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The surprising evolutionary history of South American deer

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ABSTRACT

To clarify the systematic relationships and evolutionary history of South American deer, we conducted a comprehensive phylogenetic analysis using representative species of all of the genera of Neotropical deer. Our results revealed high levels of molecular and cytogenetic divergence between groups of morphologically similar species of brockets (*Mazama*), and suggest a polyphyletic origin. At least eight ancestral forms of deer invaded South America during the late Pliocene (2.5–3 MYA), and members of the red brockets had an independent early explosive diversification soon after their ancestor arrived there, giving rise to a number of morphologically cryptic species.

1. Introduction

The evolutionary history of deer dates back almost 20 million years ago (MYA) beginning in the Miocene and Early Pliocene of Eurasia (Webb, 2000). This highly diverse group evolved as a north-temperate group of artiodactyls that retained the forest- or woodland-dwelling habit of their chevrotain-like ancestors. Meanwhile by the early Pliocene true cervids became identifiable in North America; however, the evolutionary history of deer in North America and in the neotropics is still somewhat obscure. In the late Pliocene, approximately 2.5–3 MYA, the uplift of the Panamanian land bridge allowed deer to spread south, as participants in the "Great American Interchange" between North and South America (Stehli and Webb, 1985). These were the first deer to enter the Southern Hemisphere, and their surprising success in South America may be attributed in part to the absence of any other ruminants (Webb, 2000).

Deer of Central and South America fit into two major morphological forms. Adults of the smaller deer species are less than 60 cm at the shoulder and males develop unbranched spike antlers. Their small size and simple antlers are morphological adaptations to move efficiently in densely vegetated forests and closed ecosystems (*Mazama* and *Pudu*). The remaining species inhabit more open forested areas, grasslands, pampas and wetlands and are much larger in stature and the males have branched antlers (*Odocoileus, Hippocamelus, Ozotoceros*, and *Blastocerus*; Eisenberg, 2000; Merino et al., 2005).

The evolution of the Cervidae, especially in the Neotropics, remains unclear partly because the fossil record is incomplete and rather scarce (Webb, 2000). Although phylogenetic studies of this group have been undertaken with several approaches, including morphology (Merino et al., 2005), isozymes (Smith et al., 1986), cytogenetics (Duarte and Merino, 1997), and DNA sequences (Randi et al., 1998, 2001; Pitra et al., 2004; Gilbert et al., 2006), a great deal of confusion still remains with regard to their evolutionary history and taxonomy. These studies resulted in conflicting hypotheses about the phylogenetic relationships within the Neotropical cervids and hence, inducing uncertain evolutionary interpretations.

The family Cervidae appears to have one of the highest karyotypic evolutionary rates in mammals due to extreme chromosomal fragility (Vargas-Munar, 2003). In addition, the taxonomy of brocket deer has been very problematic mostly because the low levels of morphological differentiation are not correlated with the wide karyotypic diversification among the species in this genus (Groves and Grubb, 1987, 1990; Duarte and Merino, 1997). Descriptions based on morphological taxonomic revisions of the genus *Mazama* have generated between 6 (Czernay, 1987) and up to 18 (Allen, 1915) species. Taxonomic revisions of this group based on

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cytogenetic data have proven to be more useful in comparison with morphological approaches in recognizing new cryptic species of red brockets from Mexico (*Mazama temama*; Groves and Grubb, 1987), and more recently from Brazil (*Mazama bororo*; Duarte and Jorge, 2003).

Recent phylogenetic studies of old world deer using cytochrome *b* sequence data showed a close relationship among the Odocoileinae, *Odocoileus virginianus* and *Odocoileus hemionus* as sister groups together with *Mazama* sp., whereas *Pudu* and *Blastocerus* clustered in a separate clade (Pitra et al., 2004). A phylogenetic study using isozyme data suggested that the red brocket, *Mazama americana*, was more closely related to the white-tailed deer, *O. virginianus*, than to the gray brocket, *Mazama gouazoubira* (Smith et al., 1986). Phylogenetic studies based on cytogenetic data suggested that the white-tailed deer (*O. virginianus*; Spotorno et al., 1987) and the gray brocket (*M. gouazoubira;* Duarte and Merino, 1997) had the two most ancestral karyotypes. However, these studies were unable to clearly resolve the evolutionary history of the entire group of New World deer species. Therefore, in order to resolve the controversial systematics of this group, we undertook a molecular analysis of cytochrome *b* gene sequences of all the extant representative genera of neotropical deer and we compared our results to external morphology and cytogenetic data.

Surprisingly, our results show that members of morphologically cohesive genera like *Mazama* and *Hippocamelus* do not form monophyletic groups but they represent separate radiation from two unrelated lineages with high levels of molecular and cytogenetic divergence (data only for *Mazama*).

2. Materials and methods

2.1. Samples and specimens examined

Samples were selected from a variety of sources tissue, blood and hair from free ranging or captive individuals. In total, we examined 250 animals and most of the specimens used in the study were initially identified based on morphology, but some cryptic species like *M. bororo* were subsequently identified by cytogenetics and a subset of those were used in the sequence analysis. (see Appendix A-Supplementary materials).

2.2. Morphology

We took 11 body measurements (head length, head width, distance between eyes, ear length, width of the mandible base, metacarpi length, metatarsi length, body length, thorax circumference, height, and weight) from 138 anesthetized animals (27 *M. americana*, 5 *M. bororo*, 61 *M. gouazoubira*, 36 *Mazama nana*, 8 *Mazama nemorivaga* and 1 *Ozotoceros bezoarticus*) following the procedures described in Duarte and Jorge (2003). A cluster analysis was performed to measure the similarity among the morphology of individuals and discriminate species and populations (Sneath and Sokal, 1973). The tree plot was obtained using the Complete Linkage of Amalgamation Rule and with a Euclidian Distance as distance measurement; we included one *O. bezoarticus* to compare *Mazama* with another genus.

2.3. Cytogenetics

Cytogenetic data were obtained from fresh blood or frozen lymphocytes (Duarte et al., 1999) and fibroblast cultures (Duarte and Jorge, 2003), from 213 animals (33 *M. americana*, 3 *M. bororo*, 81 *M. gouazoubira*, 45 *M. nana*, 6 *M. nemorivaga*, 9 *O. bezoarticus*, and 36 *Blastocerus dichotomus*). We analyzed at least 10 good quality metaphases for each specimen, and karyotypes were performed following methods in Duarte and Merino (1997). The supernumerary (B) chromosomes were not considered in the diploid number ($2n$) or in the fundamental number (FN).

2.4. Molecular data

Procedures for DNA extraction from tissue samples (blood and hair) were performed following Medrano et al. (1990). For most of the tissues samples, the entire cytochrome *b* gene (1140 bp) was amplified using primers ML103 and MH104 (Randi et al., 1998). For some of the hair and blood samples that were too degraded for the amplification of the entire cytochrome *b* gene, we performed partial amplifications using L14724 and H15149 (480 bp) (Irwin et al., 1991), and a set of newly designed primers CYTB FAR-L 5′-CCATGAGGACAAATATCATTCTGAT-3′ and CYTB FAR-H 5′-TCCAATAGTAATAAAGGGGTGTTCA-3′ (660 bp). The purified PCR products were then sequenced using the above two sets of primers and the ABI Big Dye Ready reaction kit and ran on ABI 377 and 3100 automated sequencers (Applied Biosystems). Cytochrome *b* sequences were obtained from 82 individuals belonging to: *M. americana* (22), *M. bororo* (5), *M. gouazoubira* (15), *M. nana* (5), *M. nemorivaga* (4), *Mazama* sp. (1), *O. bezoarticus* (10), *B. dichotomus* (15), and *Hippocamelus bisulcus* (5).

2.5. Molecular data analyses

Although the entire cytochrome *b* was originally amplified for most of the tissue samples, the majority of the sequences were obtained by concatenating data from the two fragments using the internal primers L14724 and H15149 for the 3′ end of the cytochrome *b* and CYTB FAR-L and FAR-H for the 5′ end of the cytochrome *b* and totaled 934 bp in length. Our sequences were first aligned manually and then using clustal X (Thompson et al., 1997) and the alignments for all the haplotypes were unambiguous. All unique haplotypes defined by the 934 bp sequences from 82 individuals were subsequently submitted to genbank (Accession Nos: DQ789173–DQ789231). In addition to our sequences, the genbank database was queried for other available South and North American deer sequences (*B. dichotomus* (AY607038), *Hippocamelus antisensis* (DQ379307), *Mazama* sp. (AJ000027), *Pudu puda* (AY607039), *O. virginianus* (AY607035), and *O. hemionus* (AF091630). The data set was tested for the most appropriate model of nucleotide substitution using the likelihood ratio test implemented in MODELTEST 3.06 (Posada and Crandall, 1998). Nucleotide sequence data were analyzed using maximum parsimony (MP), neighbor joining (NJ), and bayesian inference (BI) with the software packages PAUP*4.0b10 (Swofford, 1999), MEGA 3.1 (Kumar et al., 2004), and MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). The group support in the NJ and MP tree was evaluated with 1000 bootstrap pseudoreplicates and parametric posterior probabilities (PP) were generated for the bayesian analysis and are shown on the linearized tree (Fig. 3). For the BI the preferred model was HKY+ Γ with parameters set to nst = 2 with a proportion of invariable sites and a gamma-shaped rate variation. The bayesian analysis was run using 5,000,000 generations along four chains with two replicates at a temperature of 0.05. Sample frequency was set to 1000 with a burn in of 100.

Mean pairwise differences between and within groups and estimates of molecular divergence (Kimura 2-parameter) were generated in MEGA 3.1 (Kumar et al., 2004). A distance tree was generated using the HKY85 model with a constant rate applied across the tree. Divergences were estimated using the fossil calibration point used by Pitra et al. (2004) for the node of separation between *Blastocerus/Pudu* and *Mazama/Odocoileus* (5 MYA). *Rangifer tarandus* (AJ000029) was used as an outgroup.

3. Results and discussion

Our analysis of the morphological, cytogenetic and molecular data showed an astounding and complex evolutionary pattern of phylogenetic relationships among neotropical deer, particularly in brocket deer (*Mazama*).

3.1. Morphology

The external morphology (body size, and the absence of branched antlers in males) has been used to group species of gray and red brocket deer together and to distinguish them from other large neotropical cervids that have branched antlers such as *Blastocerus* and *Ozotoceros*. In addition, within the brocket deer, the red brockets and the gray brockets can be distinguished using general pelage coloration patterns and body size and shape.

Gray brocket species exhibit a wide inter-individual pelage color variation from drab brown to yellowish gray brown (Allen, 1915). In comparison, the different species of red brocket deer have very similar coloration, that ranges slightly from dark chestnut red to yellowish red with the mid-dorsal region, head, and neck ranging from black to blackish to dark brown (Allen, 1915). This similarity has caused numerous mistakes in identification of the different species in the genus *Mazama*. For instance, a morphological study based on coloration and craniometrics of an extensive sampling of the red brocket species from Brazil failed to distinguish two different species of red brockets (*M. americana* and *M. bororo;* Rossi, 2000). Furthermore, the identification of these cryptic species becomes more difficult in areas where several species occur in sympatry (Duarte and Jorge, 2003). The external morphometric analysis alone also showed low resolution power to discriminate within the brockets (Fig. 1). The morphological distance tree placed some *M. gouazoubira* mixed with *M. nemorivaga* and *M. nana*, and *M. bororo* mixed with *M. americana* as shown by Rossi (2000) using craniometry. In addition, based on coloration pattern, members of the genus *Mazama* can be split into two groups, known respectively, as gray brockets (*M. gouazoubira* and *M. nemorivaga*) and red brockets (*M. americana*, *M. nana*, and *M. bororo*).

3.2. Cytogenetics

The chromosome polymorphism found in the genus *Mazama* is remarkable and resembles that found in the genus *Muntiacus* (Groves and Grubb, 1990). The levels of karyotypic differentiation between all species of *Mazama* characterized to date are high (Duarte and Jorge, 2003). The diploid number in the genus varies from 32 chromosomes in *M. bororo* to 70 chromosomes in *M. gouazoubira*. The brockets can be grouped according to chromosomal numbers as follows: (a) gray brockets with a diploid number of $2n = 66–70$ chromosomes with low intraspecific polymorphism, close to karyotypes of *B. dichotomus* and *O. bezoarticus* ($2n = 66$ and $2n = 68$, respectively) and (b) red brockets with diploid number ranging from $2n = 32$ (*M. bororo*) to $2n = 54$ (*M. americana*), and with high levels of intra- and inter-specific chromosomal variation (Duarte and Jorge, 2003; Abril and Duarte, 2008).

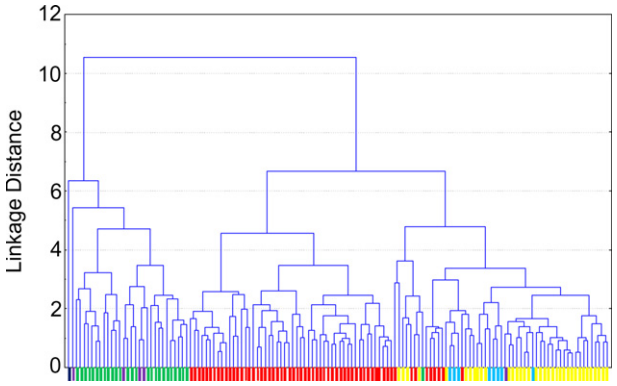

Fig. 1. Cluster analysis tree of the morphometric data of South American deer. *Ozotoceros bezoarticus* (black), *Mazama nana* (yellow), *Mazama nemorivaga* (blue), *Mazama americana* (green), *Mazama bororo* (violet), *Mazama gouazoubira* (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

The high karyotypic differences among these species followed by a decreasing trend in chromosome number are suggestive of a synapomorphy that appeared in the red brocket species group, which is not correlated with the rate of morphological and molecular differentiation. The chromosome polymorphism found in *M. americana* is correlated with geographic location, and cytotypes from different sampled localities have been identified. However, these cytotypes could not be correlated with external morphological differentiation. For instance, two red brocket deer females from different localities show low levels of morphological differentiation but are very distinct karyologically (Fig. 2). Another example where cytogenetics have been used to distinguish morphologically cryptic species is the case of the small red brocket deer (*M. bororo*), which was considered the same species as the red brocket (*M. americana*) by many authors based on morphological characters (Czernay, 1987; Rossi, 2000). The small red brocket was later recognized based on the enormous karyotypical distance ($2n = 32–34$ in *M. bororo* vs. $2n = 50–53$ in *M. americana* of the same geographic region; Duarte and Jorge, 2003).

3.3. Molecular genetics

We identified 59 different haplotypes from the 82 individuals sequenced. The values for mean pairwise divergence within each putative species group were relatively low in *M. nana* (0.36), and *B. dichotomus* (0.43); intermediate in *O. bezoarticus* (0.99) and high in *M. nemorivaga* (2.59) and *M. americana* (3.51). This suggests that the haplotypic diversity within *M. americana* is three orders of magnitude higher than *O. bezoarticus* for which we included samples encompassing the entire range of this species distribution. The mean pairwise divergence between each of the putative species groups ranged from (8.6–13.9%) and *M. americana* also showed the highest mean values between groups of 12.2% (range = 11.5–13.9%). Although this is the first study that has included representatives from multiple samples from all the different species of South American cervids, our results are in agreement with previous studies based on cytochrome *b* sequences (Randi et al., 1998, 2001; Pitra et al., 2004; Gilbert et al., 2006).

The phylogenetic analysis of cytochrome *b* sequences using all three phylogenetic methods (MP, NJ and BI) were highly concordant in topology and showed two well-supported main clades (posterior probabilities (PP) > 97 and bootstrap support (BST) > 77) that diverged approximately 5 MYA (Fig. 3). One includes gray brockets *M. gouazoubira* and *M. nemorivaga*, marsh deer (*B. dichotomus*), huemul (*H. bisulcus*), Taruka (*H. antisensis*) and pampas deer (*O. bezoarticus*). The other clade includes the red brocket group species (*M. bororo, M. nana, M. americana*, and *M. temama*) and the genus *Odocoileus*. This unexpected partition of the species of the genus *Mazama* into two separate clades was also recently reported by Gilbert et al. (2006) in a phylogenetic analysis of the Cervidae using mitochondrial and nuclear DNA markers and a small sampling of South American cervid species. The relationships revealed by these phylogenetic analyses suggest that what is now considered the genus *Mazama* actually corresponds to a polyphyletic arrangement, one clade including only the red brocket group and the other the gray brocket group. The gray brocket

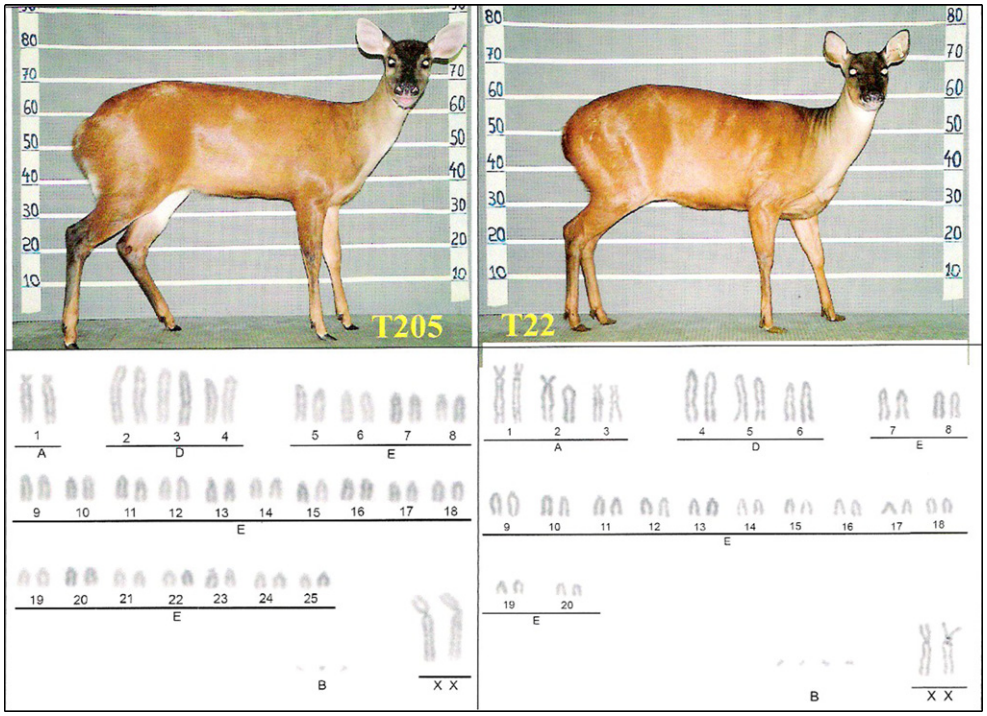

Fig. 2. Two *Mazama americana* females with very similar external morphologies show high degree of karyotypic differences. T205—an animal from the State of Paraná, Brazil and its karyotype; T22—an animal from the State of Rondônia, Brazil and its karyotype.

(*M. gouazoubira*) and the small gray brocket (*M. nemorivaga*) should be considered separate genera from the genus *Mazama*, whose type species is the red brocket *M. americana.*

Within the gray brocket clade, a node with high statistical support (100 PP/100% BST) consists of a highly diverse group of haplotypes of gray brocket deer (*M. gouazoubira*). The other five nodes with high posterior probability and bootstrap values are composed of: (a) the marsh deer haplotypes; (b) the huemul haplotypes; (c) the pampas deer haplotypes; (d) the Taruka haplotype; and (e) a basal clade that includes the four *M. nemorivaga* haplotypes. The molecular and cytogenetic results support *M. nemorivaga* as a separate species from *M. gouazoubira* as recently suggested by morphology (Rossi, 2000). Furthermore, the amount of genetic differentiation between the two gray brockets (*gouazoubira* and *nemorivaga*) and the fact that they do not cluster as a monophyletic group (Fig. 3) supports their recognition as separate genera. The same occurs in the genus *Hippocamelus* where the Huemul (*H. bisulcus*) and the Taruka (*H. antisensis*) are positioned separately in the phylogenetic tree also suggesting a polyphiletic origin and the need for taxonomic reevaluation for the genus *Hippocamelus.*

The red brocket deer clade forms a well-supported monophyletic group (100 PP/99%BST) that includes haplotypes from *O. virginianus, O. hemionus*, *M. americana, M. nana, M. bororo, Mazama* sp. from GenBank and a *Mazama* sp. from Bolivia. The haplotype that was obtained from GenBank did not have a species designation. We can only speculate that it belongs to *M. temama* because the sample was collected from an animal from the San Diego Zoo where this species was kept in captivity for many years (Jorge and Benirschke, 1977). This clade shows low resolution between red brocket haplotypes that suggests a rapid radiation event that started 2.5 MYA. This date coincides with the land mammal invasion from North to South America (Webb, 2000; Gilbert et al., 2006), that probably included at least eight different ancestral deer forms (Fig. 3). The red brocket group quickly differentiated within South America during the Pleistocene in glacial refugia (Haffer, 1987). *M. bororo* and *M. nana* separated from the ancestral red group at the beginning of the Pleistocene (1 MYA), and other *M. americana* variants began separation probably in the late Pliocene (2 MYA). The low ecological plasticity of red brocket species (Duarte, 1996) made it difficult for them to move between Pleistocene forested refugia resulting in isolation and genetic diversification after their invasion into South America. On the other hand the gray brocket species (*M. goauzoubira* and *M. nemorivaga*) had higher ecological plasticity (Pinder and Leeuwenberg, 1997) that allowed them to move across the landscape during the wet and dry cycles of the Pleistocene resulting in reduced levels of geographic differentiation and speciation. The high levels of genetic and morphological variation that we observed in *M. gouazoubira* are probably due to an enormous demographical and geographical expansion in South America beginning in the Pleistocene (1 MYA).

Our phylogenetic analyses placed *O. virginianus* closely related to red brockets supporting earlier biochemical studies (Smith et al., 1986). In addition, Pitra et al. (2004) and Gilbert et al. (2006) found similar patterns but had less resolution because of the limited number of samples of neotropical deer species representatives included in their phylogenetic analysis of old world deer.

The most recent clade that diverged from *M. americana* approximately 1 MYA consists of three *M. bororo* haplotypes (100 PP/99%) and three *M. nana* haplotypes (99 PP/99%). The estimated divergence time between *M. bororo* and *M. nana* is approximately 0.7 MYA. These results support the previous suggestion that *M. bororo* and *M. nana* were cytogenetically separate but closely related species (Duarte and Jorge, 2003; Abril and Duarte, 2008).

The high levels of genetic diversity and diversification of haplotypes in the red brocket clade (Fig. 3) are supported by cytogenetic data and suggest the presence of a complex of cryptic species within *M. americana.* For instance, two female *M. americana* that appear to be morphologically very similar, have very different karyotypes (Fig. 2) and fall in two very divergent lineages in the mtDNA tree (T205 and T22; Fig. 3), corroborating that they are not closely related, and perhaps not even the same species, in spite of their close morphological similarities. In addi-

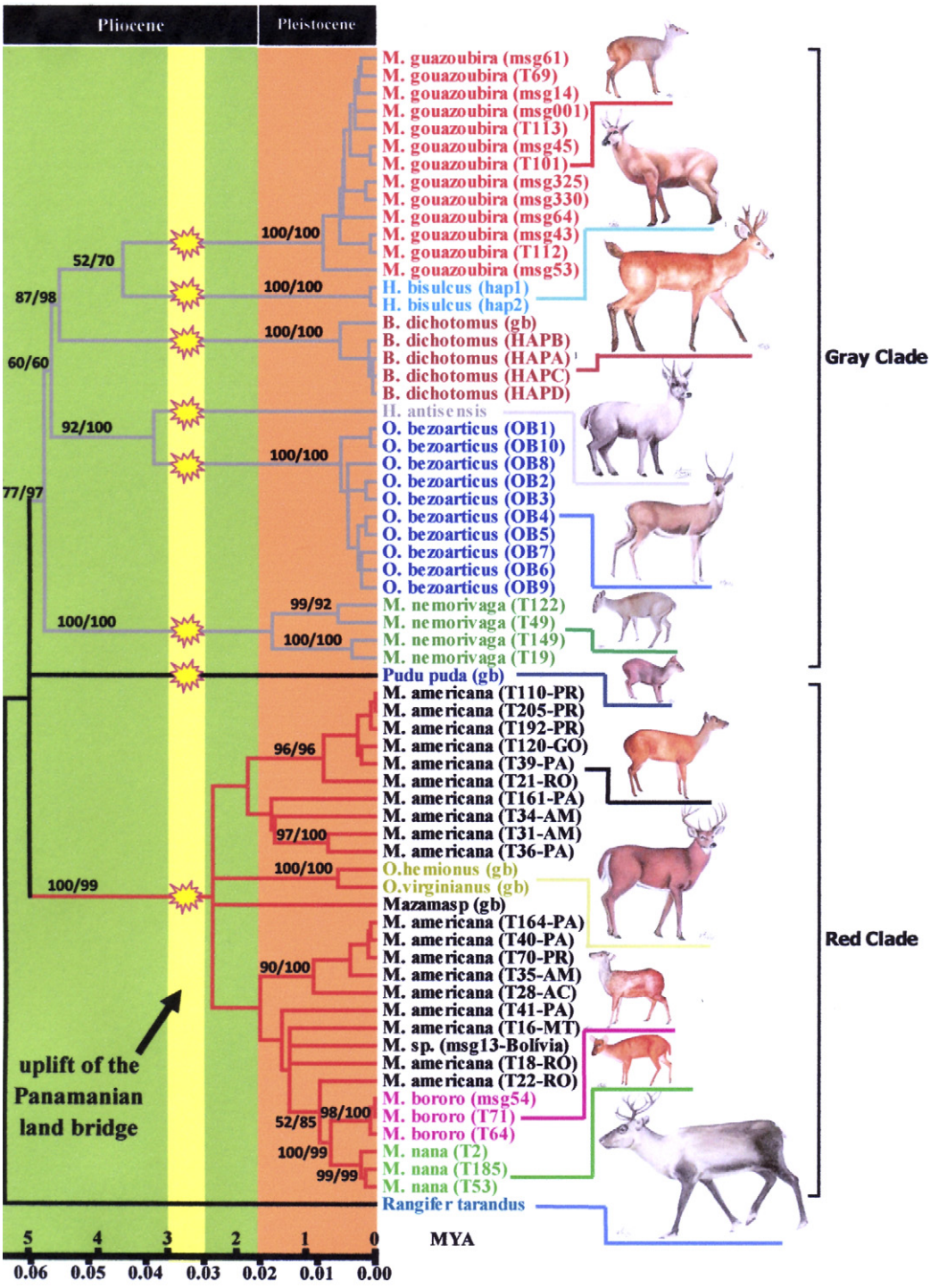

Fig. 3. Linearized tree showing phylogenetic relationships among 59 deer haplotypes derived from a 934 base pair fragment of the mitochondrial cytochrome *b* compiled with the computer program MEGA 3.1 (Kumar et al., 2004) after performing a branch length test (Takezaki et al., 1995) to test for differences in base substitution rates. The scale on top corresponds to the time scale while the scale below corresponds to the observed mean sequence divergence using the substitution model K2P (Kimura, 1980). Numbers correspond to sample IDs in the sample list (Appendix). Similar topologies were obtained using Maximum Parsimony (MP), Neighbor joining (NJ), and Bayesian Inference (BI) (not shown). Bootstrap values (1000 replicates) and Bayesian posterior probabilities (>50%) are denoted above nodes. The geographic location is denoted with abbreviations for each of the following Brazilian states: PA, Pará; RO, Rondônia; GO, Goiás; PR, Paraná; AM, Amazonas; and AC, Acre. (gb), GenBank. Yellow bar refers to the timing of the uplift of the formation of the land bridge and timing of the entry of cervids into South America. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

tion, we cannot further determine the degree of introgression that has occurred between the different groups within *M. americana*. Detailed analysis of the entire *M. americana* species complex is needed to resolve this problem. Obtaining large numbers of samples from different localities and species is very difficult, and cytogenetic, molecular and morphological data are needed from a wide geographic area in order to clarify the complex systematic relationships within this group.

3.4. Comparisons among techniques

Our cytogenetic data showed high levels of intra- and inter-specific chromosomal variation particularly within the genus *Mazama*. Our mtDNA analyses demonstrated high levels of phylogenetic distinction between various forms of morphologically similar brocket deer, especially between the red and the gray brocket deer clades. This is an interesting example of morphological convergence between groups of cervids adapted to live in densely vegetated habitats. The gray brocket species generally occupy densely vegetated savanna environments whereas the red brocket species are restricted to dense rainforest habitats. The presence of spiked antlers in the brockets has been previously proposed as an adaptation to inhabit dense vegetation habitats (Groves and Grubb, 1990) and it is not surprising that this and other characters such as size and body shape may also be the result of morphological convergence. Our results suggest that cervid phylogenetic relationships based on external morphological characters such as pelage coloration and body size and shape are problematic because of extensive homoplasy associated with this type of evidence. Consequently, using external morphology (alone or in combination with molecular data) can be misleading when used to infer the phylogenetic relations of members of the genus *Mazama*. The cytogenetic and molecular approaches used here provide new insights into the evolutionary history of this interesting group of cervids.

In summary, the present study describes one of the most amazing cases of morphological convergent evolution and cryptic species system in mammals where brocket deer with very similar external morphologies showed high levels of molecular and cytogenetic diversification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.07.009.

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