THE ROYAL SOCIETY

Evolutionary affinities of the enigmatic saola (*Pseudoryx nghetinhensis*) in the context of the molecular phylogeny of Bovidae

Alexandre Hassanin[1*] **and Emmanuel J. P. Douzery**[2]

[1]*Laboratoire de Préhistoire UMR 6569 CNRS and Service de Systématique Moléculaire GDR 1005 CNRS, Muséum National d'Histoire Naturelle, 43 rue Cuvier, 75231 Paris Cedex 05, France*
[2]*Laboratoire de Paléontologie, Paléobiologie et Phylogénie, Institut des Sciences de l'Evolution UMR 5554 CNRS, Université Montpellier II—CC064 Place E. Bataillon, 34 095 Montpellier Cedex 05, France*

To elucidate the systematic status of the enigmatic saola (*Pseudoryx nghetinhensis*), a new bovid genus recently discovered in Vietnam, and to investigate phylogenetic relationships within the family Bovidae, four distinct DNA markers were sequenced. Complete mitochondrial cytochrome *b* (1143 bp) and 12S rRNA (956 bp) genes and non-coding regions from the nuclear genes for aromatase cytochrome P-450 (199 bp) and lactoferrin (338 bp) have been compared for 25 bovid species and three Cervidae and Antilocapridae outgroups. Independent and/or combined analyses of the four nucleotide matrices through maximum parsimony and maximum-likelihood methods indicated that Bovidae consists of two major lineages, i.e. Bovinae which contains the tribes Bovini, Boselaphini and Tragelaphini, and Antilopinae which encompasses all other bovids. Within Bovinae, the tribe Bovini is divided into buffalo Bovini (*Bubalus* and *Syncerus*) and cattle Bovini (*Bos* and *Bison*) and Tragelaphini are possibly related to Boselaphini. *Pseudoryx* is shown to be (i) robustly nested within Bovinae; (ii) strongly associated with Bovini; and (iii) tentatively sharing a sister-group relationship with cattle Bovini. Within Antilopinae, three robust clades are in evidence: (i) *Hippotragus* and *Damaliscus* are linked to *Ovis*; (ii) *Aepyceros* joins *Neotragus*; and (iii) *Cephalophus* clusters with *Oreotragus*.

Keywords: *Pseudoryx*; saola; Bovidae; phylogeny; mitochondrial DNA; nuclear markers

1. INTRODUCTION

In 1992, a new living genus of bovid, *Pseudoryx nghetinhensis* (Mammalia: Artiodactyla) was discovered in the restricted mountainous jungle which separates Vietnam from Laos (Dung *et al.* 1993). Only a few hundred specimens of this endangered species, the saola as called by local hunters, survive in the wild. The horn cores of adult saola are exceptionally long (*ca.* 40–50 cm) and the generic name *Pseudoryx* refers to their superficial resemblance to those of *Oryx* (tribe Hippotragini). *Pseudoryx* differs significantly from all described bovid genera in appearance and morphology and two contradicting hypotheses have been proposed concerning its phylogenetic status within the family Bovidae.

(i) A first analysis, based on partial sequences (249 bp) of the cytochrome *b* (*cyb*) gene, indicated that the new species clusters with members of the subfamily Bovinae rather than other bovids (Dung *et al.* 1993). However, this molecular conclusion was limited in scope for three major reasons: the taxonomic sampling was very reduced with only five out of the 13–15 tribes traditionally recognized (Gentry 1992), most nodes were not robustly supported by bootstrap analysis and it was not possible to specify clearly to which tribe of Bovidae, if any, the species belongs. However, the authors proposed to incorporate the saola into the tribe Boselaphini on the basis of some morphological characters listed as primitive for bovids.

(ii) A second analysis, conducted by Thomas (1994) and based on morphology of the skull and dentition, suggested a close relationship of the saola with some Caprinae, the serows and gorals of Asia.

Since these studies, molecular investigations based on ribosomal (Gatesy *et al.* 1997) or *cyb* (Hassanin & Douzery 1999; Matthee & Robinson 1999) mitochondrial sequences of a large taxa sample have established that Bovidae is composed of two major subfamilial clades. The first corresponds to Bovinae and assembles members of the tribes Bovini (cattle and buffaloes), Tragelaphini (African spiralled-horned bovids) and Boselaphini (nilgai and chousingha). The second clusters all other bovids, i.e. Antilopinae which is composed of Alcelaphini (hartebeest and allies), Caprini *s.l.* (goats, chamois, musk-ox and relatives), Hippotragini (horse-like antelopes), Antilopini (gazelles), Reduncini (waterbuck group), Aepycerotini (impala) possibly linked to *Neotragus* (suni) and Cephalophini (duikers) possibly linked to *Oreotragus* (klipspringer) (Gatesy *et al.* 1997; Hassanin & Douzery 1999).

*Author for correspondence (hassanin@mnhn.fr).

Received 7 December 1998 *Accepted* 26 January 1999

However, the basal branching patterns within the subfamilies Bovinae and Antilopinae still remain poorly known, probably because of the rapid tribal radiation which occurred in the middle Miocene between 12 and 15 million years before present (Hassanin & Douzery 1999).

It has been shown that phylogenetic information for deciphering Bovidae evolution can be found in mitochondrial (e.g. Allard *et al.* 1992; Groves & Shields 1996; Tanaka *et al.* 1996; Hassanin *et al.* 1998*b*) and nuclear sequences (Cronin *et al.* 1996; Pitra *et al.* 1997). In this paper, two complete mitochondrial genes (*cyb* and 12S rRNA) and two non-coding regions from nuclear genes, aromatase cytochrome P-450 (*cyp*19) and lactoferrin (*Lf*) (Pitra *et al.* 1997), were analysed for a taxonomic sample including at least one representative of the major bovid lineages previously identified (Gatesy *et al.* 1997; Hassanin & Douzery 1999). The aims of this study are to examine phylogenetic relationships among bovid lineages in order to understand better the evolution of this well-diversified group of mammals and to clarify the systematic status of *P. nghetinhensis*.

2. MATERIAL AND METHODS

(a) *DNA sequencing*

In addition to *P. nghetinhensis*, 24 bovid taxa belonging to the major lineages evidenced in previous analyses (Allard *et al.* 1992; Gatesy *et al.* 1997; Hassanin & Douzery 1999) were included in this study: *Bos taurus*, *Bos grunniens* and *Bison bison* (subtribe cattle Bovini), *Bubalus bubalis*, *Bubalus depressicornis* and *Syncerus caffer* (subtribe buffalo Bovini), *Boselaphus tragocamelus* and *Tetracerus quadricornis* (tribe Boselaphini), *Tragelaphus angasii*, *Tragelaphus eurycerus*, *Tragelaphus imberbis*, *Tragelaphus scriptus*, *Tragelaphus spekii*, *Tragelaphus strepsiceros* and *Taurotragus oryx* (Tragelaphini), *Aepyceros melampus* (Aepycerotini), *Cephalophus dorsalis* (Cephalophini), *Damaliscus pygargus* (Alcelaphini), *Gazella granti* (Antilopini), *Hippotragus niger* (Hippotragini), *Neotragus moschatus* and *Oreotragus oreotragus* (polyphyletic Neotragini), *Ovis aries* (Caprini *s.l.*) and *Redunca fulvorufula* (Reduncini). Three additional taxa belonging to the Pecora families Antilocapridae (*Antilocapra americana*) and Cervidae (*Cervus elaphus* and *Odocoileus hemionus*) were used as outgroups to root the trees.

Total DNAs were extracted from blood, hair, skin, muscle and bone fragments of museum specimens following the procedures described by Winnepenninckx *et al.* (1993) and Hassanin *et al.* (1998*b*). The entire *cyb* gene was amplified by the polymerase chain reaction with the primers given in Hassanin & Douzery (1999). The complete 12S rRNA gene was amplified with three couples of primers: (i) 5′-AAAGCAAGGCACTGAAAATGCCTAGA-3′ (position L624 in the human mitochondrial genome (accession M58503)) and 5′-CATAGTGGGGTATCTAATCCCAGTT-3′ (H1069); (ii) 5′-TCGTGCCAGCCACCGCGGTCA-3′ (L906) and 5′-GAAAATGTAGCCCATTTCTT-3′ (H1354); and (iii) 5′-TATACCGCCATCTTCAGCAAACC-3′ (L1280) and 5′-TCTTCTGGGTGTAGGCCAGATGCTTT-3′ (H1620). Partial sequences of the nuclear gene encoding aromatase cytochrome P-450 (i.e. positions 2992–3185 of the *B. taurus* sequence (accession Z32741)) were obtained for all taxa except *Gazella* using the oligonucleotides determined by Pitra *et al.* (1997). The promotor segment of the lactoferrin-encoding gene (positions 322–647 of the bovine sequence (accession L19985)) was generated using four primers: (i) 5′-CACAAAACAACACAAGGGGTAG-3′ (position 321) (Pitra *et al.* 1997); (ii) 5′-GGTTCTGTTTTCTGGGAGCTGT-3′ (403); (iii) 5′-CTCAGTGCCTCCTAGAGAGC-3′ (648, reverse); and (iv) 5′-GCAGGGGTCCTARGGTGAATCT-3′ (616, reverse). Both strands of all amplicons were directly sequenced using the Thermo Sequenase cycle sequencing kit (Amersham). Sequences have been deposited in EMBL/GenBank/DDBJ (DNA database of Japan) databases under accession numbers AF091629–091635 (*cyb*), AF091636–091660 and AF091694 (*Lf*), AF091662–091685 and AF091695 (*cyp*19) and AF091686–091693 and AF091696–091710 (12S).

(b) *Homoplasy and saturation analyses*

Homoplasy and saturation levels were measured for each type of nucleotide substitution (i.e. two different transitions and four different transversions) on the complete sequences for the two nuclear markers, distinguishing between each of the three codon positions for *cyb* and between stems and loops of the secondary structure (Springer & Douzery 1996) for 12S rRNA. For 12S rDNA, *cyp*19 and *Lf* promotor sequences, insertions and deletions were coded as I and D according to Barriel (1994). For each substitution type and indels, the amount of homoplasy and degree of saturation were respectively estimated using the consistency index (CI; Kluge & Farris 1969), excluding uninformative characters and the slope of the linear regression (*S*) obtained from the saturation graphs (i.e. number of differences observed against number of changes inferred) (Hassanin *et al.* 1998*a*,*b*).

(c) *Phylogenetic analyses*

Sequences were aligned using the MUST package (Philippe 1993). Alignments have been deposited in EMBL (ftp://ftp.ebi.ac.uk/pub/databases/embl/align/) under alignment numbers DS36479 (12S), DS36480 (*cyb*), DS36481 (*cyp*19) and DS36482 (*Lf*). Indels were recoded following Barriel (1994), with the introduction of I and D character states and question marks representing the methodological consequences of gap coding. Independent and combined phylogenetic analyses of the four nucleotide matrices were conducted with the maximum-parsimony (MP) method (PAUP 3.1.1; Swofford 1993), with either equal weighting or differential weighting of the character-state transformations using the product of $CI \times S$ (Hassanin *et al.* 1998*b*). Robustness of the nodes was assessed by (i) the method of Bremer (1994) using topological constraints, with branch support values (b_r) rescaled with respect to the equally weighted tree length, and (ii) the bootstrap method (Felsenstein 1985) with bootstrap percentages (BP) computed after 1000 replicates of heuristic search with the closest stepwise addition of taxa option.

Congruence between markers was evaluated using the incongruence length difference (ILD) test of Farris *et al.* (1994), with 1000 randomizations in the ARNIE program (Random Cladistics package; Siddall 1996). In the case of the combined analysis of the four markers, branch support values were partitioned according to Baker & DeSalle (1997).

Maximum-likelihood (ML) reconstructions were performed using the quartet puzzling method (PUZZLE 4.0; Strimmer & Von Haeseler 1996), with the Tamura & Nei (1993) model of sequence evolution, a fraction of sites allowed to be invariable, an eight-category gamma distribution of the substitution rates across variable sites (Yang 1996) and the removal of all sites where indels occurred. Robustness of the nodes was assessed by reliability percentages (RP), i.e. the number of times the group appears after 10 000 ML puzzling steps (PUZZLE 4.0;

Strimmer & Von Haeseler 1996). The tests of the relative log likelihoods of alternative topologies were conducted by the ML method of Kishino & Hasegawa (1989).

3. RESULTS

(a) *Base composition, levels of homoplasy and saturation of the four molecular markers*

The mean base compositions of the *cyb*, 12S rDNA, *cyp*19 and *Lf* promotor were 31.7, 36.8, 33.0 and 18.8% for A, 28.8, 22.5, 18.0 and 29.1% for C, 13.2, 17.9, 15.3 and 28.1% for G and 26.3, 22.8, 33.7 and 24.0% for T, respectively.

For *cyb*, high levels of homoplasy and saturation were measured in first codon positions for C–T and A–G transitions (respectively, CI/S = 0.331/0.443 and 0.382/0.511) and in second codon positions for C–T transitions (CI/S = 0.375/0.492). In third codon positions, C–T transitions were more homoplastic and saturated than A–G transitions (CI/S = 0.202/0.220 and 0.367/0.612) and transversions involving A were more affected than those involving G (e.g. CI/S = 0.421/0.651 and 0.818/0.893 for A–C and C–G substitutions). For 12S rDNA, different patterns of homoplasy and saturation were evidenced between loops and stems. In single-stranded regions, A–G transitions were less concerned by homoplasy and saturation than C–T transitions (CI/S = 0.360/0.593 versus 0.239/0.381) and transversions involving G were less affected than those involving A (e.g. CI/S = 0.882/0.971 and 0.590/0.809 for A–T and G–T substitutions). Moreover, relatively high levels of homoplasy and saturation were found for indels (CI/S = 0.545/0.879). In contrast, in double-stranded regions, A–G and C–T transitions behaved similarly (CI/S = 0.374/0.483 and 0.367/0.519) and both transversions and indels were (almost) not homoplastic and saturated.

In *Lf* promotor sequences, both transitions were identically affected by multiple hits and no homoplasy was discovered for indels and transversions except a low level measured for G–T substitutions (CI/S = 0.800/0.967). In *cyp*19 sequences, no homoplasy was found for all substitution types and indels, except for C–T transitions (CI/S = 0.667/0.355).

(b) *Independent phylogenetic analyses of the four markers*

The MP analyses using the weights derived from the homoplasy and saturation levels (values are recapitulated in electronic Appendix A at (http://www.pubs.royalsoc.ac.uk/publish/pro_bs/rpb1422.htm)) produced the trees presented in figure 1. Both mitochondrial genes yielded topologies which are perfectly congruent despite a lower robustness of resolution for the 12S rDNA. Bovidae was monophyletic (BP = 61 and 54, respectively, for *cyb* and 12S rDNA) and the highest support (BP = 98–100) was provided for monophyly of the tribes Boselaphini (*Boselaphus* and *Tetracerus*) and Tragelaphini (*Tragelaphus* including *Taurotragus*) and the subtribes cattle Bovini (*Bos* and *Bison*) and buffalo Bovini (*Bubalus* and *Syncerus*). The relationships between these clades were not resolved by these mitochondrial molecules. However, they clustered in a relatively robust Bovinae clade which also included *Pseudoryx* (BP = 84 and 54, respectively, for *cyb* and 12S rDNA). Three other nodes emerged from the *cyb* analysis, but not from the 12S rDNA analysis: (i) *Aepyceros* and *Neotragus* (BP = 54); (ii) *Hippotragus* and *Damaliscus* (BP = 63); and (iii) *Cephalophus* and *Oreotragus* (BP = 66).

Both nuclear markers (*Lf* promotor and *cyp*19) produced phylogenies which were fully congruent and confirmed most of the relationships inferred from the mitochondrial molecules (figure 1). Both analyses were in agreement with the monophyly of Boselaphini (BP = 97 and 61, respectively, for *Lf* promotor and *cyp*19) and cattle Bovini (BP = 90 and 69). The close relationship between *Damaliscus* and *Hippotragus* was evidenced with *Lf* promotor (BP = 78) and the *cyp*19 topology depicted a multifurcation including those genera plus *Ovis* (BP = 86). Moreover, in the *Lf* promotor analysis, the family Bovidae and the tribe Tragelaphini were shown to be monophyletic (BP = 95 and 100, respectively) and *Neotragus* joined with *Aepyceros* (BP = 71). Three new nodes emerged from the bootstrap analysis of the *Lf* molecule relative to mtDNA markers: (i) *Pseudoryx* appeared to be robustly enclosed in the tribe Bovini (BP = 98); (ii) Boselaphini and Tragelaphini were associated (BP = 50); and (iii) *Gazella* was related to *Cephalophus* and *Oreotragus* (BP = 77). Within Tragelaphini, the phylogenetic positions of *T. angasii* relative to *T. imberbis* and *T. strepsiceros* relative to *T. oryx* (figure 1) conflicted between the *Lf* promotor and mitochondrial analyses.

(c) *Combination of the mitochondrial and nuclear markers*

Congruence between the four markers has been evaluated with the following number of variable sites: 511 for *cyb*, 366 for 12S rDNA, 150 for *Lf* promotor and 51 for *cyp*19. Pairwise ILD tests indicated that none of the six following comparisons exhibited significant incongruence: 12S/*cyb* (p = 0.39), 12S/*Lf* (p = 0.74), 12S/*cyp*19 (p = 1.00), *cyb*/*Lf* (p = 0.79), *cyb*/*cyp*19 (p = 1.00) and *Lf*/*cyp*19 (p = 0.08). Combination of the four molecules also did not evidence significant incongruence (p = 0.99). Thus, the two mitochondrial and the two nuclear markers were combined and submitted to phylogenetic analyses.

The weighted ML and MP analyses based on the four combined molecular markers provided the highest-likelihood and most-parsimonious phylograms reported in figure 2. All phylogenetic relationships previously identified with one or several isolated markers were recovered with similar or higher support except for the two conflicting nodes involving *T. imberbis* and *T. strepsiceros* within Tragelaphini. Four new nodes or receiving increased support were evidenced by MP and ML total evidence analyses: (i) *Pseudoryx* was the sister group of *Bos* plus *Bison* (BP = 85, RP = 62); (ii) Boselaphini and Tragelaphini clustered together (BP = 79); (iii) the subfamily Antilopinae, which associates all bovids other than Bovinae, appeared to be monophyletic (BP = 82; RP = 85); and (iv) *Ovis* was the sister group of the clade *Damaliscus* plus *Hippotragus* (BP = 82, RP = 72). One discrepancy remained between MP and ML analyses: *Gazella* joined respectively with *Cephalophus* plus *Oreotragus* (BP = 60) or with *Redunca* (RP = 84). One should note that the equally weighted MP analysis produced two MP trees (length = 2618 steps) which were topologically identical to those obtained by ML and weighted MP analyses, again except for the position of

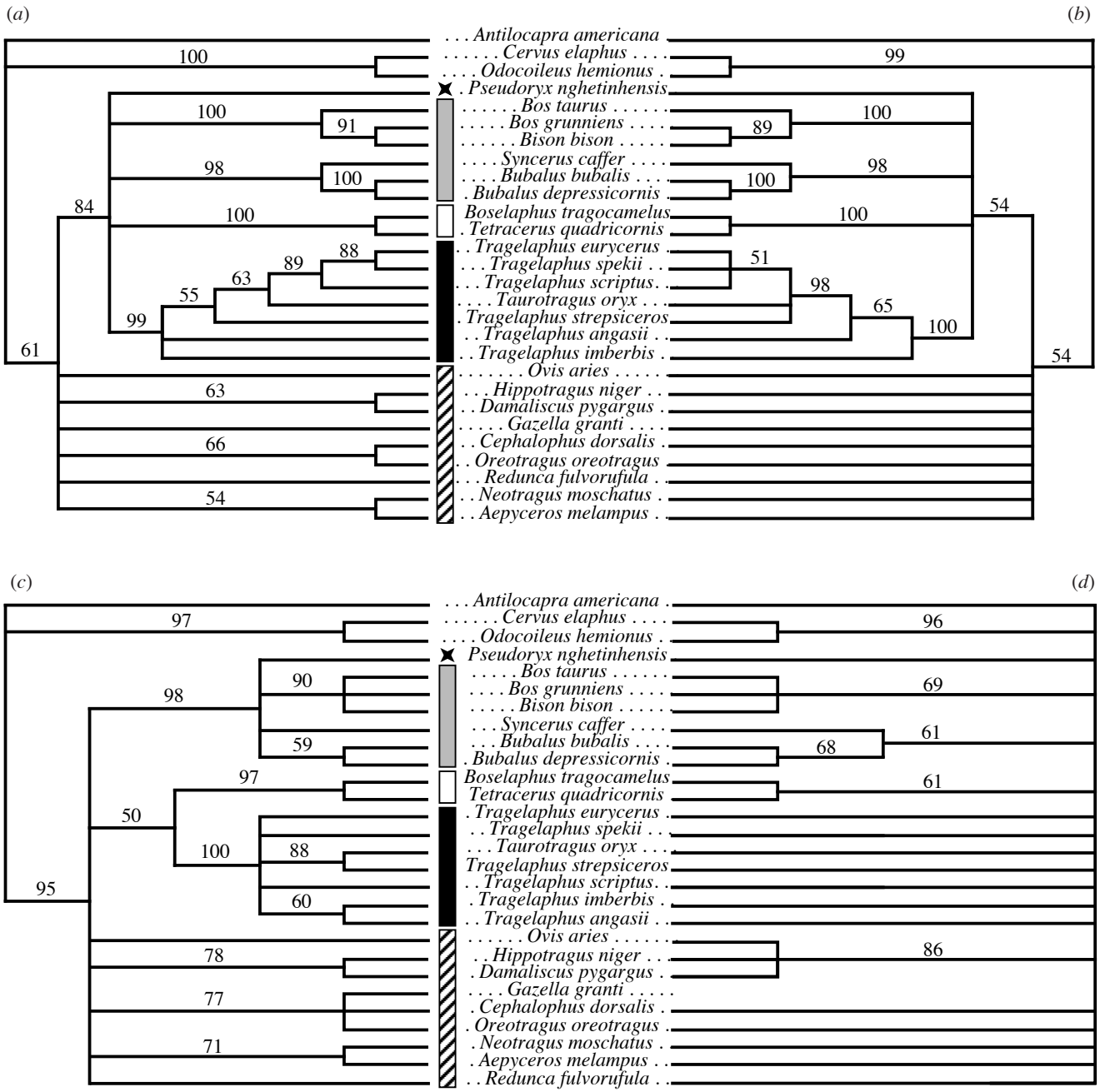

Figure 1. Bootstrap majority-rule consensus tree of weighted maximum parsimony analyses reconstructed from (*a*) *cyb*, (*b*) 12S rDNA, (*c*) *Lf* promotor, and (*d*) *cyp*19 nucleotide sequences. Bootstrap percentages (> 50%) were obtained after 1000 replicates. Vertical lines refer to the taxonomic frame recognized by Kingdon (1997): Bovini (grey), Boselaphini (white), Tragelaphini (black), Antilopinae (hatched) and the crux is for the saola.

Gazella and *Redunca* and some relationships within Tragelaphini (data not shown).

Because ILD results may be swamped by the huge degree of support for some nodes (e.g. Cervidae, Bovinae and Tragelaphini), we partitioned the branch support values to identify potential local incongruence between the four markers. A phylogenetic signal to place *Pseudoryx* inside Bovinae and Bovini and close to *Bos* and *Bison* was brought about by *cyb*, 12S, *cyp*19 and *Lf* sequences and all markers contributed to reject the Thomas (1994) hypothesis of a placement of the saola within caprines (table 1). Even if the *Lf* marker was removed from the concatenated analysis, because of its significant contribution to the saola placement (see table 1), the three remaining molecules clustered *Pseudoryx* within Bovinae (BP = 93, RP = 90; trees not shown) and Bovini (BP = 62, RP = 56) and with *Bos* plus *Bison* (BP = 69, RP = 54).

Kishino & Hasegawa's (1989) tests also unambiguously indicated that *Pseudoryx* should be included in the tribe Bovini (table 1). Other alternative positions of *Pseudoryx* produced significantly less likely topologies, e.g. *Pseudoryx* clustered with either Tragelaphini, Boselaphini or Antilopinae or, in particular, Caprini as represented by *Ovis* (table 1). We noted that base compositions (and also transition:transversion and pyrimidine:purine ratios) varied dramatically between the four mitochondrial and nuclear sequences. Thus, the ML parameters used for the combined analysis (see table 1) did not reflect the

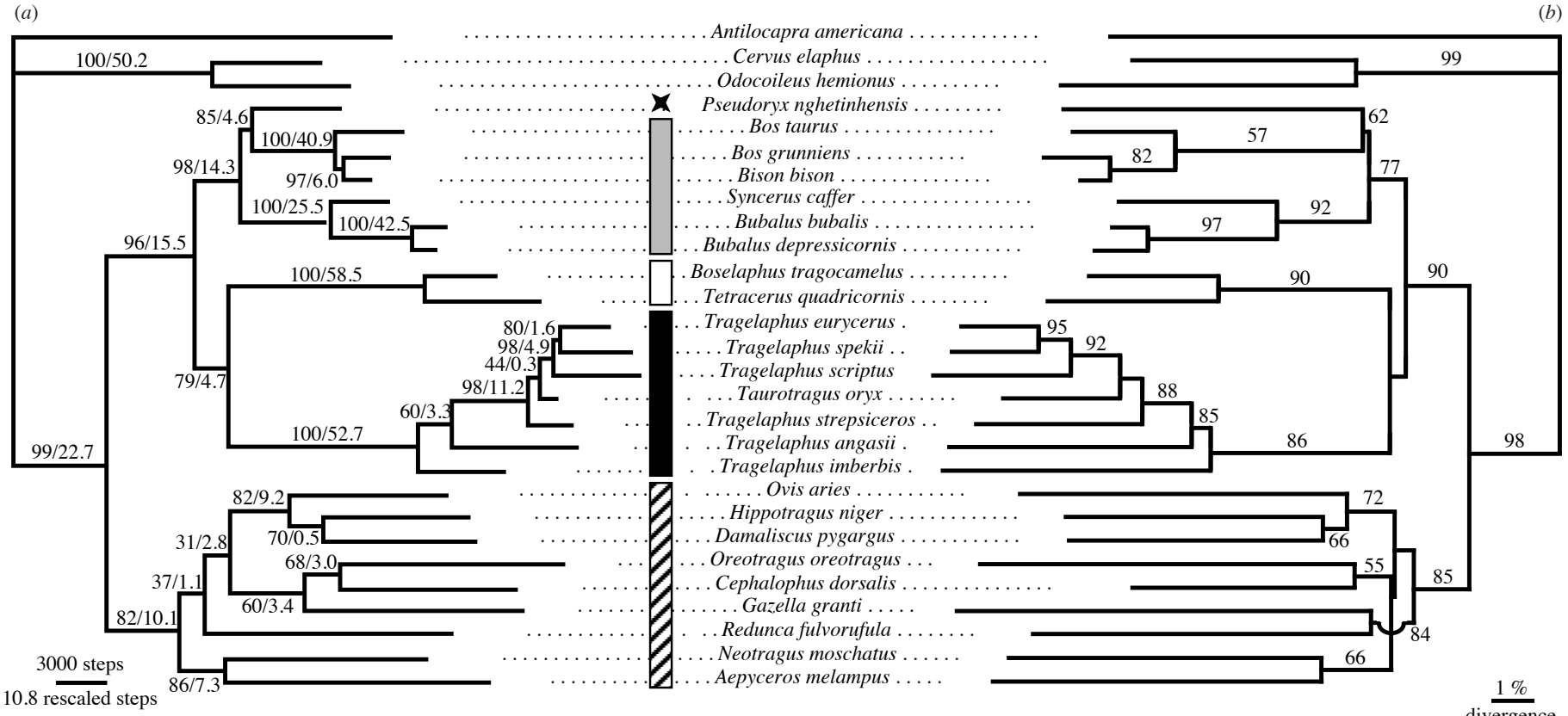

Figure 2. Phylogenetic trees reconstructed from the combination of the four mitochondrial and nuclear markers. (*a*) Weighted maximum-parsimony analysis based on the product of homoplasy by saturation indices (length = 728 402 steps), with bootstrap percentages (to the left of the slash) and rescaled branch support values (to the right of the slash). (*b*) Maximum-likelihood analysis with the reliability percentages (>50%) (see table 1 for additional information). Vertical lines refer to the taxonomic frame recognized by Kingdon (1997): Bovini (grey), Boselaphini (white), Tragelaphini (black), Antilopinae (hatched) and the crux is for the saola.

Table 1. *Tests of alternative placements of* Pseudoryx *within Bovidae*

(The four molecules *cyb*, 12S rDNA, *cyp*19 and *Lf* were combined, yielding 2575 aligned sites. Weighted maximum-parsimony searches based on the product of homoplasy by saturation indices and performed under topological constraints were used to calculate the partitioned rescaled extra steps required to observe the clade under focus (e.g. Bubalina + *Pseudoryx* means that the saola is the sister group to Bubalina). These values have been compared to the partitioned Bremer (1994) support calculated for the nodes involving *Pseudoryx* in the most parsimonious cladogram (MP). The highest-likelihood tree (lnL = −18 004.71) was found with the following parameters: 32.0% A, 25.8% C, 16.9% G and 25.3% T for base composition (see results for the detailed base compositions), 6.35 for transition:transversion ratio, 2.15 for pyrimidine:purine ratio and 0.21 for the substitution rate heterogeneity parameter (the topology is reported in figure 2*b*). These parameters represent a mean over two mitochondrial and two nuclear markers with contrasted patterns of molecular evolution and their use may have affected the branch length estimates. Differences in log likelihood (ΔlnL) and standard error (s.e.) of alternative phylogenetic hypotheses relative to the best topology (ML) were compared using the Kishino & Hasegawa (1989) procedure.)

	partitioned Bremer supports or partitioned extra steps						
topologies evaluated	*cyb*	12S	*cyp*19	*Lf*	total	ΔlnL	s.e.
Pseudoryx + Bovina (MP)	+ 3.3	+ 1.3	0.0	0.0	+ 4.6	ML	—
Pseudoryx into Bovini (MP)	+ 1.0	+ 3.3	+ 0.9	+ 9.1	+ 14.3	ML	—
Pseudoryx into Bovinae (MP)	+ 13.4	+ 2.1	0.0	0.0	+ 15.5	ML	—
Bubalina + *Pseudoryx*	+ 4.9	+ 3.4	0.0	− 3.5	+ 4.8	− 13.7	9.7
all Bovini + *Pseudoryx*	+ 5.3	+ 3.2	0.0	− 3.5	+ 5.0	− 13.7	9.7
all Bovinae + *Pseudoryx*	+ 3.8	+ 0.9	+ 0.9	+ 12.6	+ 18.2	− 33.7[a]	13.2
Boselaphini + *Pseudoryx*	+ 3.8	+ 2.5	+ 0.9	+ 16.2	+ 23.4	− 33.7[a]	13.2
Tragelaphini + *Pseudoryx*	− 0.6	+ 5.3	+ 0.9	+ 16.2	+ 21.8	− 33.7[a]	13.2
Ovis (Caprini) + *Pseudoryx*	+ 26.3	+ 11.6	+ 8.0	+ 9.0	+ 54.9	− 139.2[b]	23.0
all Antilopinae + *Pseudoryx*	+ 15.7	+ 5.9	+ 0.9	+ 12.6	+ 35.1	− 65.4[b]	17.3
all Bovidae + *Pseudoryx*	+ 17.0	+ 6.8	+ 0.9	+ 9.1	+ 33.8	− 64.7[b]	17.5

[a] Significant at $p < 0.05$: ΔlnL/s.e. > 1.96.
[b] Highly significant at $p < 0.001$: ΔlnL/s.e. > 3.29.

base compositions of any of the four markers: this might have affected the phylogenetic inferences, but probably not the conclusions of the Kishino & Hasegawa (1989) tests.

All the previous results were confirmed by MP searches under topological constraints (e.g. the grouping of *Pseudoryx* with Tragelaphini, Boselaphini or *Ovis* required 21.8, 23.4 or 54.9 additional rescaled steps; table 1). Several nuclear exclusive synapomorphies were also shared by the saola and Bovini, i.e. for *Lf* promotor G to A (on position 101), A to T (285) and A or G to C (289) substitutions, as well as a striking and unambiguously aligned deletion of seven nucleotides (140–146), and for *cyp*19 a C to T transition (54).

The exact phylogenetic position of the saola relative to other Bovini was not settled as its alternative groupings with cattle Bovini or buffalo Bovini did not involve significant log-likelihood differences (table 1). However, the preferred MP and ML hypothesis was an association of *Pseudoryx* with *Bos* plus *Bison* (figure 2, table 1) and a molecular signature defined this clade: an A to T (94) substitution in the 12S rRNA loop connected to stem 8/8′ (according to Springer & Douzery (1996)).

Relationships between the three tribes of Bovinae are another highly debated phylogenetic question. MP and ML analyses showed a sister-group relationship between Boselaphini and Tragelaphini (figure 2) but alternative topologies required only seven or nine rescaled extra steps to group Bovini with Boselaphini or Tragelaphini, respectively. No molecular signature characterized these latter two topologies while one exclusive synapomorphy was found for the grouping of Boselaphini with Tragelaphini, i.e. for *Lf* promotor A or G to C (269) substitution.

4. DISCUSSION

(a) *Is* Pseudoryx *a true Bovini?*

At the subfamily level, two contradicting conclusions have been proposed for the phylogenetic position of *Pseudoryx* within Bovidae: the first suggested an affinity with Bovinae (Dung *et al.* 1993; Schaller & Rabinowitz 1995; Robichaud 1998), whereas the second indicated a close relationship with Caprinae (Thomas 1994), i.e. the tribe Caprini *s.l.* in the present study. Our analyses show without any ambiguity that *Pseudoryx* belongs to Bovinae (figure 2, table 1). At the tribe level, Dung *et al.* (1993) placed the saola with the Boselaphini because it shares the presence of pre-orbital glands and white markings of the pelage with the nilgai. Schaller & Rabinowitz (1995) noted that these two character states are widely found among ruminants: pre-orbital glands have been observed in fossil Tragelaphini and a pattern of white markings on the head and/or body is also found in the mountain anoa and some Tragelaphini (bushbuck and bongo). These authors suggested that the saola exhibits some characters which show stronger affinities with Bovini rather than Boselaphini or Tragelaphini: (i) the frontal sinus of the skull extends well into the base of the horn cores; (ii) the incisors of *Pseudoryx* are equally sized; and (iii) the body conformation and the shape of hooves and horns are similar to those of anoas. This view is upheld by our analyses which show, with high confidence levels, that *Pseudoryx* is a true Bovini (figure 2): BP = 98, b_r = 14.3, RP = 77 and a shared unambiguous deletion of seven nucleotides in the *Lf* promotor.

Living members of the Bovini do not possess long horns but the fossil record indicates that some Pliocene–Pleistocene

Bovini of East Africa, e.g. *Simatherium shungurensis*, share a few cranial characters with *Pseudoryx* (Geraads 1995), such as the long face and the horns, which are long, slender, more backwardly inclined and weakly divergent. According to Geraads (1992), the evolutionary trends in the Bovini are linked with changes in horn cores, which become more divergent, less upright, more curved and closer to the back of the skull. Except for the latter, these trends are not found in the saola and some Pliocene Bovini from East Africa. These morphological features suggest including the saola in a separate tribe as previously mentioned by Schaller & Rabinowitz (1995), although our data rather suggest a subtribal status and a sister position for *Pseudoryx* relative to cattle Bovini (BP = 85, b_r = 4.6 and RP = 62, with a diagnostic synapomorphy in the 12S rRNA gene).

(b) *Mitochondrial and nuclear phylogeny of Bovini*

Within cattle Bovini, the systematic position of *B. grunniens* remains confused because both morphological and molecular studies have suggested a sister-group relationship with either the cow (e.g. Bohlken 1958; Janecek *et al.* 1996) or the bison (e.g. Groves 1981; Miyamoto *et al.* 1989; Pitra *et al.* 1997). As hybridizations between yak and cow give sterile male but fertile female offspring, the mitochondrial genome of *B. taurus* could be integrated into some yak populations by introgression and may explain the mitochondrial result of Janecek *et al.* (1996). As our mitochondrial and nuclear data show that *B. grunniens* clusters with *B. bison* (BP = 97, b_r = 6.0 and RP = 82), we conclude that the specimen used for this study does not issue from a lineage of yak–cow hybrids and that it should be preferable to synonymize the genus *Bison* with *Bos*.

Within buffalo Bovini, the anoa (*B. depressicornis*) is traditionally placed close to other Asian buffaloes (Tanaka *et al.* 1996). In a recent study based on *cyp*19 and *Lf* sequences, Pitra *et al.* (1997) suggested an alternative placement of the anoa with a boselaphine, the nilgai. Our mitochondrial and nuclear sequences show the anoa clustering with the Asian wild water-buffalo *B. bubalis* (BP = 100, b_r = 42.5 and RP = 97). The sequences of the anoa and nilgai provided by Pitra *et al.* (1997) are identical for *cyp*19 while only nine differences are observed for the *Lf* promotor. These two *Lf* sequences were integrated in our phylogenetic analyses and both cluster with our sequences of Boselaphini (i.e. *B. tragocamelus* and *T. quadricornis*). All differences between the anoa and nilgai sequences of Pitra *et al.* (1997) correspond to autapomorphies in our MP tree (data not shown). Therefore, we conclude that the authenticity of the anoa sequences generated by Pitra *et al.* (1997) is questionable and that anoas are closely related to Asian buffaloes as attested by other studies (e.g. Bohlken 1958; Tanaka *et al.* 1996).

(c) *Intertribal relationships among Bovidae*

Between Bovinae tribes, the phylogeny remains confused and Boselaphini are grouped either with Bovini on the basis of morphology (Groves 1981; Gentry 1992) and highly repeated DNA families (Modi *et al.* 1996) or with Tragelaphini according to mtDNA studies (Allard *et al.* 1992; Gatesy *et al.* 1997). Our data provide mixed support for a sister-group relationship between Boselaphini and Tragelaphini (figure 2). The fossil record shows a strong East African bias in the distribution of Tragelaphini in the Pliocene while numerous species of Boselaphini have been described in the Miocene of Eurasia (Thomas 1984). Therefore, an origin of tragelaphines from Eurasia looks probable. This view is corroborated by the absence of transverse ridges of the horn cores which aligns Tragelaphini with Boselaphini and by some Miocene–Pliocene fossils of East Africa exhibiting boselaphine and tragelaphine characters (Gentry 1990).

Among Antilopinae tribes, our study revealed that Hippotragini and Alcelaphini are closely linked and share sister-group relationships with Caprini *s.l.* (figure 2). Furthermore, the placement of *Neotragus* with *Aepyceros* and *Oreotragus* with *Cephalophus* supports the polyphyly of Neotragini. All these results confirm, with greater robustness, the conclusions previously reached with mtDNA sequences (Gatesy *et al.* 1997; Hassanin & Douzery 1999; Matthee & Robinson 1999).

(d) *Taxonomic conclusions*

Our molecular analyses evidenced some new taxonomic results which allow us to propose the following tentative classification for *P. nghetinhensis* within the family Bovidae Gray, 1821.

Subfamily Bovinae Gray, 1821
- Tribe Bovini Gray, 1821
  - Subtribe Bubalina Rütimeyer, 1865
    - *Bubalus* Smith, 1827
    - *Syncerus* Hodgson, 1847
  - Subtribe Bovina Gray, 1821
    - *Bos* Linnaeus, 1758
  - Subtribe Pseudoryina nov.
    - *Pseudoryx* Dung *et al.*, 1993

The present molecular study shows that the evaluation of mammalian biodiversity is still in progress and that new species, genera or even subtribes can be discovered at the end of the 20th century.

We are grateful to Herbert Thomas for skin samples from *P. nghetinhensis*; to Jean-Luc Berthier, Jacques Rigoulet, Claire Rejaud, Gérard Dousseau and Jean-François Marjarie for blood and hair samples from *B. depressicornis*, *B. grunniens* and *T. angasii*; to Jim Heffelfinger for *A. americana* and *O. hemionus* ssp. *crooki* tissues; to Philippe Chardonnet, Bertrand des Clers, Françoise Hergueta-Claro and Jean-Claude Thibault for other muscle, skin or blood samples; and to Jacques Cuisin, Jean-Marc Pons and Michel Tranier for the bone samples from museum specimens. We thank François Catzeflis, Thierry Oberdorff and Hervé Philippe for the laboratory environment, the computer management and the MUST improvements. We also thank Véronique Barriel, Cyril Gallut, Herbert Thomas, Simon Tillier and three anonymous reviewers for their helpful comments on the manuscript. This work was supported by the 'Groupement de Recherche et d'Etude des Génomes' (GREG, décision no. 82/94 of 19 July 1994). This is contribution no. 99-004 of the Institut des Sciences de l'Evolution de Montpellier (UMR 5554—CNRS).

REFERENCES

Allard, M. W., Miyamoto, M. M., Jarecki, L., Kraus, F. & Tennant, M. R. 1992 DNA systematics and evolution of the artiodactyl family Bovidae. *Proc. Natl Acad. Sci. USA* **89**, 3972–3976.

Baker, R. & DeSalle, R. 1997 Multiple sources of character information and the phylogeny of Hawaiian drosophilids. *Syst. Biol.* **46**, 654–673.

Barriel, V. 1994 Phylogénies moléculaires et insertions–délétions de nucléotides. *CR Acad. Sci. Paris, Sér. III* **317**, 693–701.

Bohlken, H. 1958 Vergleichende Untersuchungen an Wildrindern (Tribus Bovini Simpson, 1945). *Zool. Jb. (Abt. Allg. Zool.)* **68**, 113–202.

Bremer, K. 1994 Branch support and tree stability. *Cladistics* **10**, 295–304.

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

Dung, V. V., Giao, P. M., Chinh, N. N., Tuoc, D., Arctander, P. & MacKinnon, J. 1993 A new species of living bovid from Vietnam. *Nature* **363**, 443–445.

Farris, J. S., Källersjö, M., Kluge, A. G. & Bult, C. 1994 Testing significance of incongruence. *Cladistics* **10**, 315–319.

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

Gatesy, J., Amato, G., Vrba, E., Schaller, G. & DeSalle, R. 1997 A cladistic analysis of mitochondrial ribosomal DNA from the *Bovidae*. *Mol. Phylogenet. Evol.* **7**, 303–319.

Gentry, A. W. 1990 Evolution and dispersal of African Bovidae. In *Horns, pronghorns, and antlers* (ed. G. A. Bubenik & A. B. Bubenik), pp. 195–227. New York: Springer.

Gentry, A. W. 1992 The subfamilies and tribes of the family Bovidae. *Mamm. Rev.* **22**, 1–32.

Geraads, D. 1992 Phylogenetic analysis of the tribe Bovini (Mammalia: Artiodactyla). *Zool. J. Linn. Soc.* **104**, 193–207.

Geraads, D. 1995 *Simatherium shungurense* n. sp., un nouveau Bovini (Artiodactyla, Mammalia) du Pliocène Terminal de l'Omo (Ethiopie). *Ann. Paléontol. (Invert.–Vert.)* **81**, 87–96.

Groves, C. P. 1981 Systematic relationships in the Bovini (Artiodactyla, Bovidae). *Z. Zool. Syst. Evolutionsforsch.* **19**, 264–278.

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

Hassanin, A. & Douzery, E. J. P. 1999 The tribal radiation of the family Bovidae (Artiodactyla) and the evolution of the mitochondrial cytochrome *b* gene. *Mol. Phylogenet. Evol.* (In the press.)

Hassanin, A., Lecointre, G. & Tillier, S. 1998*a* The 'evolutionary signal' of homoplasy in protein-coding gene sequences and its consequences for *a priori* weighting in phylogeny. *CR Acad. Sci. Paris, Sér. III* **321**, 611–620.

Hassanin, A., Pasquet, E. & Vigne, J.-D. 1998*b* Molecular systematics of the subfamily Caprinae (Artiodactyla, Bovidae) as determined from cytochrome *b* sequences. *J. Mammal. Evol.* **5**, 217–236.

Janecek, L. L., Honeycutt, R. L., Adkins, R. M. & Davis, S. K. 1996 Mitochondrial gene sequences and the molecular systematics of the artiodactyl subfamily Bovinae. *Mol. Phylogenet. Evol.* **6**, 107–119.

Kingdon, J. 1997 *The Kingdon field guide to African mammals.* London: Academic Press.

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. Mol. Evol.* **29**, 170–179.

Kluge, A. G. & Farris, J. S. 1969 Quantitative phyletics and the evolution of anurans. *Syst. Zool.* **30**, 1–32.

Matthee, C. A. & Robinson, T. J. 1999 Cytochrome *b* phylogeny of the family Bovidae: resolution within the Alcelaphini, Antilopini, Neotragini and Tragelaphini. *Mol. Phylogenet. Evol.* (In the press.)

Miyamoto, M. M., Tanhauser, S. M. & Laipis, P. J. 1989 Systematic relationships in the artiodactyl tribe Bovini (family Bovidae), as determined from mitochondrial DNA sequences. *Syst. Zool.* **38**, 342–349.

Modi, W. S., Gallagher, D. S. & Womack, J. E. 1996 Evolutionary histories of highly repeated DNA families among the Artiodactyla (Mammalia). *J. Mol. Evol.* **42,** 337–349.

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

Pitra, C., Fürbass, R. & Seyfert, H.-M. 1997 Molecular phylogeny of the tribe Bovini (Mammalia: Artiodactyla): alternative placement of the Anoa. *J. Evol. Biol.* **10**, 589–600.

Robichaud, W. G. 1998 Physical and behavioral description of a captive saola, *Pseudoryx nghetinhensis*. *J. Mammal.* **79**, 394–405.

Schaller, G. B. & Rabinowitz, A. 1995 The saola or sprindle-horn bovid *Pseudoryx nghetinhensis* in Laos. *Oryx* **29**, 107–114.

Siddall, M. 1996 *Random cladistics, version 4.0.* Gloucester Point, VA: Virginia Institute of Marine Science.

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

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

Swofford, D. L. 1993 *PAUP: phylogenetic analysis using parsimony, version 3.1.1.* Champaign, IL: Illinois Natural History Survey.

Tamura, K. & Nei, M. 1993 Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. 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.

Thomas, H. 1984 Les Bovidae (Artiodactyla: Mammalia) du Miocène du sous-continent indien, de la péninsule arabique et de l'Afrique : biostratigraphie, biogéographie et écologie. *Palaeogeogr. Palaeoclimat. Palaeoecol.* **45**, 251–299.

Thomas, H. 1994 Anatomie crânienne et relations phylogénétiques du nouveau bovidé (*Pseudoryx nghetinhensis*) découvert dans la cordillère annamitique au Vietnam. *Mammalia* **58**, 453–481.

Winnepenninckx, B., Backeljau, T. & Dewachter, R. 1993 Extraction of high molecular weight DNA from molluscs. *Trends Genet.* **9**, 407.

Yang, Z. 1996 Among-site rate variation and its impact on phylogenetic analyses. *Trends Ecol. Evol.* **11**, 367–372.

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