

The investigation of cytochrome b gene in order to elucidate the taxonomic uncertainties between European bison (*Bison bonasus*) and its relatives

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Abstract

This study presents some observations concerning the phylogenetic relationship between Bovinae subfamily taxons, in order to solve some taxonomic uncertainties, by focusing on three main objectives. The first one was to clarify the taxonomic position of some species (*Pseudoryx nghetinhensis*, *Boselaphus tragocamelus*, *Tetracerus quadricornis* and *Bison bonasus*). The second objective was to find the pairwise differences between European bison and its relatives and to calculate the divergence time for the analyzed species. The last objective was to verify if the European and the American bison could be included in the *Bos* genus or considered a separate genus. The entire cytochrome b mitochondrial gene (1140 base pairs), for 24 species, was included in the investigation. In order to obtain a phylogenetic reconstruction with a high support, four calibration points were used. The cytochrome b gene proved to be useful in solving taxonomic uncertainties within the Bovinae subfamily and the results confirmed some of the recent studies in this field.

Keywords: phylogenetic relationships, divergence time, European bison, *cyt b*

1. Introduction

Bovidae family (infraorder *Pecora*, order *Artiodactyla*) consists of more than 300 extant and approximately 137 living species (oxen, sheep, goats, antelopes and allies), (SAVAGE & RUSSELL, [37]). Many of the bovids live in Africa, but some of them are found in Europe, Asia and North America. The taxonomy and phylogenetic relationships in this family have been controversial for a long time, as it is not easy to elucidate and comment on the results of morphological and molecular investigations (GATESY & ARCTANDER, [12]). The mitochondrial genome of most animals consists of 37 genes, but for phylogenetic research only a single mitochondrial gene is used to assess population or to establish low level of taxonomic relationships (LUCA & al. [29]). As a result, improper gene selection prone the phylogenetic studies to errors and mismatches. Numerous versions of bovid classification are currently provided without a consensus, due to the mismatches between the molecular data regarding the phylogenetic relationships and the morphological classification (HASSANIN & DOUZERY, [20]). LENSTRA & al. [28] have suggested five divisions of bovids which include the following subfamilies: *Bovinae* (cattle, nilgai and eland), *Caprinae* (sheep, goats and related animals), *Cephalophinae* (duikers), *Hippotraginae* (roan antelope) and *Antilopinae*

(gazelles, chiru and blackbuck). Confusions in the classification of cattle and its relatives have emerged with the first studies concerning the genetic relationships (GEORGESCU & al. [15]). The *Bovinae* subfamily has been divided into three tribes: *Tragelaphini*, *Boselaphini* and *Bovini* (KINGDON, [26]). The first uncertainty within this subfamily was the taxonomic position of saola (*Pseudoryx nghetinhensis*). KISHINO & HASEGAWA'S [27] have indicated that *Pseudoryx sp.* should be included in the *Bovini* tribe. DUNG & al. [11] confirmed this hypothesis and indicated that *Pseudoryx* may be incorporated inside *Bovini* clade, into *Boselaphine* tribe. GRUBB [17] proposed a new subtribe within *Bovinae* subfamily, for *Pseudoryx nghetinhensis*. A surprising hypothesis was developed by THOMAS [44] and suggested a close relationship between saola and *Caprinae*, based on dentition and skull morphology. A recent study, developed by F. BIBI [6], has placed the saola basal as a subtribe in *Bovini*. The author has analyzed the entire mitochondrial genome of the important species from the *Bovidae* family. The second uncertainty concerns the position of the *Boselaphine* tribe within the *Bovinae* subfamily. This tribe includes two monospecific genera from India: *Tetracerus* (four-horned antelope) and *Boselaphus* (nilgai). Currently, based on molecular analysis, three alternative topologies are recognized: *Tragelaphini* with *Boselaphini* (ALLARD & al. [1]; GATESY & al. [13]; HASSANIN & DOUZERY, [21]); *Bovini* with *Boselaphini* (JANECEK & al. [25], ARCTANDER, [2]) or *Boselaphini* as basal clade within the *Bovinae* subfamily (F. BIBI [6]). Morphologists indicated that *Boselaphini* may be grouped with *Tragelaphini* (KINGDON, [26]) or with *Bovini* (GENTRY, [14]; GROVES, [16]). The last uncertainty concerns the American bison (*Bison bison*) and the European bison (*Bison bonasus*). PRUSAK & al. [34] suggested that *Bison* genus is paraphyletic, both species (American bison and European bison) being grouped into two main separate clades with individuals from *Bos* genus. High genetic identity between *Bison bonasus* and its relatives from *Bos* genus (*Bos indicus*, *Bos primigenius* and *Bos taurus*) and between *Bison bison* and its relatives (*Bos javanicus*, *Bos grunniens*) has determined many researchers to consider the European bison and the American bison as species belonging to *Bos* genus (JANECEK & al. [26]; YANG & al. [52]; BIBI [6]). Thus, the morphological characteristics and the common ancestor of these two species (*Bison priscus*) contradict this hypothesis (T. P. SIPKO, [39]) and suggest that *Bison* genus is paraphyletic and should not be included in the *Bos* genus. In the present study, the analysis of the entire cytochrome b gene was carried out, in order to infer the phylogenetic relationships within the *Bovinae* subfamily. In order to elucidate the taxonomic position of *Pseudoryx nghetinhensis* and *Boselaphine* tribe we used Bayesian phylogenetic Inference method, with four fossil calibration points. The second objective of this study was to identify the pairwise differences between European bison (*Bison bonasus*) and its relatives and the last objective was to estimate the divergence time between the analyzed species.

2. Materials and Methods

DNA extraction, amplification and sequencing: Genomic DNA extraction was performed, starting from blood or hair samples from ten European bison individuals, by proteinase K treatment followed by phenol chloroform extraction (AUSUBEL & al. [3]). DNA was eluted in TE buffer (pH=8.0) and kept at -20°C. The complete cytochrome b gene (cyb, 1140bp) was amplified by polymerase chain reaction, with the primers described by WATANOBE & al. [49]. The primer sequences were mitL1 5'-ATCGTTGTCATTCAACTACA-3', mitH2 5'-CTCCTTCTCTGGTTTACAAG-3'. Amplifications were performed in a 25µl volume, using 12.5µl of Green Master Mix, 10 ×PCR Buffer with MgCl₂, 0.5µl 10µM of each primer, 9µl of nuclease free water and DNA template. The standard PCR conditions consisted of an initial

denaturation step at 94°C for 4 min, followed by 40 cycles of 94°C for 30 sec, 50°C for 45 sec, 72°C for 1 min and a final extension step at 72°C for 10 min.

The sequencing process was based on the Sanger method (SANGER & al. [36]), using the CEQ 8000 Genetic Analysis System (Beckman Coulter), according to the manufacturer's protocol.

Phylogenetic analyses: The phylogenetic reconstruction of *Bovinae* subfamily was carried out by comparing the European Bison's cytochrome b sequences with those derived from 20 *Bovinae* representative species (accession numbers are listed in Table 1). Two distant taxa, *Antilocapra americana* and *Tragulus kanchil*, were used as out groups. The complete sequences were aligned using Clustal W algorithm (THOMPSON & al. [45]) in the MEGA 5.0 (TAMURA & al. [43]) phylogenetic package. Phylogenetic relationships and divergence time were estimated using the Bayesian inference approach in Beast v. 1.7.4 software (DRUMMOND & al. [10]). A Yule speciation process was applied to the tree inference through the MCMC (Markov chain Monte Carlo) with a UPGMA starting tree and a TPM2uf+I+G site model determined by BIC (Bayesian Information Criteria) in jModel Test 3.0.6 (POSSADA & CRANDALL, [33]).

Table 1. The sequence's GenBank accession numbers for the 56 species used in phylogenetic analysis

Species	GenBank no.	Species	GenBank no.
<i>Tetracerus quadricornis</i>	EF536355	<i>Bubalus bubalis</i>	NC006295
<i>Tetracerus quadricornis</i>	NC020788	<i>Bubalus bubalis</i>	AF547270
<i>Boselaphus tragocamelus</i>	EF536350	<i>Bubalus bubalis</i>	AY488491
<i>Boselaphus tragocamelus</i>	NC020614	<i>Bos taurus</i>	DQ124411
<i>Taurotragus derbianus</i>	EF536354	<i>Bos taurus</i>	DQ124417
<i>Taurotragus derbianus</i>	NC020618	<i>Bos indicus</i>	JN817302
<i>Tragelaphus simberbis</i>	EF536356	<i>Bos indicus</i>	JN817330
<i>Tragelaphus imberbis</i>	NC020619	<i>Bos primigenius</i>	JQ437479
<i>Tragelaphus angasii</i>	NC020748	<i>Bosprimigenius</i>	GU985279
<i>Tragelaphus angasii</i>	JN632702	<i>Pseudoryx nghetinhensis</i>	NC020616
<i>Tragelaphus scriptus</i>	JN632705	<i>Pseudoryx nghetinhensis</i>	EF536352
<i>Tragelaphus scriptus</i>	JN632707	<i>Bison bison</i>	GU946999
<i>Tragelaphus eurycerus</i>	NC020749	<i>Bison bison</i>	GU946997
<i>Tragelaphus eurycerus</i>	JN632703	<i>Bison bison</i>	GU947006
<i>Tragelaphus spekii</i>	EF536357	<i>Tragulus kanchil</i> (Out Group)	JN632709
<i>Tragelaphus spekii</i>	NC020620	<i>Tragulus kanchil</i> (Out Group)	NC_020753
<i>Tragelaphus strepsiceros</i>	JN632708	<i>Antilocapra americana</i> (Out Group)	JN632597
<i>Tragelaphus strepsiceros</i>	NC020752	<i>Antilocapra americana</i> (Out Group)	NC_020679
<i>Taurotragus oryx</i>	JN632704	<i>Bison bonasus</i>	KP866252
<i>Taurotragus oryx</i>	NC020750	<i>Bison bonasus</i>	KP866253
<i>Bos grunniens</i>	JQ437480	<i>Bison bonasus</i>	KP866254
<i>Bos grunniens</i>	AY684273	<i>Bison bonasus</i>	KP866255
<i>Bubalus depressicornis</i>	EF536351	<i>Bison bonasus</i>	KP866256
<i>Bubalus depressicornis</i>	NC020615	<i>Bison bonasus</i>	KP866257
<i>Bos javanicus</i>	JN632605	<i>Bison bonasus</i>	KP866258
<i>Bos javanicus</i>	NC012706	<i>Bison bonasus</i>	KP866259
<i>Syncerus caffer</i>	JQ235541	<i>Bison bonasus</i>	KP866260
<i>Synceru caffer</i>	JQ235547	<i>Bison bonasus</i>	KP866261

Calibration points: The usage of multiple fossil calibration points seems to be the best method to scale the nodes height estimation, better than single fossil calibration point or mutation rate (DRUMMOND & al. [53], MEREDITH & al. [54]). The phylogenetic reconstruction timeframe was estimated using multiple calibration points, listed in Table 2.

Table 2. Fossil calibration points

Calibrated point	Age Ma	Age type	95% range	Fossil reference
Stem <i>Tragelaphus euryceros</i>	3.4	Minimum	3.4-4.5 Ma	WOLDEGABRIEL&. al [49]
Stem <i>Tragelaphus scriptus</i>	3.4	Minimum	3.4-4.5 Ma	WOLDEGABRIEL&. al [49]
Crown <i>Tragelaphine</i>	5.72	Approximate	4.7-6.7 Ma	DEINO& al. [9]
Crown <i>Bovini</i>	8.8	Approximate	7.0-11.0 Ma	BARRY&. al [4]

The first three calibration points constrain and scale the *Tragelaphini* tribe evolution while the last calibration point limits the divergence time of the *Bovini* tribe, excluding *Pseudoryx nghetinhensis*, to avoid a topological constraint.

3. Results and Conclusions

3.1. Phylogeny analysis of the European bison and their relatives based on cytochrome b gene

The Bayesian phylogenetic tree illustrates that cytochrome b sequences from *Bovinae* subfamily are separated into four major clades: *Tragelaphini*, *Boselaphini* and two *Bovini* clades (buffalo-bovini and cattle-bovini). The *bovini* clades consisted of cattle species, domesticated and wild buffalo. The obtained phylogeny confirms the results of previous studies, including the yak (*Bos grunniens*), the American bison (*Bison bison*) and the banteng (*Bos javanicus*) on a major clade in *Bovini* tribe (HASSANIN & DOUZERY [21]; VERKAAR & al. [48]). A similar phylogenetic arrangement was observed on another major clade which includes the European bison (*Bison bonasus*), aurochs (*Bos primigenius*) and taurine cattle (*Bos taurus* and *Bos indicus*), which is in agreement with TROY& al. [46] study. The buffalo-bovini clade incorporates five genera: *Tetracerus* (ex. Four-horned antelope), *Boselaphus* (ex. Nilgai or blue bull), *Bubalus* (ex. domestic water buffalo and Lowland anoa), *Syncerus* (ex. African buffalo) and the *Pseudoryx* (saola). In our investigation, the saola (*Pseudoryx nghetinhensis*) was situated into buffalo-bovini clade within *Bovini* main clade, thus confirming the hypothesis developed by KISHINO & HASEGAWA'S [27], DUNG & al.[11]. Based on the analysis of the entire cytochrome b sequences, we consider that the hypothesis developed by THOMAS [44] is not valid, saola being situated in *Bovinae* subfamily, nowise in *Caprinae* subfamily. Our results are in agreement with GRUBB's opinion [17], this species (*Pseudoryx nghetinhensis*) showing characteristics that justify the introduction into a new subtribe, within *Bovinae* subfamily. The analysis of cytochrome b enabled us to clarify the phylogenetic relationship between *Boselaphine* and the *Bovinae* subfamily species. The *Boselaphus tragocamelus* and the *Tetracerus quadricornis* were situated basal, into *Bovini* clade. The topologies elaborated by ALLARD & al. [1], GATESY & al. [13], HASSANIN & DOUZERY [21], in which *Boselaphine* are situated alongside *Tragelaphine*, was not confirmed by our study, but was supported by the study conducted by YANG & al. [52]. Our analyses on cytochrome b gene confirmed the hypothesis presented by JANECEK& al 25], GATESY & ARCTANDER [12] and BIBI [6], which places the *Boselaphini* together with *Bovini*, as a sister group to the buffalo-bovini clades. The topology obtained by YANG & al. [52] may contain errors because the phylogenetic relationships within a group could be affected by the number of analyzed species or by the use of an inadequate molecular marker. The complete mitochondrial genome analysis may sometimes compromise the phylogenetic accuracy (HERNANDEZ-FERNANDEZ & VRBA, [24], HASSANIN & al. [19]). We consider that the phylogeny highlighted in our study enables us to describe the evolutionary history of this group and our results are in agreement with other previous studies.

3.2. Genetic distance between European bison (*Bison bonasus*) and their relatives

The pairwise differences analysis between European bison (*Bison bonasus*) and its relatives was conducted in MEGA6 (TAMURA et al [42]) using the Maximum Composite Likelihood model (TAMURA et al [41]). The rate variation among sites was modeled with four gamma distribution categories. The analysis focused on 54 nucleotide sequences with a total length of 1140 base pairs. The pairwise differences between European bison (*Bison bonasus*) and its relatives are illustrated in Figure 1. The European bison showed a high level of similarity with *Bos indicus* (zebu, a sub-species of domestic cattle originating in South Asia) with a minimum value of 0.06 differences. The pairwise differences between American bison and European bison are 0.072, higher than those between European bison and *Bos taurus* (0.069) or *Bos primigenius* (0.068). This indicates that the wisent is closer to *Bos* genera (*Bos indicus*, *Bos primigenius* and *Bos taurus*) than its alleged relatives from *Bison* genera (*Bison bison*). In 2004, PRUSAK & al. [34] suggested that *Bison* genus is paraphyletic, both species (American bison and European bison) being grouped into two main separate clades with individuals from *Bos* genus. However, the cytochrome b sequences belonging to the American bison (*Bison bison*) and some species of *Bos* genus (*Bos grunniens*, *Bos javanicus*) contain a small number of differences (0.002), these species having a high genetic identity. Members of the *Boselaphini* tribe show an important number of differences in comparison with the European bison (0,147). The highest number of pairwise differences was found between wisent and the *Tragelaphini* tribe (values between 0.140 and 0.170).

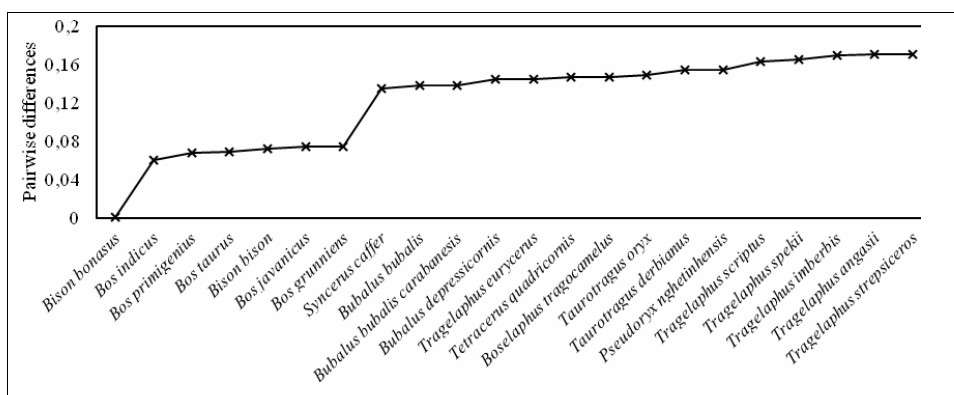


Figure 1. The estimates of evolutionary divergence between sequences using the Maximum Composite Likelihood model

3.3. Estimation of divergence times among Bovinae subfamily

Divergence time of the *Bovinae* subfamily was estimated using Bayesian method with a lognormal relaxed clock model, using 4 fossil calibration points (Figure 2). According to our results, the split between crown *Tragelaphini* and crown *Bovini* occurred during the middle Miocene, approximately 12.04 million of years ago (Ma). A previous study of WILLOWS-MUNRO & al. [50], estimated the split of *Tragelaphini* clade ancestor during the last 14.1 million years. This clade includes spiral-horned antelopes, a total of nine different species. Our data confirm previous molecular studies which included the *Taurotragus* genera (*Taurotragus oryx* and *Taurotragus derbianus*) into *Tragelaphus* main clade, supporting the idea that these two genera are closely related (HASSANIN & DOUZERY [21], MOODLEY & BRUFORD, [30]). The *Tragelaphini* diversification started 5.87 Ma ago, by splitting into two major clades. The first clade, including *T. imberbis*, *T. scriptus* and *T. agnasia* species, 12120

evolved in the last 4.49 Ma. The second clade dates about 4.54 Ma ago, in the late Pliocene. This estimated age is also supported by a previous study developed by BIBI [6]. The Tragelaphine clade contains six species: *T. scriptus*, *T. spekii*, *T. eurycerus*, *T. strepsiceros*, *Taurotragus derbianus* and *Taurotragus oryx*. The assumed age for the divergence of Bovini clade into two sister subclades, buffalo-bovini and cattle-bovini, seems to be around 11.2 Ma. The Early Pliocene may have been an especially critical period for buffalo-bovini subclade evolution. During this period, buffalos, including *Bubalus* species and *Syncerus* sp., shared a common ancestor. The radiation of this form in *Syncerus* and *Bubalus* genera clade took place 6.53 Ma ago. The *Bubalus* genera dates from the Middle Pliocene, about 3.743 Ma. The youngest species of these genera is the lowland anoa (*Bubalus depressicornis*), dating from 0.4 Ma. Another two young species from buffalo-bovini are *Boselaphus tragocamelus* and *Tetracerus quadricornis*, both of them having a divergence time around 2.9 Ma. The cattle-bovini clade was separated into two main clades 8.05 Ma ago. One of them includes the yak, the American bison and the banteng. The initial radiation for this complex has occurred at the beginning of the Miocene, approximately 6.82 Ma. The oldest species from this complex is *Bos grunniens*, dating from 3.345 Ma. The phylogenetic reconstruction shows that the divergence between American bison and yak took place 5.26 Ma. Based on our analysis, the second cattle-bovini clade includes three cattle species and a bison species: *Bos taurus* (*taurus* and *indicus* subspecies), *Bos primigenius* and *Bison bonasus* (the European bison). The European bison separated from its cattle relatives 5.83 Ma ago, at the beginning of the Miocene. The basal position of wisent in cattle-bovini clade is in accordance with JANECEK & al. [25] statement. It was observed that zebu cattle (*Bos taurus indicus* or *Bos primigenius indicus*) is the first subspecies that diverged from primigenius/taurus common ancestor, in comparison with its Eurasian closer relatives (*Bos primigenius* and *Bos taurus taurus*). Our results are supported by archaeological evidence which shows that *Bos primigenius* species dates from the Middle Pleistocene (HELMER & al. [22], BRADLEY & MAGEE [8], PANDOLFI & al. [32]), around 1.6 Ma ago.

3.4. Taxonomy position of the European bison into *Bovinae* subfamily

The wisent (*Bison bonasus*) belongs to *Artiodactyla* order, *Ruminantia* suborder, *Bovidae* family, *Bovinae* subfamily, *Bovini* tribe, *Bison* genus (BELOUSOVA & al. [5]). *Bison* genus includes two species: the European bison (*Bison bonasus*, Linnaeus 1758) and the American bison (*Bison bison*, Linnaeus 1758). There are three subspecies of European bison: Carpatian (Hungarian) bison (*Bison bonasus hungarorum* which is extinct), the Caucasian bison (*Bison bonasus caucasicus*, which is also extinct since 1925), (HEPTNER & al. [23]) and the Lowland bison (*Bison bonasus bonasus*). Two genetic lines can be distinguished within *Bison bonasus bonasus*: Lowland-Bialowieza line (descendants of seven individuals) and Lowland-Caucasian line (descendants of just five European bison), (PRUSAK & al. [34]).

Within the American bison, two subspecies are recognized: the plains bison (*Bison bison bison*) and the wood bison (*Bison bison athabascae*). The genetic similarity between the European bison and the American bison was confirmed by some previous studies of RYSKOV & al. [35] and SIPKO & al. [38]. BOHLKEN [7] and VAN ZYLL DE JONG [47] which consider them subspecies of *Bison* species, being able to interbreed. Considering the geographical distribution, body size and several morphological traits, JANECEK & al. [25] disputed previous affirmations and suggested that the American and the European bison are two separated species.

According to mtDNA studies, *Bovinae* subfamily is split in two main clades: one for *Tragelaphini* tribes and another one for the *Bovini* tribes (ALLARD & al. [1], GATESY & al. [13], MODI & al. [29]). The *Bovini* clade is divided into two sister groups: the buffalo-bovini clade and the cattle-bovini clade. We observed that the European bison (*Bison bonasus*) was situated basal, along the tribe *Bovini* (cattle-bovini clade). The same applies for the American bison (*Bison bison*), which was also included into *Bovini* tribe. We suggest that *Bos* and *Bison* should be integrated into a single *Bos* genus because these species show a high level of genetic similarity. The low level of pairwise differences between European bison (*Bison bonasus*) and its relatives (*Bos indicus*, *Bos primigenius*, *Bos taurus*) or between the American bison and its relatives (*Bos grunniens*, *Bos javanicus*) confirm, once again, our hypothesis.

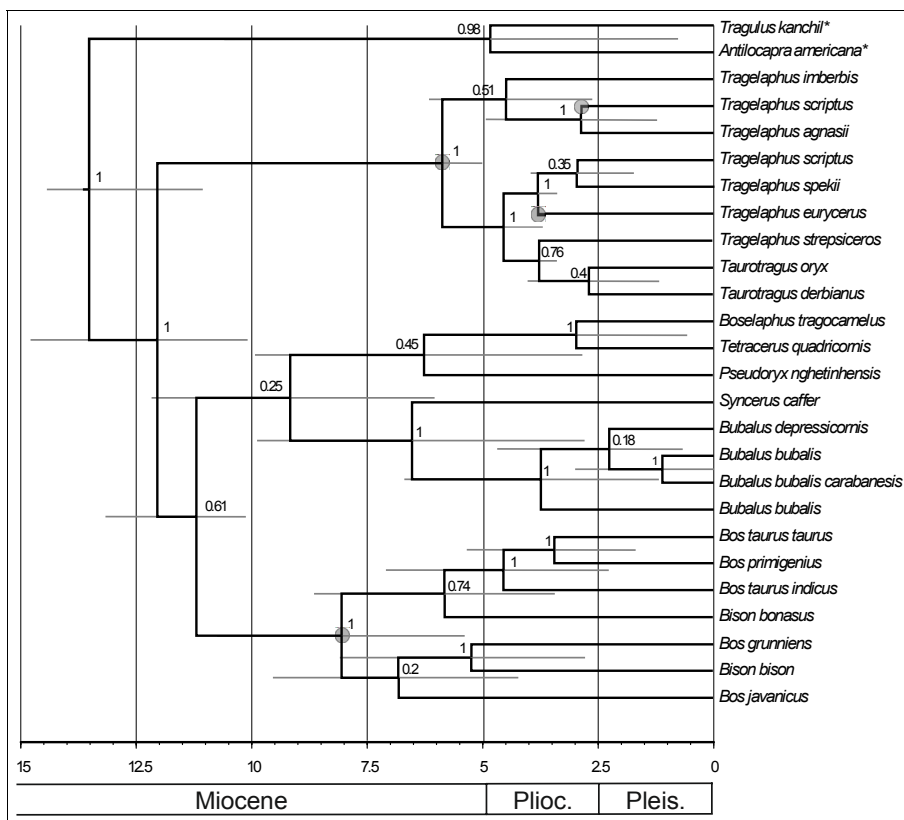


Figure 2. MCC tree resulting from Bayesian analysis of the full cytochrome b of 25 *Bovinae* species, using 4 calibration points. Node bars represent 95% intervals and node values are posterior probabilities.

In order to understand and clarify the taxonomic uncertainties within *Bovinae* subfamily, a Bayesian approach was used to infer the phylogenetic relationships based on the mitochondrial marker, cytochrome b gene. Our data has shown a common ancestor for the *Boselaphini* tribe and *Pseudoryx nghetinhensis* species, describing a basal clade within buffalo-bovini clade. Also, the inferred phylogeny has supported the paraphyletic evolution hypothesis of the *Bison* genera. Furthermore, the paraphyletic placement of *Bison* species was confirmed by

a low level of similarity between *B. bison* and *B. bonasus*. A higher similarity was observed between the European bison and *Bos* species (*Bos taurus*, *B. primigenius* and *B. indicus*), in comparison with its presumptive relative, the American bison. Determination of divergence time, by four fossil calibration points, has suggested that the *Bovinae* subfamily was formed in the Middle Miocene, followed by a radiation in Pliocene.

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