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

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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 Dicerorhinus) 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, Dicerorhinus, 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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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 Dicerorhinus 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 Dicerorhinus 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 (Promega) or in the original TA cloning kit vector using INVα F one-shot bacteria (Invitrogen). Sequencing (three different clones in the case of cloning) was performed either manually, using the thermostequence kit (Amersham) with [33P]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. AJ 245721–AJ 245725.

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
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 invariant sites) for substitution rates. Robustness was estimated through reliability percentages (RP) representing, as a percentage, how often a group appears

### Table 1

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

<table>
<thead>
<tr>
<th>12S rRNA</th>
<th>Cytochrome b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Order Perissodactyla</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Suborder Ceratomorpha</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Family Rhinocerotidae</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Subfamily Rhinocerotinae</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Tribe Rhinocerotini</strong></td>
<td></td>
</tr>
<tr>
<td>Dicerorhinus sumatrensis</td>
<td>This study, AJ245722</td>
</tr>
<tr>
<td>Rhinoceros sondaicus</td>
<td>This study, AJ245724</td>
</tr>
<tr>
<td>Rhinoceros unicornis</td>
<td>Xu et al., 1996 (X97336)</td>
</tr>
<tr>
<td><strong>Subtribe Dicerotina</strong></td>
<td></td>
</tr>
<tr>
<td>Ceratotherium simum</td>
<td>Xu and Arnason, 1997 (Y07726)</td>
</tr>
<tr>
<td>Diceros bicornis</td>
<td>This study, AJ245721</td>
</tr>
<tr>
<td><strong>Family Tapiridae</strong></td>
<td></td>
</tr>
<tr>
<td>Tapirus terrestris*</td>
<td></td>
</tr>
<tr>
<td><strong>Suborder Hippomorpha</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Family Equidae</strong></td>
<td></td>
</tr>
<tr>
<td>Equus caballus</td>
<td>Xu and Arnason, 1994 (X79547)</td>
</tr>
<tr>
<td>Equus grevyl</td>
<td>Douzery and Catzeflis, 1995 (X86943)</td>
</tr>
<tr>
<td><strong>Order Carnivora</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Suborder Feliformia</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Family Felidae</strong></td>
<td></td>
</tr>
<tr>
<td>Panthera tigris</td>
<td>Ledje and Arnason, 1996a (Y08504)</td>
</tr>
<tr>
<td><strong>Family Herpestidae</strong></td>
<td></td>
</tr>
<tr>
<td>Herpestes auriculatus</td>
<td>Ledje and Arnason, 1996a (Y08506)</td>
</tr>
<tr>
<td><strong>Suborder Caniformia</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Family Phocidae</strong></td>
<td></td>
</tr>
<tr>
<td>Phoca vitulina</td>
<td>Arnason and Johnsson, 1992 (X63726)</td>
</tr>
<tr>
<td><strong>Family Ursidae</strong></td>
<td></td>
</tr>
<tr>
<td>Ursus arctos</td>
<td>Ledje and Arnason, 1996a (Y08519)</td>
</tr>
<tr>
<td><strong>Order Artiodactyla</strong></td>
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</tr>
<tr>
<td><strong>Infraorder Suina</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Family Suidae</strong></td>
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<tr>
<td><strong>Family Tayassuidae</strong></td>
<td></td>
</tr>
<tr>
<td>Tayassu tajacu</td>
<td>Douzery and Catzeflis, 1995 (X86944)</td>
</tr>
<tr>
<td><strong>Suborder Ruminantia</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Family Bovidae</strong></td>
<td></td>
</tr>
<tr>
<td>Bos taurus</td>
<td>Sanger and Young, 1982 (V00654)</td>
</tr>
<tr>
<td><strong>Family Cetacea</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Family Balaenopteridae</strong></td>
<td></td>
</tr>
<tr>
<td>Balaenoptera physalus</td>
<td>Arnason et al., 1991 (X61145)</td>
</tr>
</tbody>
</table>

*Note.* Author references and accession numbers are indicated for each sequence. Species involved in a chimera are noted by an asterisk (*).
12S rRNA Sequence Analysis

The alignment of 12S rRNA sequences of 16 taxa was 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 = 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 = 890, 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).
Combined Analysis of the 12S RNA 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

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 clades, 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.

**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)

<table>
<thead>
<tr>
<th>Tree</th>
<th>ΔLnL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ΔLnL/SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: (((DIC, DS), RHI), TAP), EQU</td>
<td>-11357.20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Best tree</td>
<td></td>
</tr>
<tr>
<td>2: (((DS, RHI), DIC), TAP), EQU</td>
<td>0.19</td>
<td>6.91</td>
<td>0.027</td>
</tr>
<tr>
<td>3: (((RHI, DS), DIC), (EQU, TAP))</td>
<td>11.54</td>
<td>9.84</td>
<td>1.17</td>
</tr>
<tr>
<td>4: (((RHI, DIC), DS), EQU, TAP)</td>
<td>12.75</td>
<td>9.75</td>
<td>1.31</td>
</tr>
<tr>
<td>5: (((RHI, DIC), DS), TAP), EQU</td>
<td>6.38</td>
<td>4.69</td>
<td>1.36</td>
</tr>
<tr>
<td>6: (((DIC, DS), RHI), EQU, TAP)</td>
<td>10.48</td>
<td>7.07</td>
<td>1.48</td>
</tr>
<tr>
<td>7: (((DIC, DS), RHI), (EQU, TAP))</td>
<td>10.76</td>
<td>6.99</td>
<td>1.56</td>
</tr>
</tbody>
</table>

<sup>a</sup> Log-likelihood difference between the best ML tree and the evaluated topology.

<sup>b</sup> Standard error of log-likelihood difference.

<sup>c</sup> Log-likelihood value of the best ML tree.
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 cytchrome $b$ (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 equids 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.

### TABLE 4

<table>
<thead>
<tr>
<th>Clade</th>
<th>Molecular estimation* (in MYBP)</th>
<th>Paleontological estimation* (in MYBP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perissodactyla</td>
<td>56</td>
<td>56 (Upper Paleocene)</td>
</tr>
<tr>
<td>Hippomorpha (Equidae)</td>
<td>12.2 ± 2.2</td>
<td>5-3 (Lower Pliocene)</td>
</tr>
<tr>
<td>Ceratomorpha</td>
<td>46.7 ± 3.7</td>
<td>50 (Middle Eocene)</td>
</tr>
<tr>
<td>Rhinoocerotida</td>
<td>29.3 ± 1.8</td>
<td>49-37 (Middle Eocene)</td>
</tr>
<tr>
<td>Dicerorhinus-Rhinocerotina</td>
<td>17.1 ± 2.5</td>
<td>13 (Middle Miocene)</td>
</tr>
<tr>
<td>Rhinocerotina</td>
<td>11.7 ± 1.9</td>
<td>3.3-1.6 (Upper Pliocene)</td>
</tr>
<tr>
<td>Dicerorhinus-</td>
<td>25.9 ± 1.9</td>
<td>23-16 (Lower Miocene)</td>
</tr>
</tbody>
</table>

* 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.

### 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 (Cerdeno, 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 (Mereder 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, Telteaceras, which is dated between 49 and 37 Myr (Middle Eocene; Cerdeno, 1998). According to Hanson (1989), the phylogenetic position of Telteaceras is intermediate between Hyracychus (a primitive Rhinocerotidae) and all other rhinocerotids. Indeed, Telteaceras retains plesiomorphic features (presence of $I^3$/$A_3$ and canines), whereas all other rhinocerotids exhibit derived states. Thus, the first true rhinocerotid should be more recent than Telteaceras, 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 clade is also strongly supported (RP/BP = 100%, DI = +23). The clade Rhinocerotina (R. unicornis and R. sondaicus) is defined by at least 10 cranial, postcranal, and dental synapomorphies, such as an ascending ramus inclined forward, a great ocipital 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, \( I_1 \) lost, and an occipital crest angle above 100° (Groves, 1983; Cerdeño, 1995). Our molecular dating indicates a divergence date of \( 17.1 \pm 2.5 \text{ Myr} \) for the split Dicerorhinus/Ceratotherium, in agreement with the paleontological dating of 13 Myr based on the first Dicerorhinus 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–Rhinoce-rotina because it is the clade most supported from molecular and morphological data. Molecular datings suggested a split at 25.9 \( \pm \) 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 other two Asian species. Consequently, the classification of Groves (1983) is the one that best fits our results. Namely, the two African Dicerorhinus 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; J anis, 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 Cetar- morpha (“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 \( \alpha \)-globin gene cluster (Flint et al., 1990) also give support to this monoge- neric 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 postproto- crista on P3 (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 \( \pm \) 2.2 Myr, but much older than the fossil evidence.

In the present study, the clade Equidae 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
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 hip-pomorph 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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