between equids and ceratomorphs (Garland *et al.*, 1993). This date is based on the appearance of both undisputed equoids and tapiroids in the Early Eocene fossil record (Prothero and Schoch, 1989). The estimations of divergence dates for the different perissodactyl splits are listed in Table 4.

DISCUSSION

Rhinocerotidae Phylogenetic Relationships

The monophyly of Rhinocerotidae is well established from morphological and paleontological data and defined by four synapomorphies at least: a long nasal, chisel-shaped I^1, I_2 developed as a tusk, and sustentaculum of the calcaneum at a right angle (Cerdeño, 1995). In our combined molecular analysis, this clade is also well supported (RP = 100%, BP = 100%, DI = +25) with respect to Equidae and Tapiridae, its closest relatives. Based on the 12S molecular clock, divergence of Rhinocerotidae was estimated at 29.3 ± 1.8 Myr. This dating is close to the previous mitochondrial estimations by Xu *et al.* (1996) and Xu and Arnason (1997), which found 30 Myr based on the cytochrome *b* gene and 27 Myr based on mtDNA 12 protein-coding genes, respectively. Other datings, based on allozymic loci (Merelender *et al.*, 1989) or restriction mapping of the mtDNA ribosomal region (Morales and Melnick, 1994), provided 26 and 22 Myr, respectively. All these molecular estimations are more recent than the appearance of the first rhinocerotid, *Teletaceras*, which is dated between 49 and 37 Myr (Middle Eocene; Cerdeño, 1998). According to Hanson (1989), the phylogenetic position of *Teletaceras* is intermediate between *Hyrachyus* (a primitive Rhinocerotoidea) and all other rhinocerotids. Indeed, *Teletaceras* retains plesiomorphic features (presence of I^3/I_3 and canines), whereas all other rhinocerotids exhibit derived states. Thus, the first true rhinocerotid should be more recent than *Teletaceras*, that is in the Early Oligocene (between 30 and 26 Myr).

Among Rhinocerotidae, all authors consider the two living *Rhinoceros* species as sister taxa. In our molecular analysis, this cluster is also strongly supported (RP/BP = 100%, DI = +23). The clade Rhinocerotina (*R. unicornis* and *R. sondaicus*) is defined by at least 10 cranial, postcranial, and dental synapomorphies, such as an ascending ramus inclined forward, a great occipital elevation of the cranial dorsal profile, subhypsodont cheek teeth, or a long calcaneum (Groves, 1983; Cerdeño, 1995). According to our molecular estimates, the divergence of *Rhinoceros* is dated from 11.7 ± 1.9 Myr. This is much older than the paleontological estimation since the genus *Rhinoceros* is documented in the fossil record only from the Late Pliocene (between 3.3 and 1.6 Myr; Carroll, 1988).

Although less supported than Rhinocerotina, strong support (RP = 100%, BP = 84%, DI = +5) is provided for Dicerotina (*D. bicornis* and *C. simum*). This clade is well supported by the 12S and the two combined genes but not with the Cytb (with DNA parsimony and amino acid analyses, *Diceros* is the first offshoot among Rhinocerotidae). The African genera are morphologically defined by several synapomorphies, such as an astra-

galus facet more or less flattened, I_2 lost, and an occipital crest angle above 100° (Groves, 1983; Cerdeño, 1995). Our molecular dating indicates a divergence date of 17.1 ± 2.5 Myr for the split *Diceros/Ceratotherium*, in agreement with the paleontological dating of 13 Myr based on the first *Diceros* fossil (Hooijer, 1978).

The main question remains the phylogenetic position of the Sumatran rhinoceros, *D. sumatrensis*, with respect to Dicerotina and Rhinocerotina. According to our study, *D. sumatrensis* seems closer to the other Asian species than to the African species. Although ML and MP supports are relatively high (RP = 97%, BP = 77%, DI = +3), the cluster *Dicerorhinus*–Rhinocerotina is the least supported clade among Rhinocerotidae. The alternative topology in which the Sumatran rhinoceros is the sister taxon to Dicerotina shows nearly the same likelihood, indicating that the two topologies are equally likely. Although weakly supported (BP = 57%), a sister group relationship between *Dicerorhinus* and Dicerotina was also obtained by Morales and Melnick (1994) based on restriction site mapping of mtDNA. However, our results do not support the cluster *Dicerorhinus*–Dicerotina in the quartet puzzling or in the bootstrap trees (RP = 1.8%, BP = 21.4%, DI = −3).

From a morphological point of view, *D. sumatrensis* is commonly considered to retain a large number of primitive characters, which explains why it is so difficult to classify with respect to the other rhinoceros species (Groves, 1983). In the cladistic analysis of Cerdeño (1995), *Dicerorhinus* is characterized by at least five reversals to plesiomorphic states (cranial dorsal profile flattened, vertical occipital face, protocone on upper premolars and upper molars not constricted, high and narrow astragalus). Therefore, *Dicerorhinus* is clustered neither with Rhinocerotina nor with Dicerotina, but constitutes a separate lineage. According to Groves (1983), *Rhinoceros* and *Dicerorhinus* share 14 apomorphic character states (occipital crest angle under 100°, postorbital processes developed, metacone rib developed on upper cheek teeth, antecrochet lost on cheek teeth, radius shortened, . . .) whereas *Dicerorhinus* and the two African species share only 1 apomorphy (I_1 lost).

In conclusion, although the two hypotheses remain likely, we favor the cluster *Dicerorhinus*–Rhinocerotina because it is the clade most supported from molecular and morphological data. Molecular datings suggested a split at 25.9 ± 1.9 Myr between the two Asian genera, whereas the paleontological emergence of the genus *Dicerorhinus* is dated from the Lower Miocene (between 23 and 16 Myr; Carroll, 1988).

The different hypotheses proposed for the relationship of *Dicerorhinus*, on the basis of the number of horns and/or the geographical distribution (see Fig. 1), have lead to different systematic classifications, which are summarized in Table 5. Our analyses of cytochrome *b* and 12S rRNA sequences corroborate the geographic closeness hypothesis, which clusters *Dicerorhinus* with the two other Asian species. Consequently, the classification of Groves (1983) is the one that best fits our results. Namely, the two African *Diceros* and *Ceratotherium* are included in the tribe Dicerotini. The three Asian species belong to the tribe Rhinocerotini, which is then split into the subtribes Rhinocerotina (*Rhinoceros*) and Dicerorhinina (*Dicerorhinus*).

Other Phylogenetic Relationships among Perissodactyla

From morphological and molecular data, monophyly of the clade Perissodactyla is not questioned (MacFadden, 1992; Xu *et al.*, 1996). Since its first description by Owen in 1848, the Perissodactyla have been considered among systematists as a monophyletic group on the basis of several morphological characters: saddle-shaped (concave) distal navicular facet on the astragalus (Radinsky, 1966; MacFadden, 1976), mesaxonic limb symmetry coupled with the extent of reduction of lateral metapodials (MacFadden, 1976), the expended caecum in which hindgut fermentation occurs in all Perissodactyla (Mitchell, 1905; Janis, 1976), and morphology of the lower cheekteeth (MacFadden, 1992). Our study also strongly supported (RP/BP = 100%, DI = +20) the Perissodactyla with regard to Carnivora and Cetartiodactyla, its two putative sister groups. Traditionally, extant Perissodactyla are separated into two suborders: Hippomorpha ("horse-shaped"), including horses and their relatives (Equidae), and Ceratomorpha ("horn-shaped"), including tapirs (Tapiridae) and rhinoceroses (Rhinocerotidae; Wood, 1937).

In the present study, monophyly of the Equidae family is clearly evidenced (RP/BP = 100%, DI = +52). Other molecular studies based on α-globin gene cluster (Flint *et al.*, 1990) also give support to this monogeneric family. Equidae, whose morphological evolution is a school case, are morphologically defined by several characters, including the basicranium morphology (MacFadden, 1976) and the presence of a postprotocrista on P^3 (Hooker, 1989). The paleontological origin of the genus *Equus* is well documented, and the species are believed to have diverged within the past 3–5 Myr (Simpson, 1951; Lindsay *et al.*, 1975). Xu (1996) proposed 9 Myr for the divergence between donkey and horse on the basis of complete mtDNA genome, which is close to our estimation of 12.2 ± 2.2 Myr, but much older than the fossil evidence.

In the present study, the clade Ceratomorpha is well supported by both nucleotide analyses (RP = 93%, BP = 92%, DI = +11). Alternative topologies considering Tapiridae either as the sister group to Equidae (trees 3 and 7 in Table 3) or as the first offshoot in Perissodactyla (trees 4 and 6 in Table 3) are significantly worse than the highest likelihood tree by the

TABLE 5

Taxonomy of Living Rhinocerotidae at the Subfamilial, Tribal, and Subtribal Levels, According to Diverse Authors

	Diceros bicornis	*Ceratotherium simum*	*Dicerorhinus sumatrensis*	*Rhinoceros sondaicus*	*Rhinoceros unicornis*
Horn number hypothesis					
Simpson (1945)/Loose (1975)	Dicerorhininae			Rhinocerotinae	
	Dicerotini		Dicerorhinini		
Geographical hypothesis					
Pocock (1945)	Dicerinae		Rhinocerinae		
Groves (1983)	Rhinocerotinae				
	Dicerotini		Rhinocerotini		
			Dicerorhinina	Rhinocerotina	
Both hypotheses					
Guérin (1982)	Dicerotinae		Dicerorhininae	Rhinocerotinae	
Prothero and Schoch (1989)	Rhinocerotinae				
	Rhinocerotini				
	Dicerotina		Dicerorhinina	Rhinocerotina	
Cerdeño (1995)	Rhinocerotinae				
	Rhinocerotini				
	Dicerotina			Rhinocerotina	

criterion of 1.2 SE at least. Moreover, these two hypotheses are not supported in the combined bootstrap trees: RP = 3%, BP = 6%, and DI = −11 for the cluster Tapiridae–Equidae, and RP = 4%, BP = 2%, and DI = −13 for the basal position of Tapiridae within Perissodactyla. However, amino acids appear incongruent with nucleotides in strongly supporting the alternative Tapiridae–Equidae (RP = 98%, BP = 84%). An explanation for this incongruency might be the low number of informative amino acids (67) as compared to nucleotides (661 for both genes). More support to the cluster Tapiridae–Rhinocerotidae than to the clade Equidae–Tapiridae is also provided by complete mitochondrial DNA sequences of five perissodactyl species (Xu, 1996).

Tapirs and rhinoceroses were early associated as a separate clade from horses, particularly because they have more than one hoof on each foot (Simpson, 1945). Our molecular estimation provides 46.7 ± 3.7 Myr for the divergence between the ceratomorph and the hippomorph lineages. This dating is in agreement with the fossil record, which dates the appearance of the first ceratomorph-like taxon, *Hyrachyus*, in the Middle Eocene (around 50 Myr; Prothero and Schoch, 1989).

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