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Evolution

Phylogeny and evolution of antlered deer determined from mitochondrial DNA sequences

(DNA systematics/Cervidae/phylogenetic robustness/fossils/biogeography)

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ABSTRACT **Mitochondrial DNA sequences of both ribosomal RNA genes and three adjacent transfer RNA genes were obtained for the three extant subfamilies of antlered deer (Cervinae, Muntiacinae, and Odocoileinae) as well as for their antlerless sister group Hydropotinae (family Cervidae). Phylogenetic analysis of these sequences (each nearly 2.7 kilobase pairs in length) supports a cervine/muntiacine clade to the exclusion of odocoileines. These results are statistically significant, stable, and congruent with some independent data. Our mitochondrial DNA sequences, when coupled with other information, indicate that the earliest fossil antlered deer are not closely related to living muntiacines or any other contemporary subfamily. From this information, we hypothesize an Old World, Late Miocene origin of Odocoileinae.**

The subfamilies Cervinae (Old World deer), Muntiacinae (muntjacs and tufted deer), and Odocoileinae (New World deer) of the family Cervidae (order Artiodactyla, suborder Ruminantia) (table 1.V. in ref. 1) are unique in that males (as well as females of *Rangifer*) have antlers (2, 3). In the subfamilies Cervinae and Odocoileinae, antlers are usually large and complex structures, consisting of a long, multibranched distal element attached to a short nondeciduous pedicle or base. In contrast, the antlers of Muntiacinae are relatively small with a short, simple distal region connected to a long pedicle. This latter condition is considered primitive because it is found in the earliest fossil antlered deer (tribe Dicrocerini) from the Early and Middle Miocene of Eurasia (2–5). Because of their similar small, long-pedicled antlers, living muntiacines and dicrocerines have been assigned to the same subfamily Muntiacinae (= Cervulinae) (4, 6–8). However, this and other conclusions about antlered deer phylogeny are rarely supported by synapomorphies (shared derived characters) obtained from explicit cladistic analysis (9). The morphological cladogram of Groves and Grubb (10) is an important exception.

Previous phylogenetic studies of morphological and paleontological information for the suborder Ruminantia have demonstrated that the three extant subfamilies of antlered deer form a monophyletic group (3, 10). The sister group to these three subfamilies is the antlerless *Hydropotes inermis* (Chinese water deer), the sole living representative of Hydropotinae (1, 3, 5, 10, 11). Evolutionary relationships among antlered deer subfamilies remain unresolved (10).

We sequenced the 12S and 16S ribosomal RNA (rRNA) genes and three flanking transfer RNA (tRNA^{Phe}, tRNA^{Val}, and the 5′ end of tRNA^{Leu}) genes of mitochondrial DNA (mtDNA) from *Cervus unicolor* (sambar deer, subfamily Cervinae), *Muntiacus reevesi* (Reeves' muntjac, subfamily Muntiacinae), and *Odocoileus virginianus* (white-tailed deer, subfamily Odocoileinae) as well as from the closely related outgroup *H. inermis* (Chinese water deer, subfamily Hydropotinae)§. These newly obtained orthologues spanning nearly 2.7 kilobase pairs of contiguous coding mtDNA provide the large number of phylogenetically informative characters needed to resolve subfamilial relationships among antlered deer. With these results, we have reevaluated the affinities of dicrocerines and the New World origins of odocoileines.

MATERIALS AND METHODS

mtDNA from the four cervids was purified from brain or liver (12), cleaved into restriction fragments by *Bam*HI or *Eco*RI, and then cloned into the corresponding sites of plasmid vector pUC19 or pBR322 (13). Amplified plasmid DNA carrying the 2.7-kilobase-pair region of interest was isolated from transformants of DHα5 or JM109 by the alkaline extraction method (14) and then directly sequenced either by the chemical cleavage procedure [*Odocoileus* (15)] or by the dideoxy double-strand method [*Cervus*, *Hydropotes*, and *Muntiacus* (16, 17)]. The *Cervus*, *Hydropotes*, and *Muntiacus* orthologues were sequenced in both directions by a series of 14 primers (7 per complement), which were more or less evenly spaced along the two strands. Similarly, the *Odocoileus* orthologue was sequenced in both directions. The Needleman and Wunsch algorithm (18) was used to align the cervid sequences to each other and to the more distantly related outgroup *Bos taurus* [family Bovidae, suborder Ruminantia (19)]. Parsimony analysis of the aligned sequences was conducted with PAUP (20) as described (21, 22); bootstrap resampling used the program in PHYLIP (23).

Branch lengths for the most-parsimonious solution were calculated two different ways: (*i*) from unambiguous changes only (e.g., those with one most-parsimonious placement on the phylogeny) and (*ii*) from all mutations, including those with multiple assignments (24). Such ambiguous mutations were assigned to the most-parsimonious phylogeny according to the optimization procedure of Fitch (24). This approach uses all parsimonious placements of an ambiguous mutation to calculate a probability of change for each branch. These probabilities are then summed across all mutations to obtain the branch lengths. In this manner, each alternative placement contributes proportionally to the final assignments.

RESULTS AND DISCUSSION

Pairwise comparisons of the four cervid sequences indicate that *Cervus* and *Muntiacus* are most similar (Fig. 1 and Table 1). *Cervus* and *Muntiacus* differ by 5.6%, whereas *Odocoileus* differs from them by an average of 7.6%. On average,

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Abbreviation: MA, million years ago.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M35874–M35877).

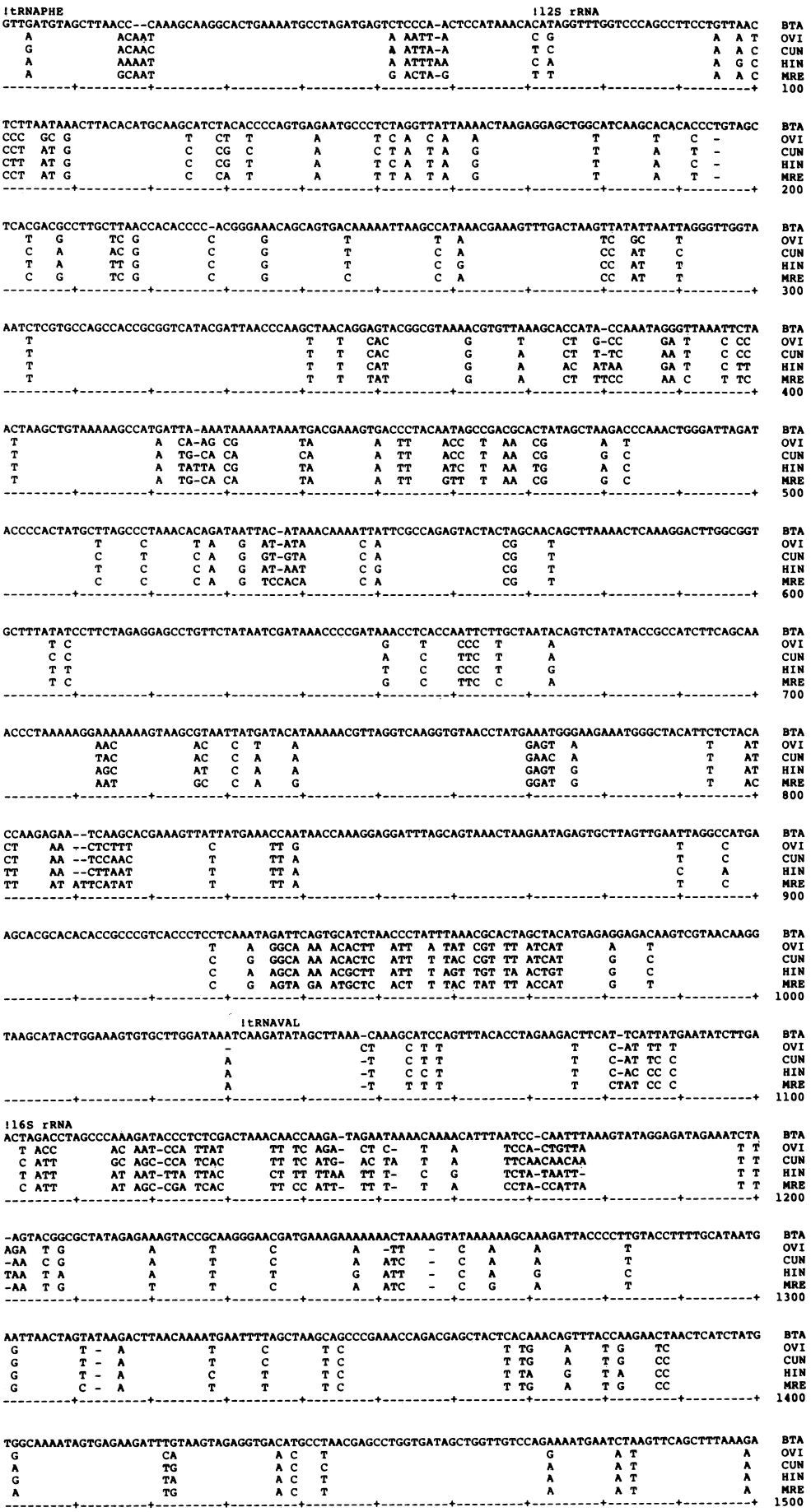

FIG. 1. *(Figure continues on the opposite page.)*

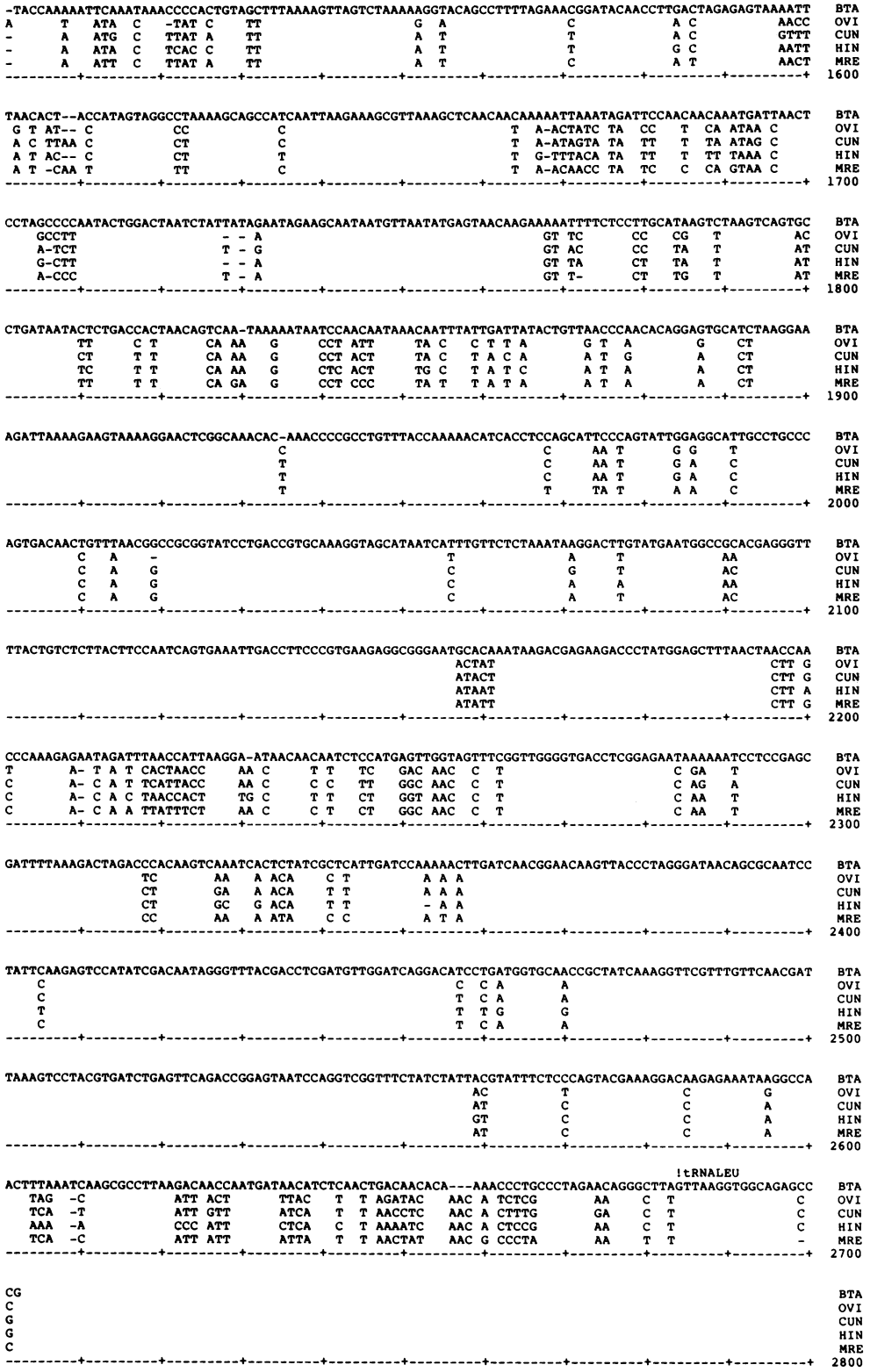

FIG. 1. Aligned mtDNA sequences of *B. taurus* [BTA (19)], *C. unicolor* (CUN), *H. inermis* (HIN), *M. reevesi* (MRE), and *O. virginianus* (OVI). Dashes refer to gaps, which have been included in the overall alignment to maximize homology (18). The *B. taurus* sequence is shown in its entirety, whereas only nucleotides at variable positions are presented for cervids. The locations of the mitochondrial tRNA and rRNA genes are indicated above the *B. taurus* sequence.

the three representatives of antlered deer differ from the outgroup (*Hydropotes*) by 7.7%.

Parsimony analysis of the three possible dichotomous solutions for the study group and outgroup demonstrates that the *Cervus/Muntiacus* arrangement is 17 and 21 mutations shorter than the *Cervus/Odocoileus* and *Muntiacus/Odocoileus* alternatives, respectively (Fig. 2) (20). The *Cervus/Muntiacus* network is supported by 37 unique mutations representing 28 transitions (positions 71, 152, 192, 204, 388, 425, 431, 480, 513, 661, 662, 772, 812, 813, 933, 949, 961, 1103, 1119, 1120, 1127, 1253, 1402, 1423, 1705, 1708, 2239, and 2665), six transversions (sites 57, 1526, 1671, 2091, 2605, and

Table 1. Pairwise comparisons of cervid mtDNA sequences

Species compared	BP	Substitutions TS	TV	TS/TV	Gaps	% divergence*
CUN/HIN	2675	141	43	3.3	11	7.3
CUN/MRE	2677	116	24	4.8	9	5.6
CUN/OVI	2673	141	41	3.4	12	7.2
HIN/MRE	2673	142	51	2.8	14	7.7
HIN/OVI	2672	156	47	3.3	13	8.0
MRE/OVI	2670	156	40	3.9	17	7.9

BP, base positions shared by both sequences; TS, transitions; TV, transversions; Gaps, insertions and deletions; CUN, *C. unicolor*; HIN, *H. inermis*; MRE, *M. reevesi*; OVI, *O. virginianus*.
*The percent sequence divergence was calculated as [(TS + TV + Gaps)/(BP + Gaps)] × 100%, where gaps are conservatively counted as single differences, regardless of length.

2649), and three gap events (one deletion at site 1201 and two insertions at positions 1608–1609 and 1728). In contrast, the *Cervus/Odocoileus* and *Muntiacus/Odocoileus* alternatives are supported by 20 and 16 unique mutations [19 transitions (positions 351, 398, 460, 776, 801, 936, 946, 963, 971, 1086, 1116, 1169, 1335, 1607, 1673, 1780, 2225, 2245, and 2664) and one deletion (site 380) versus 12 transitions (positions 208, 986, 1571, 1599, 1670, 1681, 1688, 1785, 2319, 2327, 2341, and 2663) and four transversions (sites 815, 1674, 2651, and 2701)], respectively.

The reliability of the most-parsimonious phylogeny has been evaluated by several approaches (25). The total number of unique changes in favor of the *Cervus/Muntiacus* solution is significantly greater than that for the *Cervus/Odocoileus* alternative (two-tailed sign test, $\alpha = 0.05$, 95% confidence interval of 22.96–48.67%) (26). Consequently, the same conclusion is reached for the *Muntiacus/Odocoileus* alternative. The *Cervus/Muntiacus* arrangement is stable when the sequences are resampled by bootstrapping (98.3% replication out of 1000 trials) (27). Parsimony analysis of transversion differences indicates that the *Cervus/Muntiacus* phylogeny (with 11 unique substitutions) is shorter than the *Cervus/Odocoileus* and *Muntiacus/Odocoileus* alternatives (with 0 and 5 diagnostic changes) by 11 and 6 extra mutations, respectively. When analyzed separately, the 12S and 16S rRNA gene sequences each join *Cervus* and *Muntiacus* together once again. Finally, the addition of *B. taurus* to the cervid sequence file (Fig. 1) results in the same most-parsimonious arrangement for antlered deer, with *Bos* joining *Hydropotes*. Further, this most-parsimonious solution, which is at least 7 mutations shorter than its closest competitors, supports the hypothesis that antlered deer constitute a monophyletic group (1, 3, 5, 10, 11, 28).

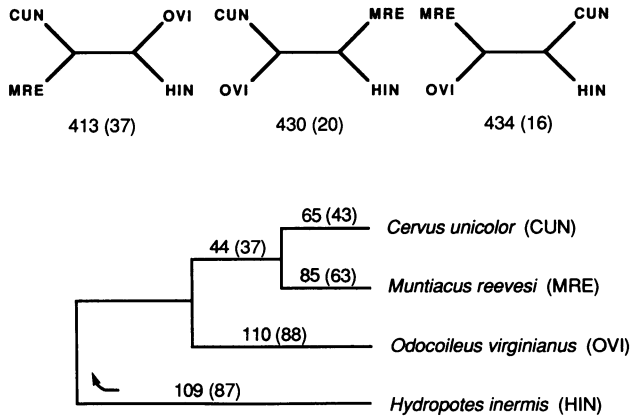

FIG. 2. (*Upper*) Tree lengths and total numbers of unique mutations (in parentheses) for the three possible dichotomous arrangements of *Cervus* (CUN), *Hydropotes* (HIN), *Muntiacus* (MRE), and *Odocoileus* (OVI). (*Lower*) Most-parsimonious phylogeny rooted using *Hydropotes* as the outgroup. The parenthetical estimates of branch length are based on unambiguous changes (e.g., those with only one most-parsimonious placement), whereas the other values are calculated from all mutations, including those with multiple assignments (see text).

The importance of using larger amounts of DNA sequence data to resolve difficult systematic problems is illustrated by the 12S and 16S rRNA orthologues of cervids (Figs. 1 and 2). When analyzed separately, neither set of gene sequences provides significant support for the *Cervus/Muntiacus* solution (sign test, 95% confidence intervals of 19.56–56.78% and 21.93–57.47% for the 12S and 16S rRNA orthologues, respectively). However, by combining the two sets of sequences, statistically significant support is obtained for the *Cervus/Muntiacus* clade (95% confidence interval of 22.73–48.96%). Thus, longer sequences of orthologous DNA offer more phylogenetically informative characters and, thereby, better resolution of difficult phylogenetic questions.

Our phylogeny is robust for mtDNA sequence data. While extensive cladistic studies based on other character systems are uncommon, some other comparative data are congruent with the phylogenetic conclusions advanced here. Based on morphological data, Groves and Grubb (10) have obtained the same cladogram for deer, although only one synapomorphy (plesiometacarpal condition of lateral metacarpals) serves to diagnose the *Cervus/Muntiacus* clade. Similarly, an amino acid replacement of serine for glycine at position 12 of fibrinopeptide A diagnoses this group (29).

Our phylogeny, when coupled with paleontological data, indicates that the tribe Dicrocerini cannot be closely related to living muntiacines. If these two groups were monophyletic, it would require that the splitting of cervines and muntiacines antedate the oldest dicrocerines. That would place the time of this split before 19–21 million years ago (MA), the oldest known age for fossil antlered deer (30, 31). However, recent muntiacines and cervines first appear in the fossil record about 6–8 MA in the Late Miocene of Asia and/or Europe (8, 32, 33). The same is true of odocoileines (8, 34, 35), a lineage of even earlier ancestry according to our phylogeny. Thus, the paleontological data, in concert with our mtDNA phylogeny, falsify the hypothesis that extant muntiacines form a monophyletic group with dicrocerines. As such, the tribe Dicrocerini should be removed from the subfamily Muntiacinae. This conclusion is not unexpected, given that the taxonomic assignments of early fossil deer have been based on primitive attributes rather than on shared derived similarities (4–6).

The most-parsimonious solution (Fig. 2 *Lower*) indicates that rates of nucleotide change among antlered deer are similar (36). This pattern is apparent either when all changes or only unambiguous changes (e.g., those with one unequivocal placement) are counted (24). By adopting a date of 6–8 MA for the cervine/muntiacine split (8, 32, 33), the time of origin for extant antlered deer may be estimated from the relatively equal amounts of change leading to *Cervus*, *Muntiacus*, and *Odocoileus* (36). Such a clock calculation suggests that odocoileines diverged from other antlered deer between 9.3 and 12.4 MA. This estimate agrees with a Late Miocene origin for the subfamily, as hypothesized from paleontological data (8, 34, 35).

In the New World, all deer belong to the subfamily Odocoileinae except for the cervine *Cervus elaphus* (6). In the Old World, this subfamily is represented today by the endemic *Capreolus* as well as by *Alces* and *Rangifer*. The earliest fossils of New World odocoileines are from North America and are younger than those from Eurasia (Early Pliocene versus Late Miocene, respectively) (8, 34, 35, 37). The biogeographic history of odocoileines is more complicated than that of other deer, which, except for *C. elaphus*,

are restricted to the Old World. Nevertheless, an Old World origin for Odocoileinae is suggested by both the geographic distributions of other cervids, as well as by the relative earliest ages of its Eurasian versus North American fossils (8, 34). The most-parsimonious interpretation of the Old World distributions of *Capreolus* [the sister group to all other living odocoileines (10)], cervines, dicrocerines, muntiacines, and more distantly related cervids is that odocoileines originated in Eurasia. Our mtDNA clock estimate places the time of this origin in the Late Miocene at 9.3–12.4 MA.

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