The Royal Society

New phylogenetic perspectives on the Cervidae (Artiodactyla) are provided by the mitochondrial cytochrome *b* gene

Ettore Randi[1*], Nadia Mucci[1], Massimo Pierpaoli[1] and Emmanuel Douzery[2]

[1]*Istituto Nazionale per la Fauna Selvatica, Via Cà Fornacetta 9, 40064 Ozzano dell'Emilia (BO), Italy* (met0217@iperbole.bologna.it) [2]*Laboratoire de Paléontologie, Paléobiologie et Phylogénie, Institut des Sciences de l'Evolution UMR 5554 CNRS, Université Montpellier II—CC064, Place E. Bataillon 34095 Montpellier Cedex 05, France* (douzery@isem.univ-montp2.fr)

The entire mitochondrial cytochrome *b* (cyt *b*) gene was compared for 11 species of the artiodactyl family Cervidae, representing all living subfamilies, i.e. the antlered Cervinae (*Cervus elaphus*, *C. nippon*, *Dama dama*), Muntiacinae (*Muntiacus reevesi*), and Odocoileinae (*Odocoileus hemionus*, *Mazama* sp., *Capreolus capreolus*, *C. pygargus*, *Rangifer tarandus*, *Alces alces*); and the antlerless Hydropotinae (*Hydropotes inermis*). Phylogenetic analyses using Tragulidae, Antilocapridae, Giraffidae and Bovidae as outgroups provide evidence for three multifurcating principal clades within the monophyletic family Cervidae. First, Cervinae and *Muntiacus* are joined in a moderately-to-strongly supported clade of Eurasian species. Second, Old World Odocoileinae (*Capreolus* and *Hydropotes*) associate with the Holarctic *Alces*. Third, New World Odocoileinae (*Mazama* and *Odocoileus*) cluster with the Holarctic *Rangifer*. The combination of mitochondrial cyt *b* and nuclear κ-casein sequences increases the robustness of these three clades. The Odocoileini + Rangiferini clade is unambiguously supported by a unique derived cranial feature, the expansion of the vomer which divides the choana. Contrasting with current taxonomy, *Hydropotes* is not the sister group of all the antlered deers, but it is nested within the Odocoileinae. Therefore, *Hydropotes* lost the antlers secondarily. Thus, the mitochondrial cyt *b* phylogeny splits Cervidae according to plesiometacarpal (Cervinae + Muntiacinae) versus telemetacarpal (Odocoileinae + Hydropotinae) conditions, and suggests paraphyly of antlered deer.

Keywords: subfamilies, Cervidae; molecular phylogeny; mtDNA; cytochrome *b*; antler evolution; *Hydropotes*

1. INTRODUCTION

The family Cervidae (order Artiodactyla, suborder Ruminantia, infraorder Pecora; Janis & Scott 1987) is characterized by deciduous cranial appendages: the antlers. Living representatives of the family include Old World deer (Cervinae), Asian muntjacs and tufted deers (Muntiacinae), Holartic moose and reindeer, New World odocoileines and Old World roe deer (Odocoileinae), and Asian antlerless monospecific *Hydropotes* (Hydropotinae) (Eisenberg 1981, pp. 199–200).

A single character-state, presence or absence of antlers, was used to define the main splitting within the Cervidae, i.e. the basal position of Hydropotinae, which were considered as the antlerless sister group of all the antlered Cervidae (Groves & Grubb 1987; Scott & Janis 1987; Janis & Scott 1987). The latter group was subdivided according to the character-state of the lateral metacarpals: the Odocoileinae show the telemetacarpal condition (the distal part of the second and fifth metacarpals persists), whereas the Cervinae plus Muntiacinae exhibit the plesiometacarpal condition (the proximal part of the second and fifth metacarpals persists: Brooke 1878; Groves & Grubb 1987). This classification of Cervidae assumes homoplasy in some morphological traits, and relies on antlers as a synapomorphy. However, cranial appendages have been shown to evolve independently at least three times in higher ruminants (Janis & Scott 1987). An alternative classification was proposed by Bouvrain *et al.* (1989), who suggested that *Hydropotes*, a telemetacarpalian, could be a sister group of Odocoileinae, or even included within that subfamily. Odocoileinae is currently subdivided into four tribes: Alcini (moose), Capreolini (roe deer), Odocoileini (New World odocoileines) and Rangiferini (reindeer), although evolutionary and taxonomic relationships among them are unclear (Eisenberg 1981, p. 200).

Several molecular investigations have been conducted on the cervid family. They involved mitochondrial and nuclear DNA or amino acid sequence comparisons: fibrinopeptides (Mross & Doolittle 1967), ribonucleases (Beintema *et al.* 1988), 12S and 16S ribosomal RNAs (rRNA; Miyamoto *et al.* 1990; Kraus & Miyamoto 1991), cytochrome *b* (cyt *b*; Irwin *et al.* 1991), κ-casein (Cronin *et al.* 1996) and mitochondrial control-region (Douzery & Randi 1997). The phylogenetic results of these studies suggested (i) strong support for a close association

*Author for correspondence.

Received 8 December 1997 *Accepted* 7 January 1998

between Cervinae and Muntiacinae (Mross & Doolittle 1967; Miyamoto *et al*. 1990; Kraus & Miyamoto 1991; Cronin *et al*. 1996); (ii) moderate-to-strong support for the monophyly of Odocoileinae (Cronin *et al*. 1996; Douzery & Randi 1997); (iii) a sister group relationship between *Alces* and *Capreolus* (Beintema *et al*. 1988; Cronin *et al*. 1996); (iv) strong support for a New World odocoileine clade (Douzery & Randi 1997), with *Rangifer* as their sister group (Cronin *et al*. 1996); and (v) an ambiguous position of *Hydropotes*, which could be either a sister group of all other cervids or a sister lineage of *Odocoileus* (Miyamoto *et al*. 1990; Kraus & Miyamoto 1991). Exploration of the phylogenetic content of the non-coding mitochondrial control region (Douzery & Randi 1997), however, suggested that *Hydropotes* may be nested within Odocoileinae, with pronounced affinities with *Capreolus*.

Because cladistic analyses detected many cases of parallelism and convergence in the evolution of morphological traits among Cervidae (Groves & Grubb 1987; Janis & Scott 1987; Scott & Janis 1987), the understanding of their pattern of character evolution requires the drawing of a phylogeny independent from those traits. A molecular approach, harvesting a large number of characters, may help to solve the unclear phylogeny of cervids. Mitochondrial DNA protein-coding genes and, in particular, the complete mitochondrial cyt *b*, have proven useful for resolving phylogenetic patterns among various artiodactyls within evolutionary time-frames lower than 20 million years (Ma): camelids (Stanley *et al*. 1994), suiformes (Randi *et al*. 1996), and ruminants (Irwin *et al*. 1991; Chikuni *et al*. 1995; Groves & Shields 1996; Tanaka *et al*. 1996). In this paper we analyse the phylogeny of 11 species representative of all living cervid subfamilies during the last 20 Ma through the comparison of complete mtDNA cyt *b* sequences. We aim to use phylogenetic inferences from these nucleotide sequences to address several questions.

1. Do the Cervidae form a monophyletic assemblage within pecoran artiodactyls?
2. What is the phylogenetic position of the antlerless Hydropotinae?
3. Are plesiometacarpal cervids, i.e. Muntiacinae and Cervinae, monophyletic as suggested by cladistic analyses of morphological traits?
4. Are patterns of cyt *b* divergence concordant with other molecular and non-molecular information, and how can they contribute to reconstructing the evolutionary history of Cervidae?

2. MATERIALS AND METHODS

The origin of DNA samples and sequences used in this study is listed in table 1. DNA was extracted from 95% ethanol-preserved tissue following standard procedures (Sambrook *et al*. 1989). The entire mtDNA cyt *b* of fallow deer, muntjac, European and Siberian roe deer, reindeer and moose were PCR-amplified (Mullis *et al*. 1986) using primers ML103 (5′-GAC TAA TGA TAT GAA AAA CCA TCG TTG-3′) and MH104 (5′-TTG TTC TTC ATC TCT GGT TTA CAA GAC-3′) (Chikuni *et al*. 1995). Amplifications were done with AmpliTaq DNA polymerase (Perkin Elmer, Foster City), 1.5–3.0 mM $MgCl_2$ in the reaction buffer, and using the following thermal cycle in a 9600 Perkin Elmer machine: 94 °C for 2 min; 94 °C for 15 s, 55 °C for 15 s, 72 °C for 1 min (30 cycles); 72 °C for 10 min. PCR products were purified by low-melting agarose gels. All sequences were obtained by double-strand DNA cycle sequencing with ABI Prism Dye Terminator procedure in an ABI 373 Sequencer, by using external and internal primers.

Amplification of cyt *b* pseudogenes translocated in the nuclear genome, rather than the functional mitochondrial sequence, may be a potential problem for mammals and birds (Arctander 1995; reviewed in Zhang & Hewitt 1996). To check for this problem, we simultaneously amplified for three taxa the cyt *b* gene and its downstream contiguous sequences towards the 12S rRNA gene. Among mammals, this latter mitochondrial molecule is one of the best known for the evolutionary patterns of the primary sequence and secondary structure (Springer & Douzery 1996): it could help to identify a nuclear mitochondrial-like amplification. For the red deer, brocket and Chinese water deer, a 3 kilobase (kb) segment of mtDNA spanning the contiguous cyt *b*, tRNA-Thr, complementary tRNA-Pro, control region, tRNA-Phe and first half of 12S rDNA sequences was therefore PCR-amplified and cloned in the plasmid pGEM-T (Promega) as described in Douzery & Randi (1997). The complete cyt *b* gene and 400 nt (nucleotides) of the 12S rDNA were dideoxy-sequenced (Sanger *et al*. 1977) on both strands using the Pharmacia kit and [α35S]dATP, with PCR and internal primers.

In addition to our sequences, we downloaded from the EMBL and GenBank databases the following orthologues: (i) pecoran outgroups: *Antilocapra americana*, *Giraffa camelopardalis*, *Capra hircus*, *Ovis aries* and *Bos taurus* (Irwin *et al*. 1991), *Ovibos moschatus* (Groves & Shields 1996), *Bos javanicus* (A. Kikkawa *et al*., unpublished data, accession D34636) and *Bubalus bubalis* (Chikuni *et al*. 1995); and (ii) tragulid outgroups: *Tragulus napu* (Irwin *et al*. 1991) and *T. javanicus* (Chikuni *et al*. 1995). Finally, the cyt *b* data set was combined with the ruminant κ-casein sequences from Cronin *et al*. (1996), after checking for congruence of the two matrices with the Mickevich–Farris index according to Farris *et al*. (1995), and using the XARN program written by J. S. Farris.

The cyt *b* sequences were analysed by using MUST (Philippe 1993) and MEGA (Kumar *et al*. 1993) packages. Tests for compositional stationarity and applicability of substitution models were applied using the program MODELS (Rzhetsky & Nei 1995). Phylogenetic reconstructions were obtained by the neighbour-joining (NJ) method (Saitou & Nei 1987), by the maximum parsimony (MP) method (PAUP 3.1.1., Swofford 1993), and by the maximum-likelihood (ML) method (quartet puzzling approach: Strimmer & von Haeseler 1996, by using PUZZLE 3.1). Robustness of the phylogenies was assessed by three different approaches: (i) the bootstrap percentage (BP) (Felsenstein 1985), with 1000 resamplings followed by an NJ reconstruction (NJBOOT program, Philippe 1993) or a maximum-parsimony reconstruction (bootstrap option in PAUP 3.1.1.); (ii) the decay index (DI), i.e. the number of extra steps required to break the corresponding node (Bremer 1988), using topological constraints enforcement with PAUP 3.1.1.; and (iii) the reliability percentages (RP), i.e. the number of times the group appears after 10 000 ML puzzling steps (Strimmer & von Haeseler 1996), under the Tamura & Nei (1993) model of sequence evolution and using PUZZLE 3.1. Relative likelihoods of alternative topologies were evaluated by the Kishino & Hasegawa (1989) test implemented in DNAML 3.572c (PHYLIP package; Felsenstein 1993).

Table 1. *Classification of living Cervidae (following Eisenberg 1981: 200; Groves & Grubb 1987; Scott & Janis 1987) with the list of the taxa studied, the origin of samples, the references and the EMBL data bank accession numbers of cyt* b *sequences used in this study*

Latin and common name	origin of specimens	references	accession numbers
subfamily Cervinae			
Cervus elaphus (red deer)[a]	Swiss Alps	this paper	AJ000021
Cervus nippon (sika deer)	unknown	Chikuni *et al.* (1995)	D32192
Dama dama (fallow deer, first individual)	unknown	Irwin *et al.* (1991)	X56290
Dama dama (fallow deer, second individual)	Castel Porziano (Roma, Italy)	this paper	AJ000022
subfamily Muntiacinae			
Muntiacus reevesi (Chinese muntjac)	Utrecht (The Netherlands)[b]	this paper	AJ000023
subfamily Odocoileinae tribe Capreolini			
Capreolus capreolus (European roe deer)	Asiago (Western Italian Alps)	this paper	AJ000024
Capreolus pygargus (Siberian roe deer)	Amur region (Russia)[c]	this paper	AJ000025
tribe Alcini			
Alces alces (moose)	Norway[d]	this paper	AJ000026
tribe Rangiferini			
Rangifer tarandus (reindeer)	Norway[d]	this paper	AJ000029
tribe Odocoileini			
Odocoileus hemionus (mule deer)	unknown	Irwin *et al.* (1991)	X56291
Mazama sp. (brocket deer)[a,e]	San Diego Zoo[f]	this paper	AJ000027
subfamily Hydropotinae			
Hydropotes inermis (Chinese water deer)[a]	San Diego Zoo[f]	this paper	AJ000028

[a]Tissues maintained in the Collection of Tissues of the Laboratory of Palaeontology, Palaeobiology and Phylogeny in Montpellier (Catzeflis 1991).
[b]Thanks to Jaap Buntier (Utrecht).
[c]Thanks to Aleksey Danilkin (Moscow).
[d]Thanks to Knud Røed (Oslo).
[e] It was not possible to distinguish between the red brocket *M. americana* and the brown brocket *M. gouazoupira*.
[f]Thanks to Oliver Ryder (San Diego Zoo).

3. RESULTS

(a) *Authenticity of mitochondrial cyt* b *sequences of Cervidae*

The mitochondrial authenticity of the sequences was checked to identify putative mitochondrial-like nuclear pseudogenes. All cyt *b* sequences have the initial ATG codon, terminate with the stop codon AGA and are 1140 nt long. An exception is the cyt *b* of reindeer which is 1143 nt long, having a subterminal GGA followed by the stop codon TAA. Neither insertions–deletions nor internal stop or nonsense codons were detected in these cervid cyt *b* sequences. Additional arguments in favour of a mitochondrial origin of our sequences came from the 12S rRNA comparisons. The comparison of 415 nt of the two *Hydropotes inermis* sequences (accession number M35876 in Miyamoto *et al.* (1990), versus this study) reveals that they are identical, except for one G to T transversion at position 104. The comparison of 413 nt of *Odocoileus virginianus* and *Mazama* sp. 12S rRNA sequences (accession M35874 in Miyamoto *et al.* (1990), versus accession AJ000030, this study) indicate 3.8% of total divergence. Among the 16 nt differences observed, there were ten transitions (Ts) and one transversion (Tv) occurring in loops (according to the secondary-structure model of Springer & Douzery (1996)), and five in stems (three are single compensatory changes, one involves a mismatch and one restores base pairing in the brocket deer sequence). The very low amount of divergence between compared sequences, the classical ribosomal patterns of substitutions as well as the phylogenetic position of the cervid sequences (the two Odocoileini taxa cluster together: data not shown) indicate the 12S rRNA sequences, and therefore the contiguous control region and cyt *b*, have a likely mitochondrial origin.

(b) *Nucleotide composition and substitutional saturation*

Nucleotide compositions at the three codon positions and for the entire cyt *b* are similar among the studied species of Cervidae. They correspond to the prevalent compositional patterns of the higher vertebrate mtDNA cyt *b*, with an excess of T over G at second positions, of A over G at third positions, and with a compositional bias increasing from first to second and third codon positions (Irwin *et al.* 1991). Third positions exhibit the higher variability in nucleotide frequencies among Cervidae. Nucleotide frequencies at first plus second positions are stationary, whereas they are not stationary when third positions are added. Tests of applicability of substitution models (Rzhetsky & Nei 1995) suggest that the Tamura & Nei (1993) model is an appropriate estimator of genetic distances among the cyt *b* of Cervidae. Saturation of Ts, and especially the third-position Ts, is apparent in

pairwise comparisons between Cervidae and the outgroups, but also among the most distantly related Cervidae. Stationarity test and saturation pattern evaluation lead to the exclusion of third-position transitions from all subsequent phylogenetic reconstructions.

Average pairwise percentages of sequence divergence (estimated by using Tamura & Nei's formula) is 12% (range 4–17%) within subfamilies of Cervidae, 15% (12–17%) between subfamilies, and 19% (15–25%) between Cervidae and the outgroups. It should be noted that divergence between the two fallow deer sequences (11%) is comparable to intersubfamiliar divergence (see § 3*c*).

(c) *Phylogenetic analyses of the mitochondrial cyt* b

An MP analysis of the cyt *b* sequences (211 phylogenetically informative sites, excluding third-position Ts) produced six most-parsimonious trees (560 steps long; consistency index CI = 0.42; retention index RI = 0.59). These six trees correspond to two alternative branchings among Caprinae and three among cervids. The NJ tree, reconstructed using the Tamura & Nei (1993) distance matrix (third-position Ts excluded), is identical to one of the six MP trees. Bootstrap analyses with NJ and MP methods yielded the single consensus topology which is shown in figure 1.

The monophyly of Cervidae is strongly supported (BP = 100/99, DI + 11). The family is subdivided into three main clades. The first clade includes *Muntiacus* (Muntiacinae) joining *Cervus* and *Dama* (Cervinae). The second clade includes *Alces* (Alcini) joining an unexpected *Capreolus* (Capreolini) plus *Hydropotes* (Hydropotinae) cluster. The third includes *Rangifer* (Rangiferini) joining *Mazama* and *Odocoileus* (New World Odocoileinae). These three clades are supported by BP and DI ranging from 65 to 79, and from + 2 to + 4, but their inter-relationships are unresolved. Constraining the monophyly of Odocoileinae involves nine additional steps, and placing *Hydropotes* as the sister group of all antlered deer involves again nine additional steps.

In a cladistic perspective, we searched for exclusive synapomorphic nucleotide substitutions defining the previous clades. The following exclusive synapomorphic nucleotide replacements unambiguously define (i) the Cervidae: A to G (position 385 in the cyt *b* gene), involving replacement of an amino acid (Met by Val), and a pyrimidine by a purine (position 525); (ii) the *Capreolus* + *Hydropotes* clade: A, C or T by G (same position 525), and A by T (position 849); (iii) the Odocoileini: A by G (position 825); (iv) the Odocoileini + *Rangifer* clade: a pyrimidine by A, twice (positions 441 and 855); and (v) the Odocoileinae: C by T (position 249). These eight diagnostic mitochondrial signatures reinforce the support for the five previously mentioned clades.

The ML tree (see figure 2) was fully congruent with MP and NJ trees. In particular, the ML tree supported the grouping of *Hydropotes* with *Capreolus* (RP = 60%), and the distinction of the three main cervid lineages: Cervinae plus Muntiacinae (RP = 82%), Capreolini, Hydropotinae and Alcini (RP = 77%), Odocoileini and Rangiferini (RP = 88%). Alternative topologies were tested for the significance of differences in likelihoods. Topologies constrained to enforce the paraphyly of Cervidae or the monophyly of antlered deer with a basal position of *Hydropotes*, exhibit significantly worse likelihoods when compared with the best tree.

The cyt *b* amino acid sequences were deduced from nucleotide sequences for the Cervidae and their outgroups. The length of the alignment was 380, with 87 variable positions, but only 55 phylogenetically informative sites. Bootstrap support for the nodes of both NJ and MP trees reconstructed from polypeptide sequence comparisons was very low, and only the two genera *Cervus* and *Capreolus* exhibited BP greater than 50% (not shown).

(d) *Divergence times for the main Cervidae groups*

We calibrated the divergence of the three cervid main clades at 20 Ma, by reference to the age of the oldest known antlered deer (Ginsburg 1988). By using the branch-length estimates of the ML tree (figure 2), we computed a mean $0.14 \pm 0.03\%$ per Ma lineage for the accumulation rate of Ts + Tv on the first and second positions and Tv only on the third codon position of the cyt *b* of cervids. Evolutionary rates actually ranged from $0.12 \pm 0.02\%$ per Ma for Cervinae + Muntiacinae, to $0.15 \pm 0.02\%$ per Ma for Odocoileini + Rangiferini, and $0.16 \pm 0.02\%$ per Ma for Capreolini + Hydropotinae + Alcini. These local molecular clocks were used to estimate the divergence times for the main cervid cladogeneses. The split of each of the three main groups (the plesiometacarpalians and the two telemetacarpalians) occurred in the Middle Miocene between 13.6 and 16.8 Ma before present (see figure 2). Separation of *Hydropotes* from *Capreolus*, and *Dama* from *Cervus*, subsequently occurred in the Late Miocene between 10.9–11.3 and 8.2–11.9 Ma before present (figure 2).

(e) *Combined analysis of mitochondrial cyt* b *and nuclear* κ*-casein genes*

The character matrices of the mitochondrial cyt *b* (third-position Ts were excluded) and the nuclear κ-casein fourth exon sequences (Cronin *et al.* 1996; all events were kept) were highly congruent ($\alpha = 100\%$ after 1000 counts with the XARN program). The resulting combined matrix includes 15 taxa and 1517 sites (211 + 54 = 265 were informative). An MP bootstrap analysis of each data set separately and the combined data set yield the same consensus topologies for cervids (*Hydropotes* was not represented in the casein study). Combination of the mitochondrial and nuclear sequences increases the support for Cervinae + Muntiacinae (BP = 94, DI = +7), *Alces* + *Capreolus* (BP = 79, DI = +4) and *Odocoileus* + *Mazama* + *Rangifer* (BP = 96, DI = +10) clades. The weakest clade is the subfamily Odocoileinae, and even after combination, the support remains low (BP = 61, DI = +2).

The Kishino & Hasegawa (1989) ML test of alternative topologies indicated that branching patterns involving cervid monophyly, the monophyly of Plesiometacarpalia (Cervinae + Muntiacinae), or the monophyly of Odocoileini + Rangiferini always exhibit significantly better likelihoods when compared with any other trees. However, no significant differences in likelihoods were recorded for alternative topologies involving either the monophyly or the paraphyly of Odocoileinae and Alcini + Capreolini.

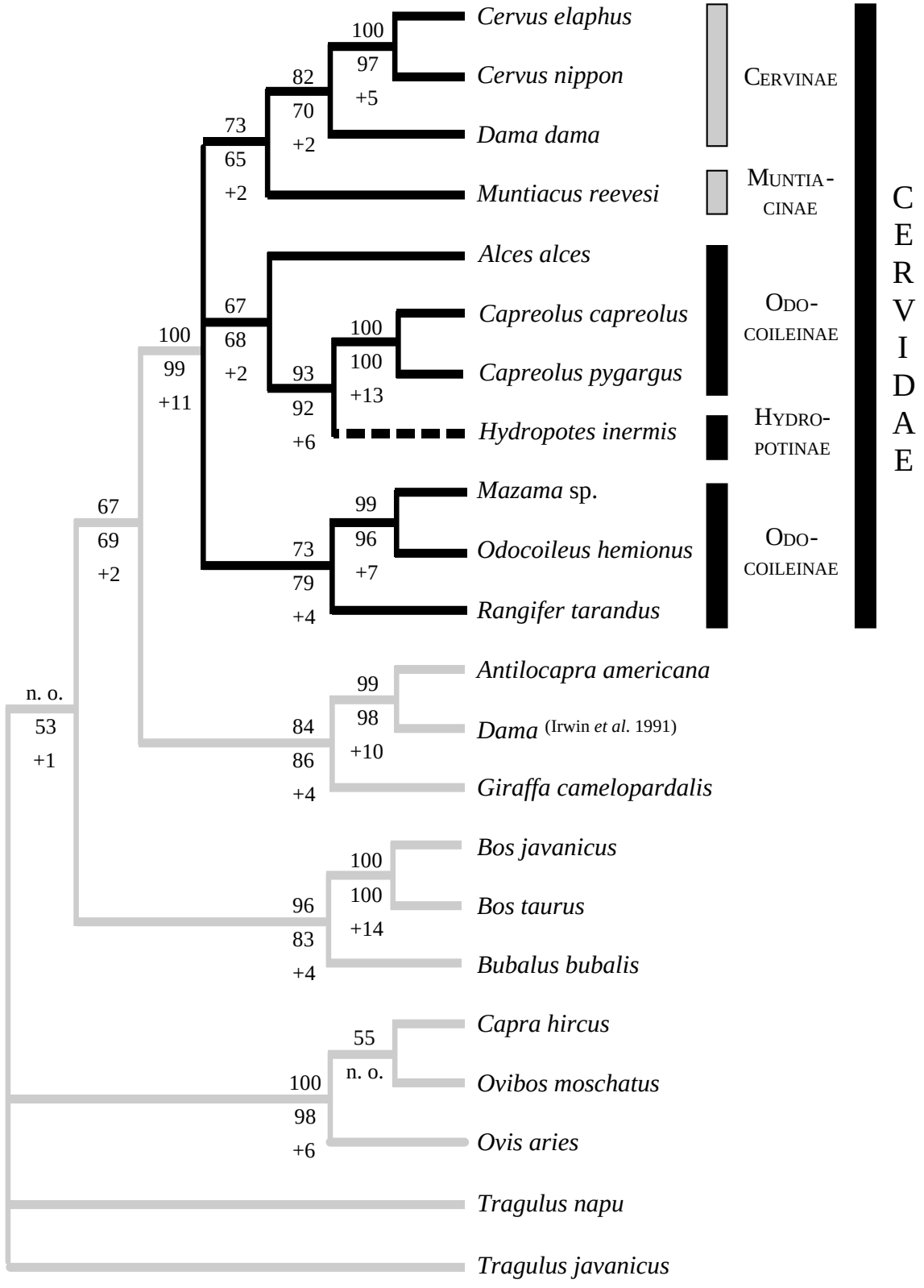

Figure 1. Majority-rule consensus tree derived after 1000 replicates of bootstrap on a matrix of 22 cyt *b* nucleotide sequences. The *Tragulus* species were used as outgroup. The NJ and one of the MP topologies were identical. Third-position transitions were excluded from the analyses, and 211 phylogenetically informative sites were kept with the MP approach. Bootstrap percentages (if higher than 50%) derived by the NJ and MP methods are indicated, respectively, above and below the branches, and decay indices are below. Some groupings were not observed (n.o.) either for NJ or MP approaches. The Cervidae systematic frame is given on the right at the subfamily level: the hatched and black boxes, respectively, depict plesiometacarpalian and telemetacarpalian cervids. The black segments of the tree denote the possession of antlers, and the dotted terminal segment indicates the secondary loss of antlers. Antlered deer and Odocoileinae are paraphyletic because of the internal position of the antlerless *Hydropotes*. Constraining the monophyly of antlered cervids involves nine extra steps, and the Kishino & Hasegawa (1989) test indicates a significantly worse likelihood of this topology relative to the best tree.

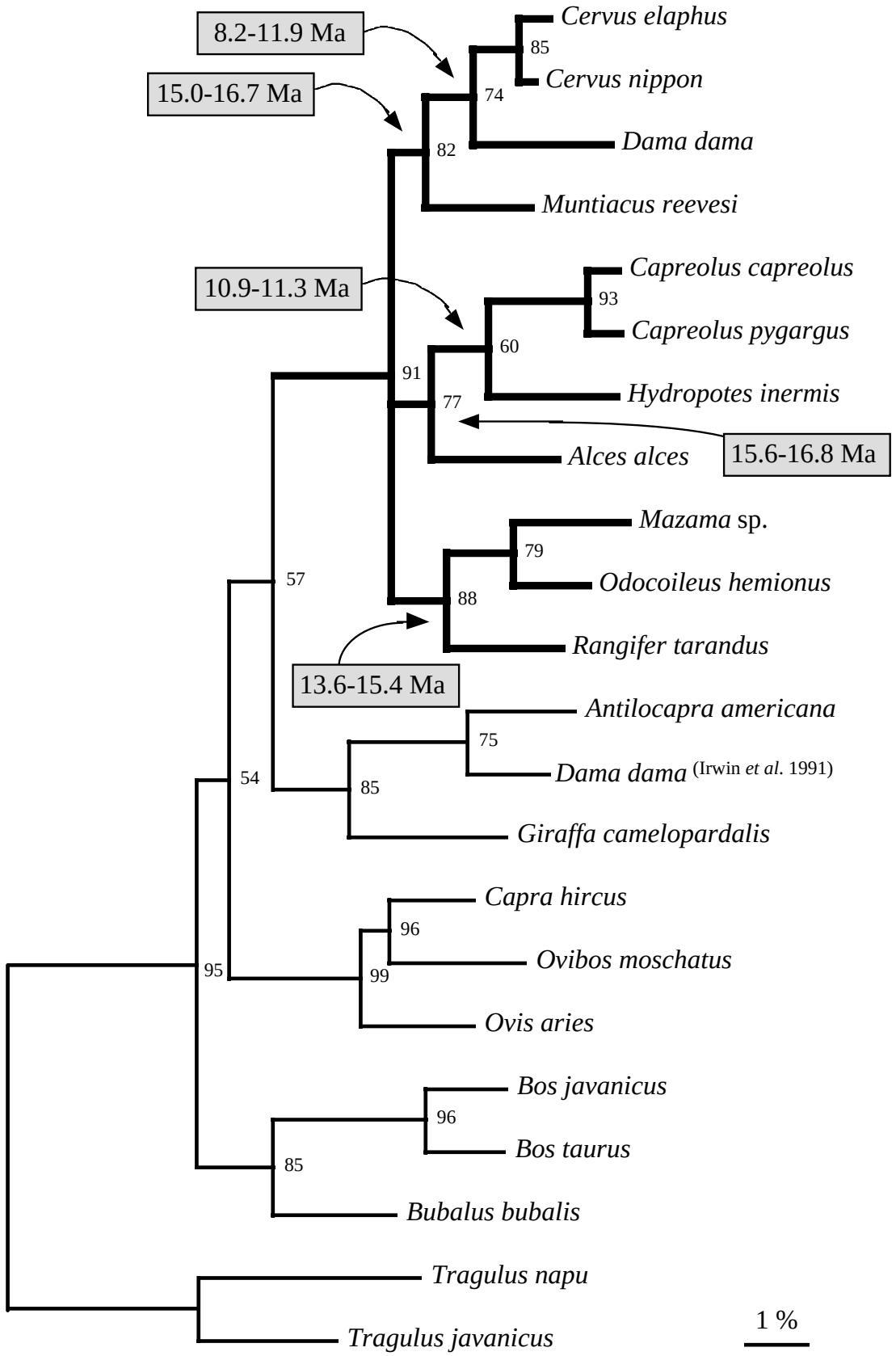

Figure 2. Maximum-likelihood phylogram derived from a matrix of 22 pecoran and tragulid cyt *b* nucleotide sequences. The ML tree (lnL = −5372.7) was generated by the quartet puzzling program PUZZLE 3.1., after exclusion of Ts on the third codon position. Reliability percentages are indicated for each node. The *Tragulus* species were used as the outgroup. Branch lengths are proportional to the per cent of expected substitutions, and the branch leading to the Pecora was arbitrarily divided into two equal parts. The thickest branches depict the Cervidae phylogeny. Local molecular clocks were computed after calibrating the divergence of the three main cervid clades at 20 Ma before present. Estimates of divergence time were then deduced for five main groups. A total of three splits occurred in the Middle Miocene between 13.6 and 16.8 Ma, and two splits occurred in the Late Miocene between 8.2 and 11.9 Ma ago.

(f) *The problem of the fallow deer cyt* b

The two fallow deer cyt *b* sequences (accession AJ000022 in this paper; and the data bank accession X56290 published by Irwin *et al*. 1991) were highly divergent. The comparison actually indicates 11% of nucleotide divergence, and a low transition–transversion ratio (R = Ts/Tv = 1.8) falling within the range of values of the most distantly related outgroups of Cervidae. The X56290 sequence clusters with that of *Antilocapra americana* (family Antilocapridae; accession X56286 in Irwin *et al*. 1991), outside the cervid clade (see figures 1 and 2).

The primary sequence of the three accessions was compared by using the Heap Big sliding window program (S. Palumbi, unpublished data). The *Dama dama* cyt *b* established by Irwin *et al*. (1991) actually represents a mosaic of at least three different sequences: some cyt *b* of the fallow deer (e.g. positions 55 to 170, or 499 to 560, are completely identical to our fallow deer sequence); some cyt *b* of the pronghorn (e.g. positions 355 to 425, or 802 to 935); and a third class of unidentified motifs showing affinities with the cyt *b* of ruminants but without clear affinity for any given taxon. This mosaic sequence (accession X56290) could have occurred through PCR recombination (see, for example, Bradley & Hillis 1997), or could represent a nuclear pseudogene. Conversely, the fallow deer cyt *b* sequence that we obtained (accession AJ000022) clusters within Cervinae (see figures 1 and 2), in agreement with ribonuclease amino acid comparisons (Beintema *et al*. 1988). It probably represents the mitochondrial cyt *b* of *Dama dama*.

4. DISCUSSION

Current morphological taxonomy splits the family Cervidae on the basis of a single character: absence (Hydropotinae) or presence (Odocoileinae + Cervinae) of antlers (Groves & Grubb 1987). However, the taxonomic value of antlers has been repeatedly questioned (see, for example, Scott & Janis 1993), and the dramatic size reduction of spike antlers in advanced South American genera *Pudu* and *Mazama* suggests that the reversal of morphological trends is possible in consequence of selection correlated with small body size (Gould 1974). Some morphological characters seem therefore to evolve with high homoplasy in ungulates (Scott & Janis 1993), and are difficult to use for determining reliable phylogenetic relationships. Nucleotide sequence comparisons of the mitochondrial cyt *b* of 11 cervids here confirm previous observations and provide significant new phylogenetic information on relationships among Cervidae.

(a) *Acquisition and secondary loss of antlers among Cervidae*

The present 11 species, representing nine genera and all the current recognized tribes, constitute a monophyletic Cervidae assemblage within pecoran artiodactyls. Cervid monophyly is strongly supported by distance, parsimony and maximum-likelihood phylogenetic analyses (BP = 100/99, DI = +11, RP = 91, significant Kishino & Hasegawa test). The presence of deciduous cranial appendages seems therefore to constitute a valid synapomorphy to define the Cervidae, and the antlers probably appeared once during the history of the group. This event took place earlier than 20 Ma before present (Ginsburg 1988).

Within Cervidae, the main evolutionary split is between Plesiometacarpalia and two Telemetacarpalia clades (Odocoileini + Rangiferini; Capreolini + Hydropotinae + Alcini). The morphological distinction between plesiometacarpals (Cervinae and Muntiacinae) and telemetacarpals (Odocoileinae and Hydropotinae) cervids was established more than a century ago by Brooke (1878), and has been confirmed recently by molecular investigations on the cervid satellite I DNA (Lee *et al*. 1997).

Our cyt *b* data confirm that Muntiacinae and Cervinae are sister lineages that originated in the Middle Miocene between 15.0 and 16.7 Ma before present (see figures 1 and 2). This agrees with most of the available morphological and molecular data (Bouvrain *et al*. 1989; Kraus & Miyamoto 1991; Cronin *et al*. 1996). Therefore, the plesiometacarpalian condition probably evolved once among cervids through the reduction of lateral metacarpals, and indicates that Cervinae + Muntiacinae represents a natural group. Further studies within Plesiometacarpalia should include nucleotide sequences of the genera *Axis*, *Elaphurus* and *Elaphodus* to confirm the monophyly of the two subfamilies.

Hydropotes inermis, the only living representative of antlerless telemetacarpal deer, was traditionally considered as the sister group of all living antlered deers (Groves & Grubb 1987). *Hydropotes* retains some other ancestral morphological characters, e.g. the presence of large upper canines, which are shared with the non-ruminant tragulids and the Muntiacini (Groves & Grubb 1987). Nevertheless, analyses of cyt *b* sequences strongly suggest that *Hydropotes inermis* is nested within the Odocoileinae and that its closest relative is the *Capreolus* clade (BP = 93/92, DI = +6, RP = 60: figures 1 and 2). Other molecular data sets support the inclusion of *Hydropotes* within the Odocoileinae. Phylogenetic analyses of complete sequences of the mtDNA control-region of Cervidae suggest the paraphyly of antlered deer because of the close association of Hydropotinae with *Capreolus* within a monophyletic Odocoileinae (Douzery & Randi 1997). Comparison of mitochondrial 12S and 16S rRNA sequences by Kraus & Miyamoto (1991) produced two different topologies: *Hydropotes* was the sister group of either all other cervids or *Odocoileus* only. A ML reanalysis of these data produces the clustering of *Hydropotes* with *Odocoileus* (Kraus & Miyamoto 1991). A basal position of *Hydropotes* relative to other cervids, and the subsequent monophyly of antlered deer is therefore unlikely with regard to these different sources of mtDNA non-coding (the control region), ribosomal (the 12S and 16S rRNA) and protein-coding (cyt *b*: figures 1 and 2) sequence data. Furthermore, the association of *Hydropotes* with Odocoileinae is supported by two morphologically derived characters: the telemetacarpal condition, and the large medial opening of the temporal canal (Bouvrain *et al*. 1989). Such an association of Hydropotinae with Odocoileinae therefore implies that antlers have been lost in Hydropotinae, as we previously came to the conclusion that antlers were probably acquired once before the radiation of modern cervids.

In conclusion, telemetacarpalians include two principal lineages, Odocoileini + Rangiferini and Capreolini + Hydropotini + Alcini, but the question remains whether or not they represent a monophyletic assemblage (figures 1 and 2). We only found one exclusive synapomorphic transition in the cyt *b* of the 22 ruminant species in favour of telemetacarpalian monophyly. The combined analyses of cyt *b* (this study) and κ-casein (Cronin *et al*. 1996) sequences provide weak support for Odocoileinae monophyly (BP = 61 and DI = + 2), but *Hydropotes* was not represented for the nuclear data set. In contrast, the study of the mitochondrial control region by Douzery & Randi (1997) gives strong support for monophyletic Telemetacarpalia, but neither *Alces* nor *Rangifer* were represented. To evaluate the question of the monophyly of Telemetacarpalia, further molecular studies should investigate all these representatives for different mitochondrial and nuclear genes, and should include the telemetacarpalian *Moschus* (family Moschidae).

(b) *The Odocoileini + Rangiferini clade*

The tribe Odocoileini, represented here by *Odocoileus* and *Mazama*, constitutes a well-defined monophyletic group (Douzery & Randi 1997; this study, figures 1 and 2). Furthermore, our analysis strongly suggests that the closest living relatives to these New World Odocoileinae are the reindeer (Rangiferini: figures 1 and 2). This Odocoileini + Rangiferini clade receives a strong transversional cyt *b* support, as its ancestral segment is defined by nine synapomorphies, with eight being Tv on third codon positions. From a morphological point of view, it is striking to note that Odocoileini and Rangiferini share a unique derived cranial feature, the posterior expansion of the vomer which divides the choanae (Brooke 1878; Bouvrain *et al*. 1989). Our cyt *b* data suggest that this character of the skull constitutes a true synapomorphy uniting New World odocoileine and reindeer. The split between Rangiferini and Odocoileini may have occurred in the Middle Miocene between 13.6 and 15.4 Ma ago (see figure 2). Furthermore, extant reindeer populations have a Holarctic distribution with three distinct taxa recognized as semispecies or subspecies (Groves & Grubb 1987). Their origins and relationships are unknown, although it has been suggested that Pleistocene relatives of *Rangifer* could be found among South American odocoileines (Groves & Grubb 1987).

(c) *The Alcini + Hydropotinae + Capreolini clade*

As previously shown, *Hydropotes* is embedded within telemetacarpalian antlered cervids. The closest relatives to Hydropotini are actually the Capreolini (figures 1 and 2), and their divergence may have occurred from an Eurasian stock in the Late Miocene around 11 Ma ago. The two roe deer species and the Chinese water deer share a guanine on the site 525 of the cyt *b*. After comparison with 199 cyt *b* sequences representing the main orders of placental mammals, this guanine was observed for only seven phylogenetically unrelated species (a whale, two rodents, two carnivores and two bovids). This replacement represents a nearly exclusive synapomorphy for the *Hydropotes–Capreolus* clade. The moose joins the latter group to form a clade of Old World tribes of Odocoileinae (Alcini, Capreolini + Hydropotini), which is moderately supported (figures 1 and 2: BP = 68/67, DI = +2, RP = 77). This clade originated during the Middle Miocene between 15.6 and 16.8 Ma ago (figure 2), and is very divergent from New World tribes of Odocoileinae (Odocoileini and Rangiferini). The combination of sequences increases the signal for a Capreolini + Alcini cluster, but *Hydropotes* was not represented, and the Kishino & Hasegawa (1989) test remains insignificant. Further investigations should evaluate these molecular results in the light of the cranio-skeletal data, to find potential synapomorphies uniting the Palaearctic roe deer and Chinese water deer, and to identify the phylogenetic position of the Holarctic moose.

We thank Aleksey Danilkin (Moscow), Oliver Ryder (San Diego), Jaap Buntjer (Utrecht) and Knut Røed (Oslo) for kindly providing us with Siberian roe deer, brocket deer (individual 'Leona') and Chinese water deer, muntjac, reindeer and moose tissue samples. Dr S. Palumbi is also thanked for providing us with the Heap Big sliding window program. E.D. gratefully acknowledges François Catzeflis for the laboratory environment, access to the collection of tissues, and a critical reading of the first draft. Jean-Yves Dubuisson and Laurent Amsellem are thanked for their kind help in computer management and analyses. Laboratory facilities were partly funded by the Service Commun de Biosystématique de Montpellier. This work was supported by the 'Groupement de Recherche et d'Etude des Génomes' (G.R.E.G.; déision number 82/94 of 19 July 1994). This is contribution 98-022 of the Institut des Sciences de l'Evolution de Montpellier (UMR 5554–CNRS).

REFERENCES

Arctander, P. 1995 Comparison of a mitochondrial gene and a corresponding nuclear pseudogene. *Proc. R. Soc. Lond.* B **262**, 13–19.

Beintema, J. J., Schüller, C., Irie, M. & Carsana, A. 1988 Molecular evolution of the ribonuclease superfamily. *Prog. Biophys. Molec. Biol. Evol.* **51**, 165–192.

Bouvrain, G., Geraads, D. & Jehenne, Y. 1989 New data relating to the classification of the Cervidae (Artiodactyla, Mammalia). *Zool. Anz.* **223**, 82–90.

Bradley, R. D. & Hillis, D. M. 1997 Recombinant DNA sequences generated by PCR amplification. *Molec. Biol. Evol.* **14**, 592–593.

Bremer, K. 1988 The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* **42**, 795–803.

Brooke, V. 1878 On the classification of Cervidae with a synopsis of the existing species. *Proc. Zool. Soc. Lond.* 883–928.

Catzeflis, F. M. 1991 Animal tissue collections for molecular genetics and systematics. *Trends Ecol. Evol.* **6**, 168.

Chikuni, K., Mori, Y., Tabata, T., Saito, M., Monma, M. & Kosugiyama, M. 1995 Molecular phylogeny based on the kappa-casein and cytochrome *b* sequences in the mammalian suborder Ruminantia. *J. Molec. Evol.* **41**, 859–866.

Cronin, M. A., Stuart, R., Pierson, B. J. & Patton, J. C. 1996 κ-casein gene phylogeny of higher ruminants (Pecora, Artiodactyla). *Molec. Phylogenet. Evol.* **6**, 295–311.

Douzery, E. & Randi, E. 1997 The mitochondrial control region of Cervidae: evolutionary patterns and phylogenetic content. *Molec. Biol. Evol.* **14**, 1154–1166.

Eisenberg, J. F. 1981 *The mammalian radiations. An analysis of trends in evolution, adaptation and behavior.* Chicago and London: The University of Chicago Press.

Farris, J. S., Källersjö, M., Kluge, A. G. & Bult, C. 1995 Testing significance of incongruence. *Cladistics* **10**, 315–319.

Felsenstein, J. 1985 Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Felsenstein, J. 1993 *PHYLIP* (*phylogeny inference package*) *version 3.5c*. Department of Genetics, University of Washington, Seattle, Washington.

Ginsburg, L. 1988 La faune des mammifres des sables miocénes du synclinal d'Esvres (Val-de-Loire). *C. R. Acad. Sci. Paris II* **307**, 319–322.

Gould, S. J. 1974 The origin and function of 'bizarre' structures: antler size and skull size in the 'irish elk', *Megaloceros giganteus*. *Evolution* **28**, 191–220.

Groves, C. P. & Grubb, P. 1987 Relationships of living deer. In *Biology and management of the Cervidae* (ed. C. M. Wemmer), pp. 21–59. Washington, DC: Smithsonian Institution.

Groves, P. & Shields, G. F. 1996 Phylogenetics of the Caprinae based on cytochrome *b* sequence. *Molec. Phylogenet. Evol.* **5**, 467–476.

Irwin, D. M., Kocher, T. D. & Wilson, A. C. 1991 Evolution of the cytochrome *b* gene of mammals. *J. Molec. Evol.* **32**, 128–144.

Janis, C. M. & Scott, K. M. 1987 The interrelationships of higher ruminant families, with special emphasis on the members of the Cervidae. *Am. Mus. Novit.* **2893**, 1–85.

Kishino, H. & Hasegawa, M. 1989 Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *J. Molec. Evol.* **29**, 170–179.

Kraus, F. & Miyamoto, M. M. 1991 Rapid cladogenesis among the pecoran ruminants: evidence from mitochondrial DNA sequences. *Syst. Zool.* **40**, 117–130.

Kumar, S., Tamura, K. & Nei, M. 1993 *MEGA*: *molecular evolutionary genetics analysis*, *version 1.01*. Pennsylvania State University, University Park, Pennsylvania, USA.

Lee, C., Court, D. R., Cho, C., Haslett, J. L. & Lin, C.-C. 1997 Higher-order organization of subrepeats and the evolution of cervid satellite I DNA. *J. Molec. Evol.* **44**, 327–335.

Miyamoto, M. M., Kraus, F. & Ryder, O. A. 1990 Phylogeny and evolution of antlered deer determined from mitochondrial DNA sequences. *Proc. Natn. Acad. Sci. USA* **87**, 6127–6131.

Mross, G. A. & Doolittle, R. F. 1967 Amino acid sequence studies on artiodactyl fibrinopeptides. II. Vicuna, elk, muntjac, pronghorn antelope, and water buffalo. *Arch. Biochem. Biophys.* **122**, 674–684.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. & Erlich, H. 1986 Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harb. Symp. Quant. Biol.* **51**, 263–273.

Philippe, H. 1993 MUST: a computer package of management utilities for sequences and trees. *Nucl. Acids Res.* **21**, 5264–5272.

Randi, E., Lucchini, V. & Diong, C. H. 1996 Evolutionary genetics of the suiformes as reconstructed using mtDNA sequencing. *J. Mammal. Evol.* **3**, 163–194.

Rzhetsky, A. & Nei, M. 1995 Tests of applicability of several substitution models for DNA sequence data. *Molec. Biol. Evol.* **12**, 131–151.

Saitou, N. & Nei, M. 1987 The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molec. Biol. Evol.* **4**, 406–425.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989 *Molecular cloning*. New York: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. & Coulson, A. R. 1977 DNA sequencing with chain terminating inhibitors. *Proc. Natn. Acad. Sci. USA* **74**, 5463–5467.

Scott, K. M. & Janis, C. M. 1987 Phylogenetic relationships of the Cervidae, and the case for a superfamily 'Cervoidea'. In *Biology and management of the Cervidae* (ed. C. M. Wemmer), pp. 3–20. Washington, DC: Smithsonian Institution.

Scott, K. M. & Janis, C. M. 1993 Relationships of the Ruminantia (Artiodactyla) and an analysis of the characters used in ruminant taxonomy. In *Mammal phylogeny*: *placentals* (ed. F. S. Szalay, M. J. Novacek & M. C. McKenna), pp. 282–302. New York: Springer.

Springer, M. S. & Douzery, E. 1996 Secondary structure and patterns of evolution among mammalian mitochondrial 12S rRNA molecules. *J. Molec. Evol.* **43**, 357–373.

Stanley, H. F., Kadwell, M. & Wheeler, J. C. 1994 Molecular evolution of the family Camelidae: a mitochondrial DNA study. *Proc. R. Soc. Lond.* B **256**, 1–6.

Strimmer, K. & von Haeseler, A. 1996 Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. *Molec. Biol. Evol.* **13**, 964–969.

Swofford, D. L. 1993 *PAUP 3.1.1*: *phylogenetic analysis using parsimony*. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois, USA.

Tamura, K. & Nei, M. 1993 Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molec. Biol. Evol.* **10**, 512–526.

Tanaka, K., Solis, C. D., Masangkay, J. S., Maeda, K.-I., Kawamoto, Y. & Namikawa, T. 1996 Phylogenetic relationship among all living species of the genus *Bubalus* based on DNA sequences of the cytochrome *b* gene. *Biochem. Genet.* **34**, 443–452.

Zhang, D.-X. & Hewitt, G. M. 1996 Nuclear integrations: challenges for mitochondrial DNA markers. *Trends Ecol. Evol.* **11**, 247–251.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute towards production costs.