



Phylogeny, Ratification of Recognized Taxa, and Determination of New Cryptic Taxa in Brocket Deer (Cervidae, Artiodactyla) using Mitochondrial DNA: a Surprising Genetic Diversity Revealed in Many New Possible Taxa

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Abstract

Brocket deer (Cervidae, Artiodactyla) constitute one of the most systematically complex groups of mammals due to their chromosomal fragility, which favors rapid speciation processes through reproductive isolation barriers, and because they represent a case of extreme morphological convergence in the mammalian world. In recent years, new genera have been distinguished in gray brocket deer (*Subulo*, *Passalites*, *Bisbalus*) and new genera and species in red brocket deer (*Andinocervus rufinus*, *Mazama reperticia*, *Mazama zetta*, *Mazama tshudii*, *Odocoileus pandora*). In the present study, we analyzed three databases with mitochondrial DNA sequences. In the first database (DB1), 157 Neotropical deer (including 120 brocket deer) were analyzed for two mitochondrial genes (mtND5 and mt12S rRNA); in the second database (DB2), 141 Neotropical deer (including 91 brocket deer) were analyzed for the mtCyt-b gene, and in the third database (DB3), 72 Neotropical deer (including 50 brocket deer) were studied for their complete mitogenomes. Some of the main results obtained are as follows: 1- The existence of the new brocket deer genera proposed in recent years (*Subulo*, *Passalites*, *Bisbalus*, *Andinocervus*) was confirmed; 2- The presence of *Subulo gouazoubira* in the Bolivian and Colombian Andes was confirmed (or at least, the presence of specimens carrying the mtDNA of *S. gouazoubira*); 3- The presence of a possible new taxon related to *S. gouazoubira* was detected in the Bolivian Amazon, and another possible new taxon related to *Subulo chunyi* was detected in the Bolivian Andes. 4- Within *Passalites nemorivagus*, at least two taxa were detected. Two specimens with phenotypes differentiated from those of *P. nemorivagus*, but related to this species, were also detected, one in the Colombian Amazon and the other on the Ecuadorian Pacific coast, which may constitute new taxa of gray brocket deer. 5- *Bisbalus citus* was originally located in Venezuela. Here we report the presence of *B. citus* along the northern coast of Colombia (Bolívar, Cesar, and Magdalena departments), thus

constituting the first record of this species for this country; 6- *Mazama rufina* (*Andinocervus rufinus*) showed that its original mitochondrial radiation occurred in the present-day Ecuadorian Andes, and there is possibility that at least two taxa exist within it; 7- The phylogenetic position of *Mazama* (*Odocoileus*) *pandora* varied depending on the database used and the different procedures employed, therefore studies with nuclear markers are required to precisely delimit its position; 8- It was confirmed that, as shown in a previous study, the *Mazama temama* complex is possibly composed of three different species. The origin of this complex was detected in the present-day Chocó region of Colombia. Also, for the first time, the presence of a taxon of the *M. temama* complex was detected molecularly in Ecuador; 9- Nevertheless, the *Mazama americana* complex presented the most complex taxonomic and systematic situation. Eight distinct groupings were detected within this complex (*M. americana* I, II-III, IV, V, VI, VII, and VIII), five of them new to science. Some of these contained significant subgroups that could constitute new brocket deer taxa (*M. americana* IV, V, and VII). 10- With all this, we detected and geographically indicated at least 16 groups of brocket deer that require karyotypic studies especially since they could constitute new genera, species and subspecies of brocket deer not yet determined by science.

Keywords: Andinocervus; Bisbalus; Brocket deer; Cervidae; Evolutionary Biology; *Mazama*; Miocene; mtCyt-b; mtND5 and mt12S rRNA; Mitogenomes; Neotropics; Passalites; Phylogeny; Phylogeography, Pleistocene; Pliocene; Subulo

Introduction

In recent decades, one of the families of medium to large sized to large mammals that has undergone the most taxonomic and systematic changes is the Cervidae. In fact, studies of the systematics of Neotropical deer have been highly controversial among different authors, with no consensus on how many genera or species may exist [1]. Among Neotropical deer, the most complex systematic approaches are found in the case of the brocket deer (which is even more complex than that of Neotropical squirrel species [2,3]; various species of the genus *Leopardus* [4]; and the Neotropical primate genera *Callicebus* [5,6] and *Aotus* [7,8]). This extreme taxonomic and systematic complexity of brocket deer is due to the fact that these species are considered to have one of the most impressive and surprising cases of morphological convergence among mammals [9-11]. Until recently, some mammalogists considered the brocket deer to be a single genus whereas others more recently considered it to consist of multiple genera and species across North, Central and South America [12].

The history of the brocket deer taxonomy and systematics is extremely long and complex. The first description of a New World brocket deer within Linnaeus's nomenclature (binomien) allocated a red brocket deer into the genus *Moschus* (*Moschus americanus* Erxleben 1777), a group that included other small ungulates, with similar morphological appearance of a small deer, already known to science at the time (chevrotains). A few years later, Kerr [13] described several species of brocket deer, placing them in the genus *Cervus*. Many of the new species' classifications and descriptions maintained the brocket deer within *Cervus* for many decades [14-16]. Rafinesque [17] was the first author who proposed exclusively a genus for the brocket

deer, *Mazama*. Hernández [18] named these deer as maçame, or teuthlamaçame, names which came from the Nahuatl word meaning deer for this Mexican pre-colombian culture. Rafinesque used the name *Mazama* three times in 1817. The first time (in September), the author considered the species *Mazama pita* (= *M. rufa*, Illiger 1815), and *Mazama bira* (= *S. gouazoubira* Fischer 1814). The second time (in October) he defined *Mazama pudu* (= *Pudu puda* Molina 1782), *Mazama orina*, and *Mazama caprina* (= *Antilocapra americana* Ord 1815). Finally, the third time (in November) he defined *Mazama tema* (= *M. temama* Kerr 1792, originally identified by the author as *Cervus temama*), and the mountain goats *Mazama dorsata* and *Mazama sericea* (= *Antilocapra americana* Ord 1815). The *M. rufa* type species was fixed subsequently by Merriam [19], who rescued the genus as the oldest available name for the brocket deer given in the September publication of 1817 made by Rafinesque. Therefore, *Mazama* was established as the traditional genus for brocket deer.

Until recently biologists considered that there were 10 species of brocket deer widely distributed throughout the neotropical region [20]. Nevertheless, there were many controversies among biologists about the number of species and subspecies that were within *Mazama*. For instance, Allen [21] proposed 18 species and 11 subspecies divided in two main groups, the red brocket deer (*M. americana*, *M. trinitatis*, *M. rufa*, *M. sheila*, *M. rufina*, *M. bricenii*, *M. sartorii*, *M. zetta*, *M. gualea*, *M. fuscata* and *M. zamora*), and the gray or brown brocket deer (*M. simplicicornis*, *M. murelia*, *M. tschudii*, *M. nemorivagus*, *M. superciliaris*, *M. cita* and *M. pandora*). Cabrera [22] reclassified the brocket deer into four species (*M. americana*, *M. gouazoubira*, *M. chunyi* and *M. rufina*) and 10 subspecies, which comprised a large portion of the full species identified by Allen [21]. Czernay

[23] defined six brocket deer species (*M. americana*, *M. gouazoubira*, *M. rufina*, *M. bricenii*, *M. chunyi* and *M. nana*) and seven subspecies. The latter species would replace the isolated population of *M. rufina* in southern Brazil. In fact, eight of the species under the red brocket deer complex (*M. americana*, *M. trinitatis*, *M. sheila*, *M. sartorii*, *M. zetta*, *M. gualea*, *M. fuscata* and *M. zamora*) defined by Allen [21] were condensed into one species composed of 9-15 subspecies by Cabrera [22] and Czernay [23]. Additionally, a morphological review of *M. americana* by Rossi [24] considered that it was only one species. Based on chromosomal differences, *M. temama* and *M. americana* were recognized as different species [1]. Nonetheless, Duarte [25] described another species of red brocket deer from the southern Mata Atlantica of Sao Paulo in Brazil. This captive animal from Capao Bonito was identified as *M. bororo* and had a chromosome number of $2n = 34$, four-five supernumerary chromosomes, and an FN of 46. In the same work, Duarte [25] described different diploid numbers within *M. americana* ($2n = 49$ to 68 chromosomes). These numbers were from his review of other studies [26-28]. The first chromosome description of *M. americana* was done by Taylor, et al. [26], who reported $2n = 68$ and FN = 74. Later, Jorge, et al. [27] analyzed three individuals of *M. americana temama* and determined that this Mexican, Centroamerican and northwestern Southamerican brocket deer had a diploid number of $2n = 50$ chromosomes and FN = 70. The X chromosome was submetacentric. The metacentric Y was the smallest chromosome. The important differences between these studies places doubt that both *Mazama* taxa were the same species. In the same sense, Neitzel [28] analyzed a Paraguayan female and described yet another pattern for an alleged *M. americana*, with $2n = 52$, plus four to five supernumerary chromosomes and FN = 56. The submetacentric X was the largest chromosome. Duarte [25] analyzed four Brazilian red brocket deer specimens, and found diploid numbers ranging from 48 to 54 chromosomes, and FN = 54 to 56, respectively. A few years later, Duarte, et al. showed, in seven specimens of *M. americana*, the existence of $2n = 48$ to 53 chromosomes, with an autosomal polymorphism in a large chromosome, with two to six supernumerary chromosomes and an FN equal to 46-57 as well as in 14 specimens of *M. gouazoubira*, the existence of $2n = 68$ -70, zero to three supernumerary chromosomes and FN = 70. Analysis of 28 *M. nana* specimens revealed the existence of $2n = 36$ to 40 chromosomes, one to seven supernumerary chromosomes and FN = 56 to 60, with the Y chromosome being metacentric and the smallest of the group. Two hybrids from *M. americana* and *M. nana* showed $2n = 45$ chromosomes, three to four supernumerary chromosomes and FN = 56. Medellín, et al. [30] differentiated *M. pandora* from *M. americana* in Mexico. Duarte [25,31], Duarte, et al. [32], and Duarte, et al. [33] recognized *M. bororo* as morphologically and karyotypically intermediate between *M. americana* and *M. nana*. With these new findings,

Duarte [34] recognized eight species of *Mazama*: *M. rufina*, *M. pandora*, *M. chunyi*, *M. americana*, *M. gouazoubira*, *M. nana*, *M. nemorivaga*, and *M. bororo*. Likely, this motivated Duarte, et al. [32] to claim that this wide chromosomal variation suggested the existence of several cryptic species under the *M. americana* morphological pattern.

Notwithstanding, the answer to the question about the number of subspecies within *M. americana* was more complex. Abril, et al. [10] and Cursino, et al. [11] determined in Brazil that *M. americana* was divided into six distinct cytotypes: Rondônia ($2n = 42\text{♀}$ -43♂ and FN = 49), Juína ($2n = 43$ -44♀/44-45♂+three to six complementary chromosomes and FN = 48), Jarí ($2n = 49\text{♂}$ and FN = 56), Carajás ($2n = 50\text{♀}$ -51♂ and FN = 54), Santarém ($2n = 51\text{♂}$ and FN = 56) and Paraná ($2n = 52\text{♀}$ -53♂ and FN = 56). These cytotypes were classified in two chromosome lineages. Lineage A included the Rondônia and Juína cytotypes, and Lineage B which comprised the Jarí, Carajás, Santarém, and Paraná cytotypes. Both lineages evolved from a common ancestor through different chromosomal rearrangements and showed a high level of chromosome differentiation suggesting, at least, the existence of two or more distinct species [10]. The karyotypes of these Brazilian *M. americana* (species complex) seem to be derived from a single common ancestral karyotype with $2n = 52/53$ + three to four supplementary chromosomes, XX/XY1Y2 and FN = 54, which arose after successive tandem fusions and an X-autosome fusion following the fixation of different rearrangements: i) Paraná cytotype, by a pericentric inversion; ii) Carajás cytotype, by a rearrangement of the Paraná cytotype with another tandem-fusion translocation; iii) Santarém cytotype, by a rearrangement of the Paraná cytotype with another centric-fusion translocation; iv) Jarí cytotype, by a rearrangement of the Santarém cytotype with another centric-fusion translocation; v) Juína cytotype, by a centric-fusion translocation and three tandem-fusion translocations; and vi) Rondônia cytotype, by a rearrangement of the Juína cytotype with another tandem fusion translocation [10]. Cursino, et al. [11] also showed that other red brocket deer that were different from the *M. americana* "sensu stricto" evolved by accumulating multiple centric and tandem fusions leading to distinct karyotypes. This was the case of *M. nana* with $2n = 36$ to 41 chromosomes and one to six complementary chromosomes and FN = 58, and independently *M. bororo* with $2n = 34$ chromosomes, four to five supplementary chromosomes and FN = 46. These authors reaffirmed that *M. temama* from Mexico was a different species from the Brazilian *M. americana* complex with a karyotype with $2n = 50$, XX/XY and FN = 72, facts that were supported by Grubb [35] and subsequent authors.

Fiorillo, et al. [22] showed that molecular analyses demonstrated that *M. nemorivaga* is a clearly a differentiated species within the gray brocket deer, although it was

traditionally classified as a subspecies of *M. gouazoubira* (*M. g. superciliaris*), being one of the most basal taxa in the evolution of the Odocoileinae. At that time, it was clear that morphology was not a useful tool for discrimination among species of brocket deer, especially from the red group, because there was a high degree of homoplasy and morphological convergence in the evolution of these deer [1,9,37], due to rapid diversification and morphological stasis [9,38], generating species complexes. In fact, some authors [10,11] affirmed for the case of *M. americana*, that it was a major challenge for today's science. For this reason, Cifuentes-Rincón [39] claimed that it was important to determine which taxa of the *M. americana* complex would correspond to the true *M. americana*. They considered that this information would be the basis for the identity of other *Mazama* species. As we aforementioned the holotype for *M. americana* was described by Erxleben [40] being the type locality Cayenne, in French Guiana. Notwithstanding, this holotype is lost. Henceforth, these authors proceeded to the description of a neotype from the same type locality, showing a karyotype of $2n = 45$ plus three complementary chromosomes and $FN = 51$. Meanwhile, the Brazilian specimens studied by Abril, et al. [10] and this neotype from French Guiana could not be differentiated by morphological analyses, they were, however, clearly differentiated by karyotypes as well as molecularly, corroborating the existence of a complex of cryptic species. This chromosomal variation produced reproductive isolation among these specimens due to sterility problems caused by chromosomal meiosis pairing between different chromosomal lineages [11,41,42]. Similarly, the Brazilian cytotypes from Lineage A located in the Brazilian western Amazon and those from Lineage B located in the eastern area of the Amazon and in the Atlantic Forest were different species inside the *M. americana* complex [11,42,43]. The last work designated a neotype for *M. rufa* and analyzed it as a possible distinct species from the *M. americana* complex. Morphological analyses from craniometry and body biometry indicated an overlap of quantitative measurements between *M. rufa* and the entire *M. americana* complex. The phylogenetic mtDNA analysis yielded a reciprocal monophyletic relationship between *M. rufa* and *M. americana*, both being identified as distinct molecular operational taxonomic units by the General Mixed Yule Coalescent species delimitation analysis. The karyotypic analysis showed *M. rufa* with a karyotype of $2n = 52$ chromosomes, and an FN of 56. Comparative analysis indicated that fifteen rearrangements separate *M. rufa* and *M. americana* (sensu stricto) karyotypes, which confirmed their substantial chromosomal divergence and probable reproduction isolation. Another relevant Brazilian study by Mantellato, et al. [44] showed, using sequences of mtCyt-*b*, that the holotype of *M. americana jucunda* was undifferentiated from the mtCyt-*b* sequences of *M. bororo*.

Therefore, the sequences of mtCyt-*b* were very informative for elucidating that *M. americana jucunda* represents the same taxon as *M. bororo*. In fact, it was suggested that the mtCyt-*b* gene has a high level of congruence within species boundaries, based on classical alpha-taxonomic studies.

We note that a major fraction of the quoted investigations was generated by Brazilian researchers. In Mexico, Escobar-Morales, et al. [46] analyzed molecularly *M. temama*, which was traditionally considered a subspecies of *M. americana* [47,48] and *M. pandora*. Originally, Merriam [49] described *M. pandora* as a new species differing in colour and body measures from *M. temama*, but the author was unable to determine its relationships with other brocket deer species. Later other authors Goldman EA, et al. [50,51] classified this taxon as a subspecies of the Central American *M. americana*, although it was previously classified as a subspecies of the gray brocket deer, *M. gouazoubira* [48]. As previously stated, *M. pandora* was elevated to full species status [30] based on cranial morphology (*M. pandora* is differentiable with respect to other two sympatric cervids, *M. temama* and *O. virginianus* because it has broader premaxillae with the posterior half of the nasals conspicuously humped in a lateral profile), antler characteristics, and skin coloration. Medellín, et al. [30] did not propose the existence of a phylogenetic relationship of this taxon with other brocket deer. The molecular analyses (mt genes *ND2*, *Cytb*, and control region) clearly suggested that both Mesoamerican brocket deer (*M. temama* and *M. pandora*) belonged to the red group and were more closely related to genus *Odocoileus* and to the other red brocket deer species but clearly differentiated from the gray brocket deer group [46].

This brings us to another transcendental question. The brocket deer really is a polyphyletic group. Smith, et al. [52] were the first to determine some evidence of the relationships of certain brocket deer with *Odocoileus*. These authors examined cranial measurements and genetic distances using isoenzymes of *O. virginianus* from southern USA and Suriname vs. *M. americana*, and *M. gouazoubira* (now *P. nemorivagus*) also from Surinam. They found a clear separation of both *M. americana* and *O. virginianus* with respect to the gray brocket deer. Additionally, *Odocoileus* from Surinam showed less similarity than expected with specimens of *O. virginianus* from the USA.

Characteristics, such as the supraorbital arches, frontal bone, space at the back of the frontal sutures, and the auditory bulla, are all larger in *Odocoileus* than in *Mazama*. Furthermore, there are obvious differences in the antlers of the males. A linear discriminant analysis, based on 31 variables, reclassified (probability of 100%) the 39 skulls into three "a priori" designated groups (*O. virginianus* from the southern

USA, *O. virginianus* from Suriname, and *M. americana* from Suriname). For example, based on 31 craniometric variables, one *M. americana* skull from Suriname was reclassified with a 100% probability to *O. virginianus* from Suriname. It was also reclassified with a 99.75% probability to *O. virginianus* from the southern USA, but not to the assemblage composed of gray brocket deer. In addition, the authors analyzed 19 biochemical loci, and found that the genetic distance between the two brocket deer species was 0.236, whereas the genetic distance was notably lower between *O. virginianus* from the southern USA and *M. americana* ($D = 0.148$), and between *O. virginianus* from Suriname and *M. americana* ($D = 0.063$). The distances between the two forms of *Odocoileus* and the gray brocket deer from Suriname were considerably greater ($D = 0.378$ and $D = 0.271$, respectively).

Gilbert, et al. [53] conducted the first molecular DNA study that showed the polyphyletic condition of the brocket deer in a chronological order. They analyzed two mt genes (*COII* and *Cyt-b*), and two nuclear introns (*PRKI* and α *LAlb*) of 25 species of cervids and found that *M. americana* and *M. gouazoubira* conformed in two well different and not reciprocally monophyletic lineages. Later, Ruiz-García, et al. [54], although the original manuscript was submitted to a journal in 2003 but the manuscript was lost for four years, using both mt control region (*D-loop*) and 16 microsatellites, also showed a polyphyletic relationship in the brocket deer. A sequence of mt*D-loop* from an individual identified as *M. americana* (this individual is actually considered *M. temama*) showed a striking relationship with several sequences of *O. virginianus* in contrast to what was expected. Nonetheless, some Bolivian *Mazama* sequences formed a clade with *Pudu puda* and *Ozotoceros bezoarticus* (the current Blastocerina clade). The analysis with DNA microsatellite loci also showed a higher genetic relationship between *M. americana* and *O. virginianus*, than between *M. americana* and *M. gouazoubira*. Duarte, et al. [9] examined the phylogenetic relationship among Neotropical cervids and confirmed the polyphyly of the brocket deer using a 934 base pair (bp) fragment of the mt*Cyt-b* gene. Hassanin, et al. [55] did the same but with complete mitogenomes. In fact, the genus *Mazama*, a polyphyletic group, is considered one of the most impressive and surprising cases of morphological convergence among mammals [9].

This necessarily introduced notable taxonomic changes, especially, in the gray brocket deer. Several authors [38,56,57] introduced the denominations subtribe *Odocoileina* (red clade) and subtribe *Blastocerina* (gray clade), which were considered more appropriate due to the many exceptions of coat color found within these groups. The subtribe *Odocoileina* contained species from tropical, subtropical, and temperate regions of the Americas whereas the subtribe *Blastocerina* contained species solely from subtropical and

tropical regions of South America [38,56,57]. Because *M. americana* is the type species of the genus and following the International Code of Zoological Nomenclature (ICZN), the genus *Mazama* should be maintained for the related taxa [38]. For this reason, the molecular and karyotype study of *M. americana* [39], was the fundamental starting point for nomenclatural changes to the genus. Taking this into account, it is obvious that the genus *Mazama* should not be maintained for taxa belonging to the subtribe *Blastocerina* which must be considered as distinct genera [38,39]. Additionally, diverse mitochondrial phylogenies demonstrated that *M. gouazoubira* and *M. nemorivaga* did not share a common ancestor and were not closely related [38,57]. Therefore, two new genera were proposed, one for the lineage that includes *M. gouazoubira*, and another for *M. nemorivaga*. Bernegossi, et al. [58] analyzed the case of *M. gouazoubira* characterizing specimens collected close to the original type locality (topotypes). The morphometric analysis did not discriminate the individuals of *M. gouazoubira*, including these topotypes, from other brocket deer species. Nonetheless, a Bayesian inference (BI) tree, using mitogenomes, confirmed the polyphyly of the genus *Mazama* and supported the need to change the genus name for gray brocket deer. These authors validated the genus *Subulo*, and fixed a type species for the genus. In the absence of the holotype, the authors denominated a neotype with a male topotype sampled in Paraguay. *Subulo* is the oldest available generic name applied to the deer currently placed into *Mazama*. *Subulo* was proposed as a subgenus of the genus *Cervus*. According to the priority principle, *Subulo* became a junior synonym of *Mazama* and the second oldest generic name available for the morphotype of the brocket deer. As Smith [59] did not designate a type species for this genus, Bernegossi, et al. [58] proposed *Cervus (Subulo) simplicicornis* as the type species for *Subulo*, and updated the nomenclature for the gray brocket deer to *Subulo gouazoubira*.

Also, *M. nemorivaga*, a gray brocket deer from the Amazon basin, had a nomenclatural genus change. Morales-Donoso, et al. [60] analyzed morphological, cytogenetic, and molecular phylogenetic data (mt*Cyt-b*, mt*COII*, and mt*D-loop* markers) of a neotype specimen of *M. nemorivaga* from its type locality (French Guiana). The morphological and cytogenetic differences between this and other Neotropical Cervidae confirmed this taxon as a unique and valid species. The phylogenetic analysis showed the basal position of *M. nemorivaga* within the *Blastocerina* clade. This taxon showed early diversification and wide divergence from the other species, suggesting that the taxon should be transferred to a different genus. Gloger [61] proposed the genus *Passalites* with *P. nemorivagus* as the only species, being the type species of *Passalites*, *Cervus nemorivagus*, by monotypy. Thus, Morales-Donoso, et al. [60] used *P. nemorivagus* as a new and correct nomenclature combination.

Sandoval, et al. [62] clarified the taxonomy and phylogenetics of *M. americana citus*. They sampled a topotype of *M. a. citus* from the eastern side of Lake Maracaibo (Zulia state, Venezuela) and characterized this specimen morphological, cytogenetical, and molecularly with a phylogenetic analysis using complete mitogenomes. The results showed the topotype to be a large gray brocket with a cinnamon band above the eyes and a karyotype of $2n = 61$ and $FN = 70$. Its karyotype differed in at least 10 rearrangements from that of *S. gouazoubira*. A BI tree recovered *M. americana citus* within the Blastocercina subtribe, separated phylogenetically from other gray brockets (100% branch value), revealing that this gray brocket to be a valid species that should be assigned to a new genus. Sandoval, et al. [62] assigned the generic names *Bisbalus*, and *Bisbalus citus* as the type species for this taxon.

On the other hand, some authors have suggested that *Mamaza pandora* could be a third species of *Odocoileus* (*Odocoileus pandora*). The first to suggest the inclusion of *pandora* in the genus *Odocoileus* were Gutiérrez, et al. [38]. Subsequently, the phylogenetic relationship between *M. pandora* and *Odocoileus* was further confirmed in a more informative analysis based on mitogenomes [62,63], suggesting the adoption of *Odocoileus pandora*.

Recently, three other studies have appeared that have contributed new taxonomic and systematic restructurings in the world of brocket deer. Escobedo-Morales, et al. [64] used genome-wide ultraconserved elements (UCE) and mitogenomes to confirm the differentiation between Mexican and Colombian specimens (they only used one of this last nationality, previously analyzed by us), in addition to differentiating within *temama* a group from southern Central America (from Nicaragua to Panama). These three groupings have been given the status of full species: *Mazama temama* sensu stricto (Mexico to Honduras), *Mazama reperticia* (Nicaragua to Panama), and *Mazama zetta* (Colombia). They also commented that this species is distributed throughout Ecuador; but no sample from that country was analyzed by them, so, based on that work, it was deduced that this hypothesis is still to be confirmed. Ramírez-Chaves, et al. [65] analyzed data from a mitogenome of *M. rufina* present in GenBank (along with mitogenomes of other Neotropical deer species, all obtained from GenBank) and from five specimens of *M. rufina*, analyzed directly by them, of Colombian origin (all from the department of Caldas), sequenced for the mtCyt-*b* gene, and compared with nine other specimens of *M. rufina* and other Neotropical deer species whose mtCyt-*b* gene sequences were extracted from GenBank. Both analyses showed that *M. rufina* was strongly differentiated from other red brocket deer taxa, and the taxon was redefined as a new genus, *Andinocervus rufinus*.

Sandoval, et al. [66] reported a supposed new species of brocket deer from La Ramada, Lambayeque department west of the Peruvian Andes. The taxon was named *Mazama tschudii*. When its karyotype was compared to other brocket species, this taxon exhibits a unique karyotypic pattern that distinguishes it from *S. gouazoubira* and *P. nemorivagus*, as well as from *M. americana*, *M. rufa*, *M. jucunda* and *M. nana*. Notwithstanding, the karyotype of the collected specimen of *M. tschudii* ($2n = 42$, $FN = 68$) closely resembles that of the *M. temama* neotype ($2n = 44$, $FN = 70$) [67], based on classical staining. Nonetheless, C-banding of *M. tschudii* revealed additional interstitial heterochromatic bands on the q-arms of one chromosome pair that are not present in the *M. temama* neotype. Comparative analyses using BAC-FISH probes further distinguished the two species: the bi-armed chromosomes in *M. tschudii* differ in structure and composition from those in *M. temama*. *M. tschudii* exhibits eleven centric and three tandem fusions not observed in *M. temama*. The phylogenetic analysis of the complete mitogenome of *M. tschudii* positioned this taxon within the Odocoileina subtribe, alongside other *Mazama* species. It is worth noting, however, that this specimen ended up on a branch of the phylogenetic tree along with a Colombian *M. temama* that we sampled in San Francisco (Huila department) many years ago, which, in turn, forms the sister group to the Mexican specimens of *M. temama*. This makes us have some doubts that *M. tschudii* is really a species not yet reported, as we will discuss later.

Therefore, the analysis of new chromosomal and molecular data of brocket deer collected from different geographical regions of the Neotropics will likely reveal an enormous amount of cryptic genetic diversity not yet revealed. To help unravel the enormous systematic and evolutionary complexity that brocket deer must exhibit outside of Brazil and Mexico (where most karyotypic and molecular studies have been primarily conducted), we analyzed three molecular datasets with different brocket deer specimens and taxa.

In the first dataset (BD1), we used two small fragments of the mitochondrial genes *ND5* and *12S rRNA*, which have been characterized, the first with high nucleotide change rates per million years [68] and the second with considerably lower nucleotide change rates per million years [69]. The combined analysis of these two fragments can, to some extent, represent the average evolution of mitochondrial DNA. These two fragments are indicated for DNA amplification from very small samples (two or three hairs, a minimal portion of bone, a drop of saliva) that likely contain a minimal amount of low-quality DNA. A significant portion of the samples obtained from brocket deer in the last 25 years were of this nature. Therefore, it was important to be able to obtain sequences

from many brocket deer specimens from different geographic origins, from which it would be more complex to obtain larger sequences or complete mitogenomes. For this first dataset, the sequences of 120 brocket deer were analyzed (104 obtained for this study and 16 obtained from GenBank).

For the second dataset (BD2), complete sequences of the mt*Cyt-b* gene were obtained. As previously mentioned, this gene has a high capacity to distinguish species based on classic alpha taxonomy studies [45]. Additionally, this mt gene has been used to determine subspecies and different species in mammals. Bradley, et al. [70] claimed that values < 2% would indicate intraspecific variation, values between 2% and 11% could correspond to different subspecies or species (requiring further studies to determine different species), and values > 11% would be indicative of clear species recognition. For this dataset, sequences were obtained from 91 brocket deer (56 obtained for this study and 35 obtained from GenBank).

For the third dataset (BD3), complete mitogenomes were obtained. Obtaining complete mitogenomes is important because they offer the maximum possible information with this type of DNA, which has significant advantages for reconstructing phylogenetic relationships between relatively closely related species or taxa. Mitogenomes are interesting for phylogenetic research because they exhibit rapid mutation accumulation, rapid coalescence time, lack introns, have a high number of copies per cell, a negligible recombination rate, and exhibit haploid inheritance [71]. Despite representing a single linked locus, selection pressures and evolutionary rates are highly heterogeneous across mitochondrial DNA [72, 73]. For all these reasons, mitogenomes are more precise in reconstructing the divergence history among closely related species, or within species, than other molecular markers [74]. For this third dataset, the complete mitogenomes of 50 brocket deer were analyzed (37 obtained for this study and 13 obtained from GenBank). Therefore, this work presents the largest number of results obtained with mitochondrial DNA in brocket deer to date in an attempt to elucidate their complex systematics and their intriguing evolutionary history.

With all of the above in mind, the main objectives of this study are: 1- To confirm whether the new genera of brocket deer proposed in recent years are discernible using the three generated mitochondrial databases; 2- To establish how many genetically differentiated groupings are found within each of the recognized brocket deer species, especially in the *M. americana* complex, using these three databases; 3- To detect possible new taxa, or species, not previously recognized within the brocket deer and which would warrant a karyotype study to determine their possible reproductive isolation from other similar taxa; 4- To

determine the phylogenetic relationships between brocket deer genera and species and other genera of Neotropical deer (*Odocoileus*, *Blastoceros*, *Ozotoceros*, *Hippocamelus*, *Pudu*, and *Pudella*), especially using the first two databases and exclusively using brocket deer and *Odocoileus* for the third database; 5- Estimate the separation times between different brocket deer taxa and how long ago the proliferation of haplotypes began within each studied brocket deer taxon using the three databases; 6- Estimate the degree of genetic heterogeneity among all recognized brocket deer taxa using the three databases, especially among the different red brocket deer taxa and among the different gray brocket deer taxa; 7- Estimate the levels of genetic diversity within each analyzed brocket deer taxon for the first two databases; and 8- Determine possible demographic changes throughout the natural history of each analyzed brocket deer taxon and establish a possible relationship with different types of geological and climatological changes during the Pliocene and Pleistocene.

Materials and Methods

Samples Analyzed

Over the past 28 years, various tissues from brocket deer and other deer species have been collected in different areas of the Neotropics. Most of the samples came from hunters' trophies (hair, small pieces of skins, antler fragments, or turbinate bone fragments) or from bushmeat consumed by locals in their respective geographic areas where the animals were hunted (muscle). A small fraction of the samples originated from museum collections in Colombia, Ecuador, and Peru (Alexander von Humboldt Institute in Villa de Leyva, Colombia; Mammalogical Collection of the National Institute of Biodiversity of Ecuador, INABIO, in Quito, Ecuador; "Gustavo Orces V" Natural History Museum of the National Polytechnic School of Quito, Ecuador; Museum of the Bolivar National College in Ambato, Ecuador; Natural History Museum of the National University of San Agustín in Arequipa, Peru; and Biological Collection of the Catholic University Santa María, Arequipa, Peru).

For BD1, sequences from the following samples were analyzed: 38 specimens of *M. americana* or very close taxa (five from GenBank), eight specimens of *M. temama* (two from GenBank), four specimens of *M. pandora* (two from GenBank), 30 specimens of *M. rufina*, seven specimens of *B. citus* (one from GenBank), nine specimens of *P. nemorivagus* (one from GenBank), two specimens of *S. chunyi*, 16 specimens of *S. gouzaoubira* (four from GenBank), and six specimens classified "a priori" as *Mazama* sp., for a total of 120 analyzed sequences of brocket deer. In this dataset, the following specimens were also studied comparatively: 24 specimens of *Odocoileus virginianus* (four from GenBank),

two specimens of *Ozotoceros bezoarticus* (one from GenBank), four specimens of *Pudella* (probably two of *P. mephistophila* and two of *Pudella carlae*), two specimens of *Blastoceros dichotomus*, two specimens of *Pudu puda*, and three specimens of *Hippocamelus antisensis*.

For BD2, sequences from the following samples were analyzed: 39 specimens of *M. americana* or very closely related taxa (14 from GenBank), six specimens of *M. temama* (5 from GenBank), three specimens of *M. pandora* (all three from GenBank), 18 specimens of *M. rufina* (three from GenBank), eight specimens of *P. nemorivagus* (two from GenBank), three specimens of *B. citus* (one from GenBank), two specimens of *S. chunyi* (both from GenBank), and 11 specimens of *S. gouazoubira* (five from GenBank), for a total of 91 sequences of brocket deer. Together, two *Odocoileus hemionus* (both from GenBank), 21 *O. virginianus* (three from GenBank), seven specimens of *Pudella* (five from GenBank),

five specimens of *P. puda* (four from GenBank), five specimens of *H. antisensis*, three specimens of *Hippocamelus bisulcus* (all from GenBank), four specimens of *B. dichotomus* (one from GenBank), and three specimens of *O. bezoarticus* were analyzed.

For BD3, the following specimens were analyzed: 24 specimens of *M. americana* or closely related taxa (five from GenBank), two specimens of *M. pandora* (both from GenBank), three specimens of *M. temama* (two from GenBank), five specimens of *M. rufina*, three specimens of *B. citus* (one from GenBank), six specimens of *P. nemorivagus*, six specimens of *S. gouazoubira* (three from GenBank), and one *Mazama* sp., for a total of 50 mitogenomes analyzed for the brocket deer. Comparatively, one *O. hemionus* (from GenBank) and 21 specimens of *O. virginianus* (two from GenBank) were analyzed. The precise geographic locations of the analyzed specimens can be seen in Table 1.

Location	BD1 (NS)	BD2 (NS)	BD3 (NS)
Species	120	91	50
<i>Subulo gouazoubira</i>	16	11	6
New specimens	12	6	3
Colombia	(n = 2)	(n = 1)	(n = 1)
Huila department	1	1	1
Norte de Santander department (Toledo, PNN Tamá)	1	-	-
Bolivia	(n = 3)	(n = 2)	-
Beni department (Mamoré River)	2	1	-
Santa Cruz department	1	-	-
La Paz department (Yungas)	-	1	-
Paraguay	(n = 3)	(n = 1)	-
Alto Paraguay department (Agua dulce)	2	1	-
Unknown place	1	-	-
Argentina	(n = 3)	(n = 2)	(n = 2)
Chaco province (Saenz Peña)	1	-	-
Tucumán province (San Pedro Colalao, El Paraíso)	2	2	2
Uruguay	(n = 1)	-	-
Durazno department	1	-	-
GenBank specimens	4	5	3
Paraguay	(n = 2); MZ350858; MZ350866	(n = 1); MZ350858	(n = 1); MZ350858
Brazil	(n = 2); KJ772514; MZ350862	(n = 2); KJ772514; MZ350862	(n = 2); KJ772514, MZ350862
Uruguay	-	(n = 2); DQ789183; DQ789186	-
<i>Subulo chunyi</i>	2	2	-
New specimens	2	-	-

Peru	(n = 2)	-	-
Cuzco department (Marcapata)	2	-	-
GenBank specimens	-	2	-
Peru	-	(n = 2)	-
<i>Passalites nemorivagus</i>	9	8	6
New specimens	8	6	6
Colombia	(n = 1)	(n = 1)	(n = 1)
Putumayo department (VillaGarzón)	1	1	1
French Guiana	(n = 1)	(n = 1)	(n = 1)
Cayenne district	1	1	1
Peru	(n = 3)	(n = 3)	(n = 3)
Loreto department (Nanay River, Nauta)	3	3	3
Brazil	(n = 3)	(n = 1)	(n = 1)
Amazonas state (Madeira and Negro rivers)	3	1	1
GeneBank specimens	1	2	-
Brazil	(n = 1); MZ350867	(n = 1); KY928657	-
Guyana	-	(n = 1); MT008225	-
<i>Bisbalus citus</i>	7	3	3
New specimens	6	2	2
Colombia	(n = 5)	(n = 2)	(n = 2)
Bolivar department (San Jacinto, El Carmen)	2	-	-
Cesar department (Valledupar, Chiriguana)	2	1	1
Magdalena department (PNN Tayrona)	1	1	1
Venezuela	(n = 1)	-	-
Zulia state (Matacán)	1	-	-
GeneBank specimens	1	1	1
Venezuela	(n = 1); OQ198443	(n = 1); OQ198443	(n = 1); OQ198443
<i>Mazama (= Andinocervus) rufina</i>	30	18	5
New specimens	30	15	5
Colombia	(n = 18)	(n = 9)	(n = 4)
Cundinamarca department (PNN Chingaza)	7	5	2
Boyacá department (PN Guanenta, Miraflores)	2	1	1
Norte de Santander department (PNN Tamá)	2	-	-
Risaralda department (Santa Rosa del Cabal)	2	1	1
Tolima department (Toche, Alto la Línea)	2	1	-
Valle del Cauca department (Florida)	1	-	-
Cauca department (Moras)	1	1	-
Putumayo department (Puerto Asis)	1	-	-
Ecuador	(n = 12)	(n = 6)	(n = 1)
Tungurahua province (Ambato)	3	1	-
Cotopaxi province (SJose de Sigchos, PN Llanganates)	2	1	-
Azuay province (PN Cajas)	1	-	-
Loja province	1	-	-
Zamora-Chinchipe province (PN Podocarpus)	1	1	1

Carchi province (La Esperanza)	1	1	-
Napo province	-	1	-
Unknown place	3	1	-
GenBank specimens	-	3	-
Ecuador	-	(n = 1); KR107039	-
Venezuela	-	(n = 2); LT546656, LT546568	-
<i>Mazama pandora</i>	4	3	2
New specimens	2	-	-
Guatemala	(n = 2)	-	-
Petén department	2	-	-
GenBank specimens	2	3	2
Mexico	(n = 2); OQ731408, OQ731409	(n = 3); OQ731408, OQ731409, KY928657	(n = 2); OQ731408, OQ731409
<i>Mazama temama</i>	8	6	3
New specimens	6	1	1
Colombia	(n = 5)	(n = 1)	(n = 1)
Chocó department (RioSucio, Serrania del Baudó)	3	-	-
Huila department (San Francisco)	2	1	1
Ecuador	(n = 1)	-	-
Pichincha province	1	-	-
GenBank specimens	2	5	2
Mexico	(n = 2); MZ362858, MZ350864	(n = 4); MZ362858, MZ350863; KC146966, KC146967	(n = 2); MZ362858, MZ350864
Colombia	-	(n = 1); HW880928	-
<i>Mazama rufa</i>	2	2	1
New specimens	1	-	-
Paraguay	(n = 1)	-	-
Canindeyú department	1	-	-
GenBank specimens	1	2	1
Brazil	(n = 1); OQ198444	(n = 2); OQ198444, DQ789201	(n = 1); OQ198444
<i>Mazama nana</i>	1	1	1
GenBank specimens	1	1	1
Paraguay	(n = 1); MZ350863	(n = 1); MZ350863	(n = 1); MZ350863
<i>Mazama jucunda</i>	1	4	1
GenBank specimens	1	4	1
Brazil	(n = 1); MZ350859	(n = 4); MZ350859; DQ789187, DQ789212; DQ789231	(n = 1); MZ350859
<i>Mazama americana</i>	34	32	22
New specimens	32	25	21
Colombia	(n = 4)	(n = 2)	(n = 2)
Cauca department (Santa Rosa)	1	1	1
Putumayo department (Puerto Asís)	1	-	-

Amazonas department (Puerto Rastrojo, Puerto Restrepo)	2	1	1
Peru	(n = 18)	(n = 17)	(n = 13)
Loreto department	9	10	8
Ucayali department	6	5	3
Madre de Dios department	1	1	1
Huánuco department (Tingo María)	1	-	-
San Martín department (Lamas)	1	1	1
Ecuador	(n = 1)	(n = 1)	(n = 1)
Orellana province (Napo River)	1	1	1
French Guiana	(n = 1)	(n = 1)	-
Cayenne district	1	1	-
Trinidad & Tobago	(n = 2)	(n = 1)	(n = 1)
Río Claro-Mayaro region	2	1	1
Brazil	(n = 4)	-	-
Amazonas state (Yavarí, Aripuaná, Amazonas rivers)	3	-	-
Pará state	1	-	-
Bolivia	(n = 2)	(n = 3)	(n = 2)
Beni department (Beni and Mamoré rivers)	2	2	2
La Paz department (Yungas)	-	1	-
GenBank specimens	2	7	2
French Guiana	(n = 1); MZ350857	(n = 2); MZ350857, MN726911	(n = 1); MZ350857
Brazil	(n = 1); MZ350356	(n = 3); MZ350356, DQ789211, DQ789219	(n = 1); MZ350356
Peru	-	(n = 1); ON721322	-
Venezuela	-	(n = 1); KY928654	-
<i>Mazama sp.</i>	6	1	1
New specimens	6	1	1
Colombia	(n = 2)	-	-
Bolívar department (El Guamo)	1	-	-
Guainía department (Inírida River)	1	-	-
Venezuela	(n = 1)	-	-
Zulia state	1	-	-
Ecuador	(n = 1)	-	-
Manabí province (Puerto López)	1	-	-
Bolivia	(n = 2)	(n = 1)	(n = 1)
Beni department (Exaltación, Mamoré River)	1	1	1
La Paz department	1	-	-

Table 1: Sources of the brocket deer species collected and analyzed at the mitochondrial (mt) *ND5* and *12S rRNA* genes (BD1), at the *mtCyt-b* gene (BD2) and at the whole mitogenomes (BD3). NS = Sample sizes at each one of the three databases (BD1, BD2, and BD3).

Laboratory Procedures with Mitochondrial DNA

We extracted and isolated DNA and sequenced BD1, 2, 3 from skin, muscle, bone, and hair samples from animals hunted and consumed by rural communities (the major fraction of the samples) and from donations from museums using the NX-48S Tissue DNA kit (NexttractorR NX-48S; Genolution, Seoul, Republic of Korea). The primers used to amplify BD1 were for the mtND5 gene, as follows: L12673: 5'-GGTGCAACTCCAAATAAAAGTA-3', and H12977: 5'-AGAATTCTATGATGG ATCATGT 3' [75]. The primers to amplify the mt12SrRNA gene were L1091: 5'-AAAAAGCTTCAAAGTGGGATTAGATACCCCACTAT-3'; H1478: 5'-TGACTGCAGAGGGTGACGGGCGGTGTGT-3' [76-78]. The total length of the analyzed sequences for BD1 was 608 base pairs (bp).

Following the protocol and primers used by Randi, et al. [79], we amplified 1,140 bp of the mtCyt-b gene (BD2), using MH104 and ML103 primers (5'-TTGTTCTTCATCTCTGGTTTACAAGAC-3' and 5'-GACTAATGATATGAAAAACCATCGTTG-3', respectively). Both mtDNA strands were sequenced directly using BigDye Terminator v3.1 (Applied Biosystems, Inc., Foster City, CA, USA). Sequencing products were analyzed on an ABI3730 DNA Analyzer system (Applied Biosystems, Inc., Foster City, CA, USA).

For the mitogenomes (BD3), only the mtDNA of 37 brocket deer specimens and 19 white-tailed deer specimens were of sufficient quality to be used in Illumina procedures. Standard Illumina fragment libraries (the dual-indexed libraries for Illumina sequencing were obtained using a SureSelectXT library preparation kit [Agilent Technologies, Santa Clara, CA, USA] with Nextera-style indices; 250-280-bp paired-end reads) were prepared for these high quality mtDNAs. They were sequenced to ~0.3× genome-wide depth of coverage on the Illumina HiSeq 2000 platform. We generated the 56 mitochondrial genome assemblies with SOAPdenovo2 v2.4.0 software [80] and evaluated a series of k-mer sizes. Additionally, we used SPAdes v3.0 to compare assemblies [81]. All raw Illumina reads were mapped to assembled contigs using BWA-MEM v2.0 [82] to assess coverage depth. Mapping results were analyzed using SAMtools v1.22 software [83], PCR duplicates were removed, and MitoFinder v1.4 [84] was used with SPAdes v3.0 [81] for assembly. Mitochondrial contigs were identified with ElasticBLAST v1.4.0 software [85] and mitogenomes were annotated with the MitoFinder and MITOS2 webserver (<http://mitos.bioinf.uni-leipzig.de/index.py>), and compared with reference mitogenomes from GenBank of two brocket deer (MZ350356 and MZ350857) and of two white-tailed deer (KM612278 and KY928667), and confirmed by read-

depth statistics. Overlapping regions were examined for irregularities such as frameshift mutations and premature stop codons with the mitogenomes of reference by using the UGENE v39.0 software [86]. A lack of such irregularities indicates an absence of contaminating numt sequences. Base frequencies, amount of missing data, and the length of the final mitogenomes were checked with a custom Python script (scikit-bio v0.5.6). The mitogenome lengths were, on average, around 16,420 bp and AT-biased. They consisted of 13 protein-coding genes, two ribosomal RNA genes, 22 transfer RNA genes, and one non-coding control region. Illumina short reads for skin, and muscle samples (around 50 million reads) were of better quality than those for hair, and bone samples (around seven million reads).

Phylogenetic and Population Genetic Analyses

Phylogenetic Trees, BAPS analyses, and Divergence Times

jModeltest v2.0 [87], Kakusan4 [88], and MEGA X v10.0.5 [89] software were applied to determine the best evolutionary mutation model for the analyzed sequences of BD1, BD2 and BD3 (including for different mt gene partitions in the last case). The Bayesian information criterion (BIC) [90], the Akaike information criterion (AIC) [91], and the maximum likelihood criterion (lnL) [92] were used to determine the best evolutionary model for the relationships among the neotropical deer taxa studied. At BD1, BIC showed that the best nucleotide substitution model was TN93 + G (BIC = 14.138,46), while for AIC and lnL the best nucleotide substitution model was GTR + G + I (11.376, 64 and -5.390,31, respectively). At BD2, BIC and AIC showed that the best nucleotide substitution model was TN93 + G (16.237,03 and 13.549,87, respectively), whereas for the lnL criteria was GTR + G (lnL = -6.487,78). At BD3, BIC showed that the best nucleotide substitution model was TN93 + G + I (BIC = 280.009,37), whereas for the AIC and the lnL criteria, the best model was GTR + G + I (265.357,14 and -131.146,83, respectively). Therefore, the nucleotide substitution model for the phylogenetic trees obtained with BD1 and BD3 was GTR + G + I, meanwhile for BD2, the model used was TN93 + G.

We constructed six mitochondrial phylogenies for aligned BD1, BD2 and BD3 with maximum likelihood (ML) and Bayesian Inference (BI) procedures. For the ML tree, we used RAxML v8.2.12 software [93]. RAxML was run with the GTRGAMMA model of nucleotide evolution and default parameters, and 1,000 bootstrap replicates to calculate node support for the three datasets. The degree of support received by individual nodes in the ML bootstrap analysis

was categorized as follows: strong if bootstrap value > 75%; moderate if bootstrap value > 50% and < 75%; negligible if value < 50% [38].

BI trees were completed with the BEAST v. 1.10.4 software [94] for the three sequence datasets. Four independent iterations were run using three data partitions (codon 1, codon 2, and codon 3) with six MCMC chains sampled every 10,000 generations for 40 million generations after a burn-in period of 4 million generations. We checked for convergence using Tracer v1.7.2 [95]. We plotted the likelihood versus generation and estimated the effective sample size (ESS >200) of all parameters across the four independent analyses to determine convergence and optimal results. The results from different runs were combined using LogCombiner v1.10.4 software [96] and TreeAnnotator v1.10.4 [97] was used to determine the best trees. A Yule speciation model and a relaxed molecular clock with an uncorrelated log-normal rate of distribution were used [98]. Posterior probability (*pp*) values provide an assessment of the degree of support of each node on the tree. *pp* values greater than 0.90 were considered highly significant. Trees were visualized in FigTree v. 1.4.4 software [99].

These BI trees, obtained with the BEAST v. 1.10.4 software, were used to estimate the time to most recent common ancestor (TMRCA) for the different nodes found in these trees. We used a prior of 5 million years ago (mya) for the initial node of divergence between the Neotropical deer [9].

Additionally, to confirm, or not, many of the results obtained with the phylogenetic trees, we conducted a Bayesian Analysis of Population Structure (BAPS) with the software BAPS v6.0 [100], using mixed and unmixed models to group genetically similar individuals into panmictic genetic clusters. Calculations were performed, with the number of *k* clusters varying from 2 to 20. Five replicates were carried out for each *k* value. The BAPS analyses were conducted with the three molecular datasets.

Genetic Heterogeneity Statistics Estimated for the Brocket Deer Taxa

For the three molecular datasets, we estimated the following statistical heterogeneity indices for different groups of red and gray brocket deer analyzed: table of contingency, H_{ST} , K_{ST} , K_{ST}^* , γ_{ST} , N_{ST} , and F_{ST} [101]. Indirect gene-flow estimates were obtained assuming an infinite island model [102]. Significance was estimated with permutation tests using 10,000 replicates. We also estimated genetic heterogeneity between brocket deer group pairs.

For this task, we used exact probability tests with Markov chains, using 10,000 dememorization parameters, 20 batches, and 5000 iterations per batch. All the heterogeneity statistics were calculated with the DNAsp v6.1 and Arlequin v3.5.1.2 software [103,104]. We also used the absolute genetic distance [Da] to determine the percentage of genetic differences among the different brocket deer taxa.

Genetic Diversity Statistics and Possible Demographic Changes in the Brocket Deer Taxa Using the *mtND5* and *mt12S rRNA* Gene Dataset and the *mtCyt-b* Gene Dataset

The following genetic diversity statistics were applied to BD1 and BD2 to determine the genetic diversity for the different brocket deer taxa studied: number of haplotypes (*H*), haplotype diversity (H_d), nucleotide diversity (π), and θ statistic by sequence. These genetic diversity statistics were calculated with the DNAsp 6.1 and Arlequin 3.5.1.2 software [103,104].

To estimate possible demographic changes for all the groups considered of brocket deer at BD1 and BD2, we used Fu and Li's D^* and F^* tests [105], Fu's F_s statistic [106], Tajima's D test [107], and R_2 statistic [108]. Ninety-five percent confidence intervals and probabilities were obtained with 10,000 coalescence permutations. Also, a mismatch distribution (pairwise sequence differences) was obtained [109]. We used raggedness (*rg*) to determine the similarity between the observed and theoretical curves. These demographic analyses were also carried out using the software DNAspv6.1 and Arlequin v3.5.1.2 software [103,104]. Based on the mismatch distribution analysis, it is possible to estimate how long ago a possible population expansion began. The following expression was used for this purpose: $\tau = 2\mu t$, where τ is a parameter calculated by the DNAsp 6.1 software, μ is the mutation rate per million years for the genes analyzed [0.69 %/million of year for BD1 (average of 1.25%/million years for the *mtND5* gene and 0.13%/million years for the *mt12S rRNA* gene, being these specific values for Cervidae, [39,110]) and 1.15%/million years for BD2 [53]) and *t* is the time in generations that have passed since the demographic change began [109]. A generation in the case of brocket deer ranges from 7-8 months to two years of life [111]. In this case, we opted to use one year as the generation period for these deer.

Additionally, we used the coalescence-based Bayesian Skyline Plot (BSP) to determine possible demographic changes in the evolution of different brocket deer taxa and in what time these changes occurred. BSP analysis was performed in BEAST v2.4.3 using the empirical base

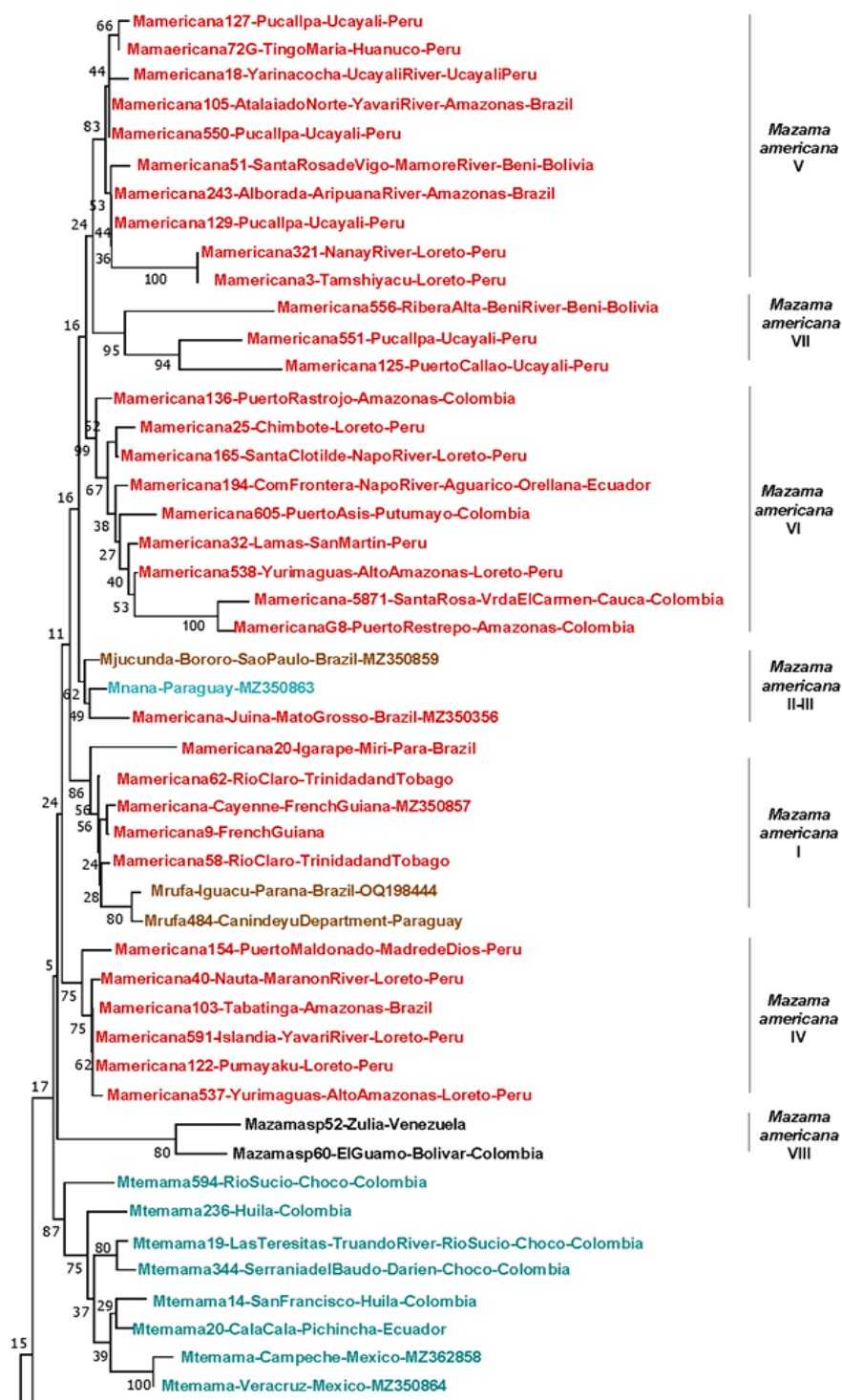
frequencies and a strict molecular clock [112]. We assumed a substitution rate of 7×10^{-8} substitutions per site and per year to obtain the time estimates in years as well as with kappa with log-normal (1, 1.25), and a skyline population size with uniform (0, infinite; initial value 80). We conducted a total of five independent runs of 40 million Markov chain Monte Carlo (MCMC) iterations following a burn-in of 10% of iterations, logging every 10,000 iterations. We selected a stepwise (constant) Bayesian skyline variant with maximum time as the upper 95% high posterior density (HPD). The total height of the tree was determined by treeModel.rootHeight. The convergence of the analysis was assessed by checking the consistency of the results over five independent runs. For each run, we used the software Tracer v1.7 to inspect the trace plots for each parameter to assess the mixing properties of the MCMCs and to estimate the effective sample size (ESS) value for each parameter. Runs were considered as having converged if they displayed good mixing properties and if the ESS values for all parameters were greater than 200. We discarded the first 10% of the MCMC steps as a burn in and obtained the marginal posterior parameter distributions from the remaining steps using Tracer v1.7. To test whether the inferred changes of effective numbers over time were significantly different from a constant population size null hypothesis, we compared the BSP obtained with the 'Coalescent Constant Population' model (CONST) implemented in BEAST v2.4.3 using Bayes Factors. Therefore, we conducted five independent CONST runs using 40 million MCMC iterations after a burn-in of 10%, logging every 10,000 iterations. We assessed the proper mixing of the MCMC and ensured that ESSs were greater than 200. We then used the Path sampler package in BEAST v2.4.3 to compute the log of marginal likelihood (logML) of each run for both BSP and CONST. We set the number of steps to 100 and used 40 million MCMC iterations after a burn-in of 10%. Bayes factors were computed as twice the difference between the log of the marginal likelihoods ($2[\text{Log ML}(\text{BSP}) - \text{Log ML}(\text{CONST})]$) and were performed for pairwise comparisons between BSP and CONST runs. As recommended, Bayes factors greater than 6 were considered as strong evidence to reject the null hypothesis of constant effective numbers throughout time. Nevertheless, all these demographic procedures have several caveats. Selection can affect the effective population size, reducing the effective number for a time and increasing

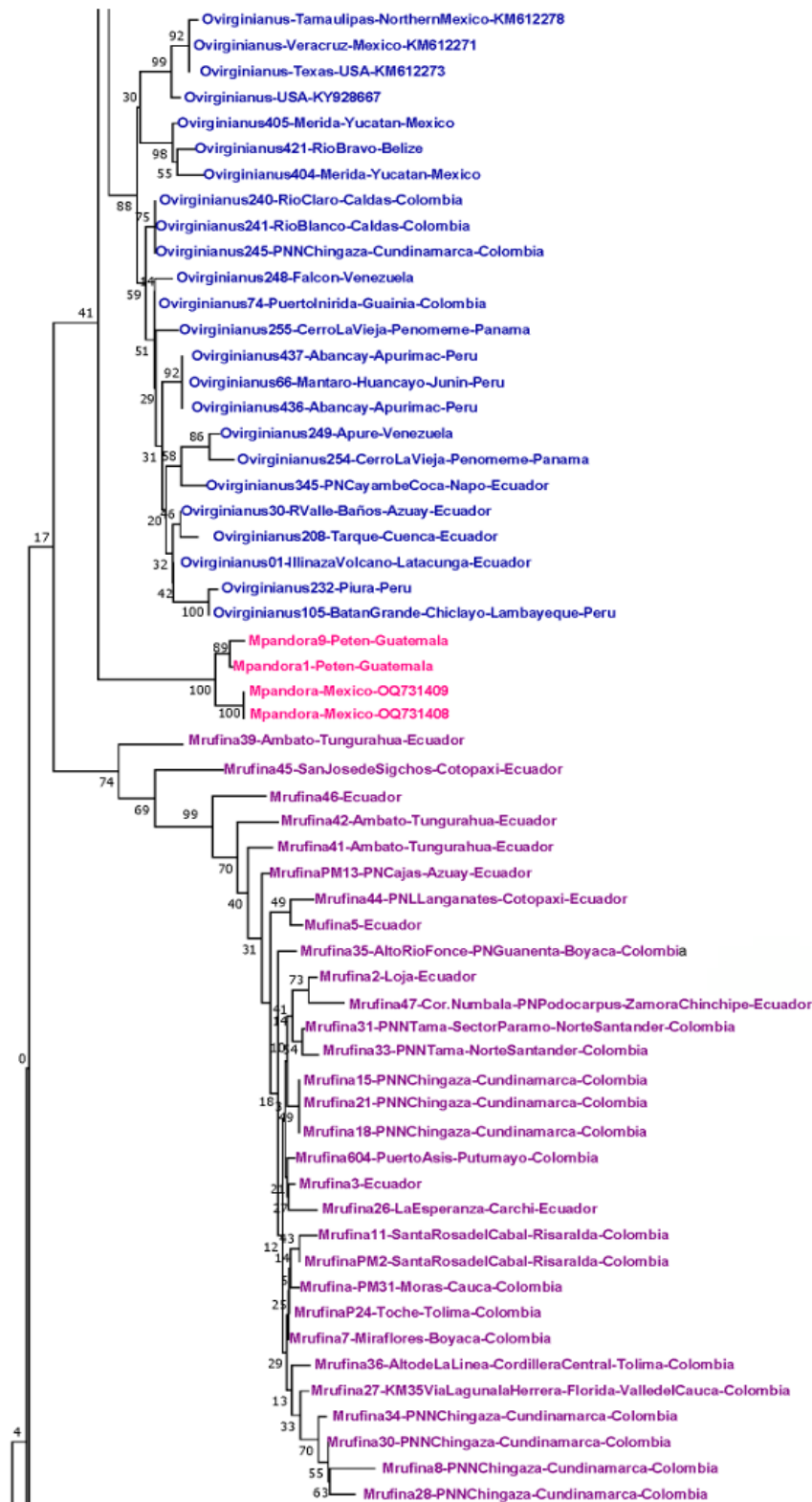
the coalescence rate later [113]. The same occurs with small changes in the mutation rate (μ), which can greatly affect the effective number and, in turn, estimated divergence time [114].

Results

Phylogenetic Analysis of the Brocket Deer with BD1

The ML tree (Figure 1) showed that the Neotropical deer clade that initially diverged was the ancestor of the genus *Subulo* (bootstrap 93%) (Blastocerina). Within *Subulo*, we can distinguish two well-identified clades: *S. gouazoubira* (94%) and *S. chunyi* (100%). As with BD2, it was clearly confirmed that the aforementioned *S. chunyi* was highly related to *S. gouazoubira* and, therefore, deserves to be part of the genus *Subulo*. On the other hand, a specimen from the Andean region of the department of La Paz (Bolivia) was detected with a phenotype similar to that of *S. gouazoubira*, but smaller in size. This specimen was strongly associated with *S. chunyi* (100%) but clearly differentiated from it. Specimens from that geographic area should be chromosomally analyzed, as they could correspond to a species or new taxon within the genus *Subulo*. Additionally, specimens of *S. gouazoubira* have been found scattered across different countries in southern South America (Argentina, various areas of southern Brazil, Bolivia, Paraguay, and Uruguay), and the presence of brocket deer carrying the mtDNA of *S. gouazoubira* in Colombia has been confirmed. One of these specimens was previously reported by Hassanin, et al. [55] from the department of Huila (Colombia). Some authors criticized the presence of *S. gouazoubira* in Colombia. Nonetheless, its sequencing was repeated several times with identical results. In the present analysis, we detected a second specimen of *S. gouazoubira* in Colombia, from the Tamá National Natural Park in the department of Norte de Santander. Therefore, two specimens of this taxon have now been detected in Colombia. It would be important to perform karyotypic analysis on one of these specimens. Within the *S. gouazoubira* clade, a specimen from Exaltación, Mamoré River, Beni (Bolivia) was included. Phenotypically, it was indistinguishable from an *S. gouazoubira* specimen, but various analyses showed diverse relationships with other Blastocerina taxa.





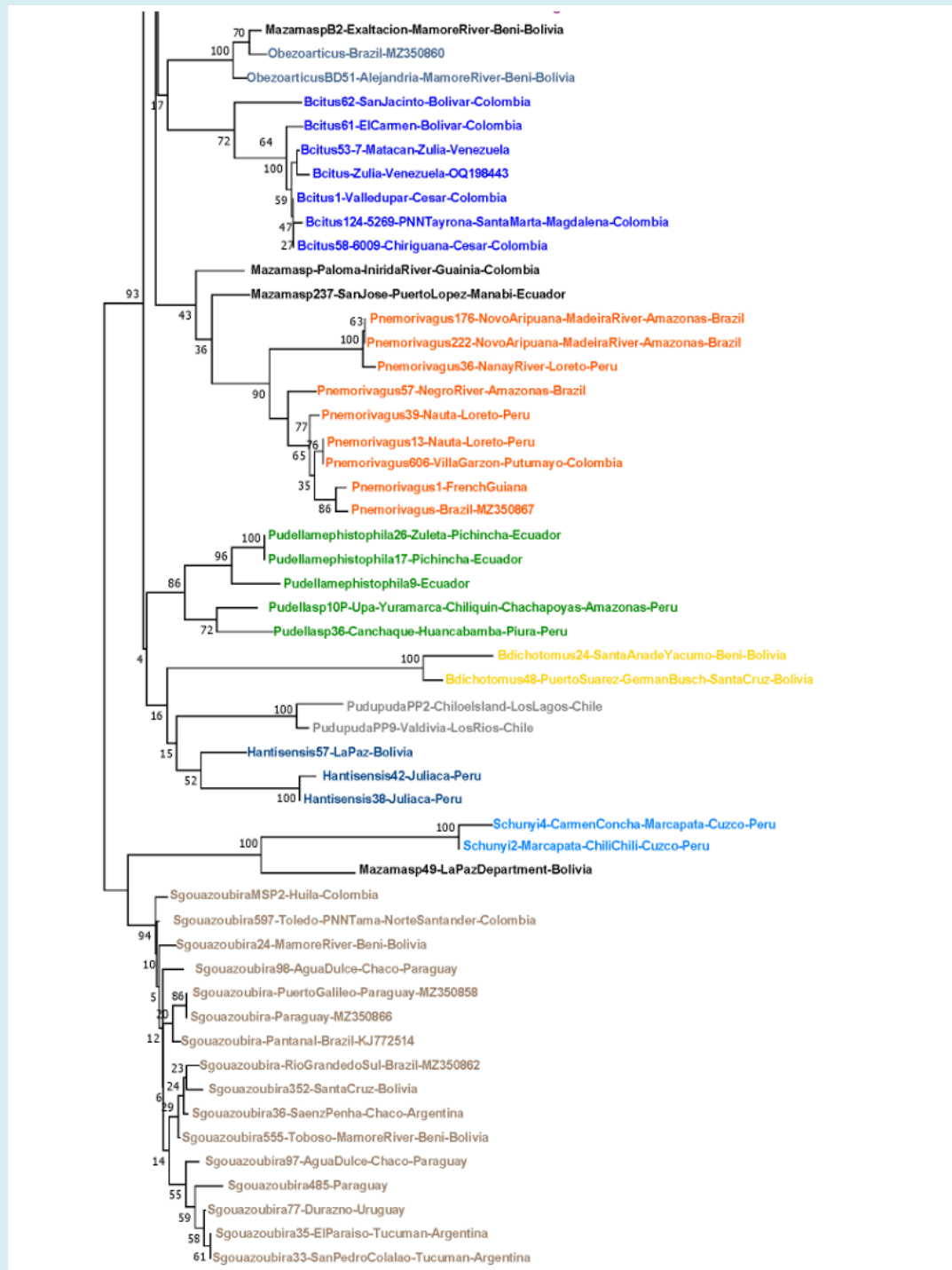


Figure 1: Maximum Likelihood (ML) tree for a total of 157 specimens of different species of Neotropical deer sequenced at the mitochondrial *ND5* and *12S rRNA* genes (BD1). Each species was indicated with different colors. A total of 120 brocket deer specimens were analyzed (*Subulo gouazoubira*, *Subulo chunyi*, *Passalites nemorivagus*, *Bisbalus citus*, *Mazama* (= *Andinocervus*) *rufina*, *Mazama pandora*, *Mazama temama*, *Mazama rufa*, *Mazama nana*, *Mazama jucunda*, and different groups of *Mazama americana*). The different groupings within the *Mazama americana* complex were marked. As outgroups, 24 *Odocoileus virginianus*, two *Ozotoceros bezoarticus*, four *Pudella*, two *Blastoceros dichotomus*, two *Pudu* and three *Hippocamelus antisensis* specimens were used. Specimens with numerical codes, their sequences were obtained in GenBank. In nodes, bootstrap percentages.

The next clade to diverge was weak and contained no brocket deer specimens. Within it, four well-defined taxa (Blastocerina) were found: *B. dichotomus* (100%), *H. antisensis* (with a relatively low bootstrap, which will be discussed later, 52%), *P. puda* (100%), and *Pudella* (86%). This finding fully confirms the results of Barrio, et al. [115] who demonstrated the phylogenetic separation of northern and southern Andean pudus into two distinct genera, as their analysis was not monophyletic (a new genus, *Pudella*, for northern pudus and the traditional genus, *Pudu*, for southern pudus). Similarly, the results of BD1 confirm the assertion by Barrio, et al. [115] that within *Pudella* there are two different species: *Pudella mephistophila* (96% in our case, with three Ecuadorian specimens north of the Huancabamba deflection in Peru) and *Pudella carlae* (72%, with two Peruvian specimens in the vicinity of the Huancabamba deflection).

The next grouping consisted of specimens of the brocket deer, *P. nemorivagus* (Blastocerina). A central core (90%) was observed with nine specimens from different countries (Brazil, Colombia, French Guiana, and Peru). Within this core area, two subgroups were detected: one extending through the central Brazilian Amazon, reaching the Loreto Department (Peru) (100%), and another extending north of the Amazon River in Brazil, Colombia, and French Guiana, overlapping with the former in the Amazonian department of Loreto (Peru) (77%). Interestingly, two other brocket deer specimens were associated with *P. nemorivagus*, but outside the core area and with low bootstrap percentages. One corresponds to a specimen hunted on the Inírida River (Guainía Department, Colombia), and the other was hunted in Puerto López, Manabí Province, on the Ecuadorian Pacific coast. Both skins exhibited a reddish coloration, which led to their initial classification as *M. americana*. These two specimens likely represent new brocket deer taxa that should be analyzed karyotypically.

The next clade to diverge (Blastocerina) contains two well-characterized species, although linked by very low bootstrap relationships. On the one hand, there is *O. bezoarticus* (100%), and on the other, another group of brocket deer that was recently characterized as a new species, *B. citus* (72%). This species was karyotypically and molecularly determined using a specimen from El Zulia (Venezuela), but we show here that it also extends across several departments in northern Colombia (Bolívar, Cesar, and Magdalena).

The next association corresponds to the most divergent clade of Odocoileina (although in some analyses it appears associated with Blastocerina). This is the Andean species, *M.* (= *Andinocervus*) *rufina*. There is a central core (99%) and a couple of somewhat more divergent specimens (74%). The most divergent specimens of the clade all had an Ecuadorian

origin, which could be related to the origin of this taxon in what are now the Ecuadorian Andes. Some of these specimens could also constitute different taxa within *M. rufina*.

Unlike what has been reported by other authors, the next Odocoileina clade to diverge was *M.* (= *Odocoileus*) *pandora* (100%). This result did not support the recent classification of this taxon in the genus *Odocoileus* since it is not the sister group of the latter genus. The next large grouping corresponds to *O. virginianus* (88%) and the remaining red brocket deer taxa. The species most closely related to *O. virginianus* was shown to be *M. temama* (87%). The Colombian specimens differed from the Mexican specimens (100%). In this analysis, we demonstrated, for the first time using molecular evidence, the presence of *M. temama* in Ecuador (Pichincha province).

The following groupings corresponded to what we called the *M. americana* complex in a broad sense. These groupings were statistically well identified (high or very high bootstraps), although the relationships between them for determining phylogenetic relationships were low. One initial association included two brocket deer from northern Colombia with a somewhat atypical phenotypic appearance because one part of the body presented a strongly gray coloration, while another part was a well-defined red (80%). Both specimens (one Venezuelan and the other from northern Colombia) were distributed in the same area where we find *B. citus*. Tentatively, we named this cluster as *M. americana* VIII. It is important to determine karyotypes of these specimens compared to the one identified as *B. citus* in Venezuela. The next detectable clade within the *M. americana* complex was designated *M. americana* IV (75%). This group is found throughout the Peruvian Amazon, reaching the border with Brazil. The next grouping consisted of the taxon that corresponded "sensu stricto" to *M. americana*, as it included the representative neotype of the original *M. americana* (Cayenne, French Guiana, MZ350857) (86%). This grouping extends across French Guiana, northern Brazilian Amazon (state of Pará), and included the brocket deer present on the Caribbean island of Trinidad. We designated this grouping *M. americana* I. Of particular interest is the presence within this group of two specimens of the karyotypically differentiated species *M. rufa*, represented by one specimen from southern Brazil (state of Paraná) and another from Paraguay. Therefore, *M. americana* I (*M. americana* "sensu stricto") was molecularly closely related to *M. rufa*. Another grouping within this complex, though not particularly robust (62%), consisted of three brocket deer specimens, each belonging to a different species due to their clear karyotypic differences: one specimen of *M. jucunda* (Brazil), one specimen of *M. nana* (Paraguay), and one specimen of *M. americana* from Juina (Mato Grosso state, Brazil). We will provisionally refer to this grouping (based on the observations from the second

dataset) as *M. americana* II-III due to its complexity and the fact that it comprises three taxa of seemingly different red brocket deer species. The next swarm of specimens with a high bootstrap percentage (99%) consisted of specimens distributed throughout north-central Peruvian, Colombian, and Ecuadorian Amazon. We named this grouping *M. americana* VI. It is important to note that within this group, a subgroup of two specimens (one from the Colombian Amazon and one trans-Andean specimen from the department of Cauca, Colombia) (100%) was identified. These specimens, along with other representatives of this group, warrant karyotypic analysis because *M. americana* VI could contain at least two distinct taxa of red brocket deer.

Subsequently, another group (95%) was detected, consisting of three specimens (two from the south-central Peruvian Amazon and one from the northern Bolivian Amazon). The Bolivian specimen showed some differentiation from the two Peruvian specimens, which, again, warrants karyotypic analysis of the specimens from that area of the Amazon because it could contain two different new brocket deer taxa. We named this group *M. americana* VII. Finally, another significant swarm of red brocket deer (83%) was detected, extending throughout the Peruvian Amazon and reaching into central Brazil and north-central Bolivia. Within this swarm, a pair of extremely closely related individuals from northern Peru were found (100%). This group was designated *M. americana* V.

The complex situation within the broadly defined *M. americana* species was thus evident. In brief, there were at least seven different groups, some with significant subgroups: 1- *M. americana* I (which includes the original *M. americana* in the strict sense and another karyotypically distinct species accepted by the scientific community, *M. rufa*); 2- *M. americana* II-III (comprising *M. americana* from Juína, Mato Grosso, Brazil, together with two other karyotypically differentiated taxa considered to be two full species, *M. nana* from Paraguay and *M. jucunda* from Sao Paulo, Brazil); 3- *M. americana* IV (located throughout the Peruvian Amazon, reaching the westernmost area of the Brazilian Amazon); 4- *M. americana* V (distributed throughout the Peruvian Amazon, reaching the north-central Bolivian Amazon and the central Brazilian Amazon, Madeira River and tributaries); 5- *M. americana* VI (basically distributed throughout the north-central Peruvian Amazon and the Colombian and Ecuadorian Amazon and which could contain another differentiated taxon in the trans-Andean zone of Cauca in Colombia); 6- *M. americana* VII (distributed throughout the south-central Peruvian Amazon and northern Bolivian Amazon and which could contain two distinct taxa); and 7- *M. americana* VIII consisting of two specimens of strange phenotypic appearance within the geographical distribution area of *B. citus*.

BI tree (Figure 2) showed aspects very similar to the previously discussed tree, but also some strongly differentiated situations. This tree perfectly discriminated between Blastocerina and Odocoileina (posterior probability, $pp = 1$), with one conspicuous exception: it included *B. citus* within the Odocoileina, especially with the *M. americana* complex + *M. temama* ($pp = 0.93$). In the case of Blastocerina, the deeper branches of the tree did not have high pp values, thus the relationship between the different genera of this grouping was unclear. There was no conspicuous differentiation between *Pudu* and *Pudella*, which form a monophyletic clade ($pp = 0.85$), although differentiation was observed between the taxon *mephistophila* (north of the Huancabamba deflection) and the taxon *carlae* (south of the Huancabamba deflection) ($pp = 0.86$). The pudus would be the sister group to the group composed of *B. dichotomus* ($pp = 1$) + *H. antisensis* (which split into two highly differentiated branches within this group; on the one hand, two specimens from southern Peru, $pp = 1$, and on the other hand, one Bolivian specimen, $pp = 0.43$) + (*S. gouzaoubira* + *S. chunyi*). The clade of *S. gouzaoubira* + *S. chunyi* was very strong ($pp = 1$). Notwithstanding, a phenomenon was observed that was not recovered in any of the other phylogenetic trees analyzed independently of the three datasets analyzed. *S. gouzaoubira* was divided into two different groups, one made up exclusively of specimens of *S. gouzaoubira* from Paraguay, Pantanal in Brazil, Bolivia, and the two Colombian specimens, and the other made up of the remaining specimens of *S. gouzaoubira* (Paraguay, Argentina, Bolivia, Uruguay and southeastern Brazil) and by the specimens *S. chunyi* ($pp = 1$) and the specimen from the Andean zone of the department of La Paz (Bolivia) which may constitute a new taxon as we discussed for the previous tree ($pp = 1$). The sister group to this large clade [(*Pudella* + *Pudu*) + (*B. dichotomus* + (*H. antisensis* + (*S. gouzaoubira* + *S. chunyi*)))] consisted of *O. bezoarticus* ($pp = 1$) + *P. nemorivagus* (central core, $pp = 0.98$, with two main subgroups within it) and a single specimen of *B. citus* (the most differentiated from the previous analysis, from San Jacinto, Bolívar Department, Colombia). Two aspects were worth highlighting. First, the *S. gouzaoubira* specimen from Exaltación (Bolivia) was strongly associated with an *O. bezoarticus* specimen from Brazil. Second, the two brocket deer specimens that were weakly associated with the *P. nemorivagus* core (one of them from the Ecuadorian Pacific coast, where *P. nemorivagus* is supposedly not distributed) and which could represent new taxa also appeared in the BI tree. Within the Odocoileina, two large, clearly differentiated clades were observed. The most divergent of these was the swarm of *M. rufina* individuals ($pp = 1$). As in the previous tree, some Ecuadorian specimens showed the oldest ancestors and could constitute a distinct taxon within *M. rufina*. The other large clade ($pp = 0.99$) was made up of two main subclades: one consisting of *O. virginianus* ($pp = 0.96$) (which, among the large clades,

showed the most recent common ancestor) and the other of all the red brocket deer taxa. The first taxon whose ancestor diverged was *M. pandora* ($pp = 1$), which did not behave as the sister group to *O. virginianus*. The next brocket deer ancestor to diverge was that of the *B. citus* group, except for the specimen that remained with the Blastocerina. With this grouping of *B. citus*, the two specimens of red brocket deer from Venezuela and northern Colombia (*M. americana* VIII) appeared in this clade (in a way that was profoundly differentiated from the ML tree), which were differentiated from the remaining groupings of the *M. americana* complex "sensu stricto". Both specimens ($pp = 1$, $pp = 0.9$) were the most divergent from the central core of *B. citus* ($pp = 1$). Therefore, a profound difference occurred with regard to the phylogenetic relationships between and within *B. citus* with the two phylogenetic trees. *M. temama* was clearly differentiated from other red brocket deer ($pp = 0.91$), with the Colombian and Ecuadorian specimens well differentiated from the Mexican specimens ($pp = 1$).

Regarding the *M. americana* complex, the *M. americana* I group (containing *M. americana* "sensu stricto" and *M. rufa*) was well established ($pp = 0.97$), as was *M. americana* VI ($pp = 0.96$), which contained a subclade (also clearly visible in the other tree) comprised of a specimen from the Colombian Amazon and a trans-Andean specimen ($pp = 1$). *M. americana* VII was divided into two groups in this case: on the one hand, the specimens from south-central Peru ($pp = 0.98$), and on the other, an independent branch not specifically associated with any taxon, the specimen from the northern Bolivian Amazon. *M. americana* V was also an established taxon ($pp = 0.88$). *M. americana* II-III, including the three fully karyotypically differentiated species, was also significantly confirmed ($pp = 0.98$), as was *M. americana* IV. Therefore, with regard to the groupings of the *M. americana* complex, the ML and BI trees discriminated the same groupings, although the relationships between them were not exactly the same. In both cases, the relationships between these different groups showed low and insignificant bootstrap percentages.

Additionally, several analyses were performed using the BAPS procedure (Figure 3). The first analysis (Figure 3a) included only specimens of *M. temama* and the *M. americana* complex. The optimal number of groupings was three [Log (marginal likelihood) of the optimal partition = -1,557.909, $p = 0.834$]. The first grouping contained all specimens of *M. americana* I, II-III, IV, V, and VI; the second grouping contained all specimens of *M. temama* plus the two "rare"

specimens from northern Colombia and Venezuela (possibly *M. americana* VIII); and the third grouping contained the three specimens of *M. americana* VII. The second analysis used all specimens from the previous analysis plus all the studied samples of *O. virginianus* and three samples of *M. pandora* (Figure 3b). The optimal number of groupings was three [Log (marginal likelihood) of the optimal partition = -2,587.077, $p = 0.998$]. The first grouping contained *M. temama* and the eight groupings of the *M. americana* complex, while the second grouping contained the 24 specimens of *O. virginianus*, and the third grouping included the three specimens of *M. pandora*. This analysis proved to be much more conservative than the phylogenetic trees, as it did not differentiate any groupings in *M. temama* + eight *M. americana* groupings, but it did differentiate *M. pandora* from *O. virginianus*. Finally, we carried out an analysis including all the specimens from the previous test plus *S. gouzaoubira*, *S. chunyi*, *P. nemorivagus*, *B. citus*, *M. rufina*, and the different *Mazama* sp. specimens detected (Figure 3c). The optimal number of groupings was seven [Log (marginal likelihood) of the optimal partition = -6,028.254, $p = 1$]. The first grouping included all specimens of *M. rufina*; the second grouping included all specimens of *P. nemorivagus*, the two specimens that could constitute new taxa (including the specimen from the Pacific zone of Ecuador) associated with *P. nemorivagus*, the "rare" specimen from Exaltación, Mamoré River (Bolivia), and the most differentiated specimen from the core of *B. citus* (San Jacinto, Bolívar, Colombia; this specimen had been differentiated from the rest of *B. citus* in tree BI); the third grouping added *M. temama* plus all the groupings of the *M. americana* complex, with the exception of the two specimens of *M. americana* VIII; the fourth grouping included all the specimens of *S. gouzaoubira* plus the specimen from the Bolivian Andes most closely related to *S. chunyi*, which appeared to be a new taxon in the phylogenetic trees; the fifth grouping added the 24 specimens of *O. virginianus* plus the three specimens of *M. pandora* (the first evidence in favor of *pandora* being associated with *Odocoileus*); the sixth grouping contained all the specimens of *B. citus* (excluding the specimen from San Jacinto, Bolívar, Colombia, associated with the *P. nemorivagus* grouping) plus the two "rare" specimens that in other analyses formed *M. americana* VIII (this fact agrees with what was observed in BI tree); and the seventh grouping coincided with the two specimens of *S. chunyi*. In general, it was observed that the BAPS procedure tends to be more conservative in the number of genetically differentiated brocket deer groupings than phylogenetic trees.

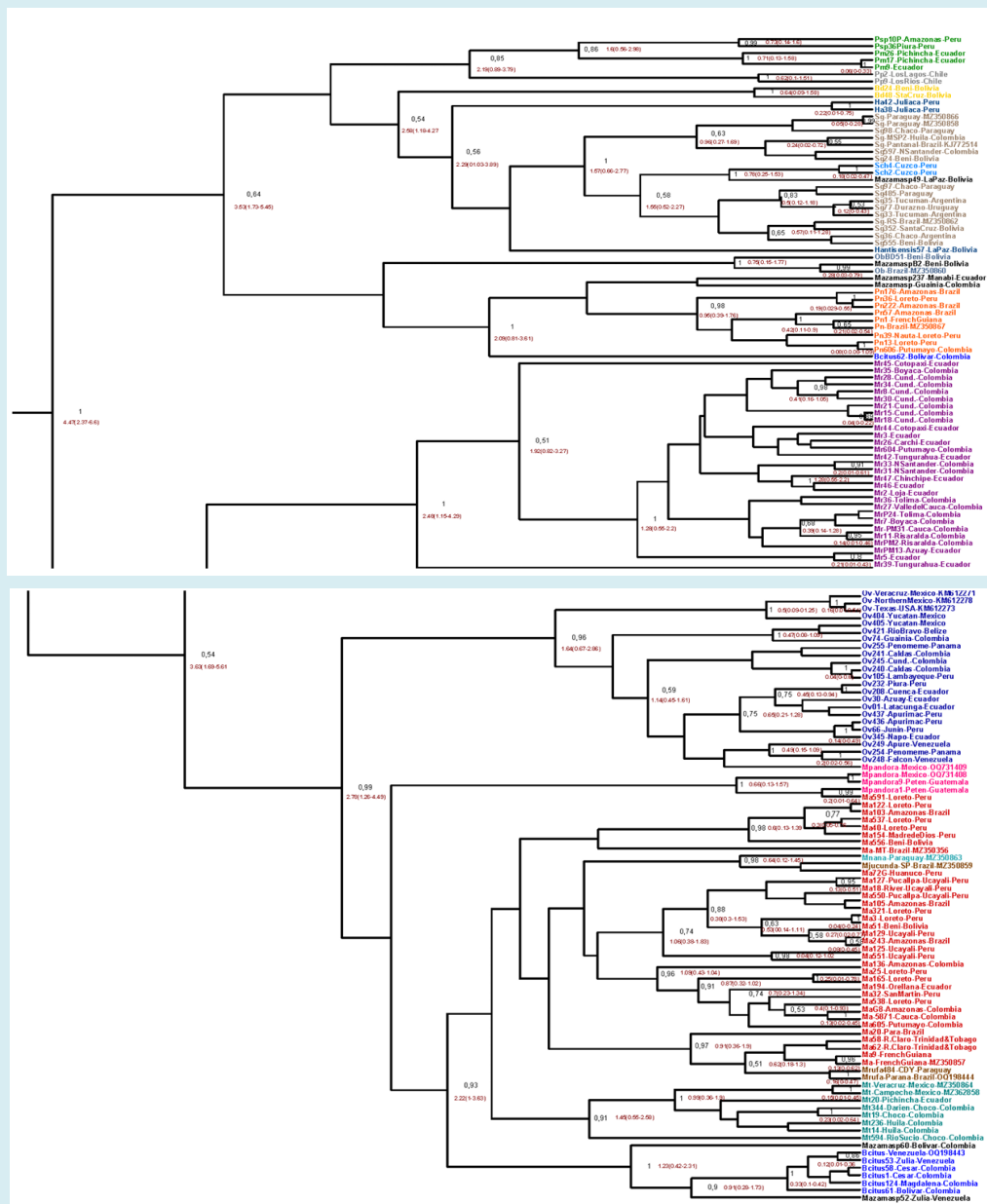


Figure 2: Bayesian Inference (BI) tree for a total of 157 specimens of specimens of different species of Neotropical deer sequenced at the mitochondrial *ND5* and *12S rRNA* genes (BD1). Each species was indicated with different colors. A total of 120 brocket deer specimens were analyzed (*Subulo gouazoubira*, *Subulo chunyi*, *Passalites nemorivagus*, *Bisbalus citus*, *Mazama* (= *Andinocervus*) *rufina*, *Mazama pandora*, *Mazama temama*, *Mazama rufa*, *Mazama nana*, *Mazama jucunda*, and different groups of *Mazama americana*). As outgroups, 24 *Odocoileus virginianus*, two *Ozotoceros bezoarticus*, four *Pudella*, two *Blastoceros dichotomus*, two *Pudu* and three *Hippocamelus antisensis* specimens were used. Specimens with numerical codes, their sequences were obtained in GenBank. In nodes, posterior probabilities in black and, in red, temporal split estimations (and into parentheses, 95% High Posterior Density, HPD, as confidence intervals; values in millions of years). Psp = *Pudella carlae*; Pm = *Pudella mephistophelica*; Pp = *Pudu puda*; Bd = *Blastoceros dichotomus*; Ha = *Hippocamelus antisensis*; Pn = *Passalites nemorivagus*; Sg = *Subulo gouazoubira*; Sch = *Subulo chunyi*; Mr = *Mazama rufina*; Mt = *Mazama temama*; Ma = *Mazama americana*; Mnana = *Mazama nana*; Mrufa = *Mazama rufa*; Mjucunda = *Mazama jucunda*; Mpandora = *Mazama pandora*; Ov = *Odocoileus virginianus*; Ob = *Ozotoceros bezoarticus*; Bcitus = *Bisbalus citus*.

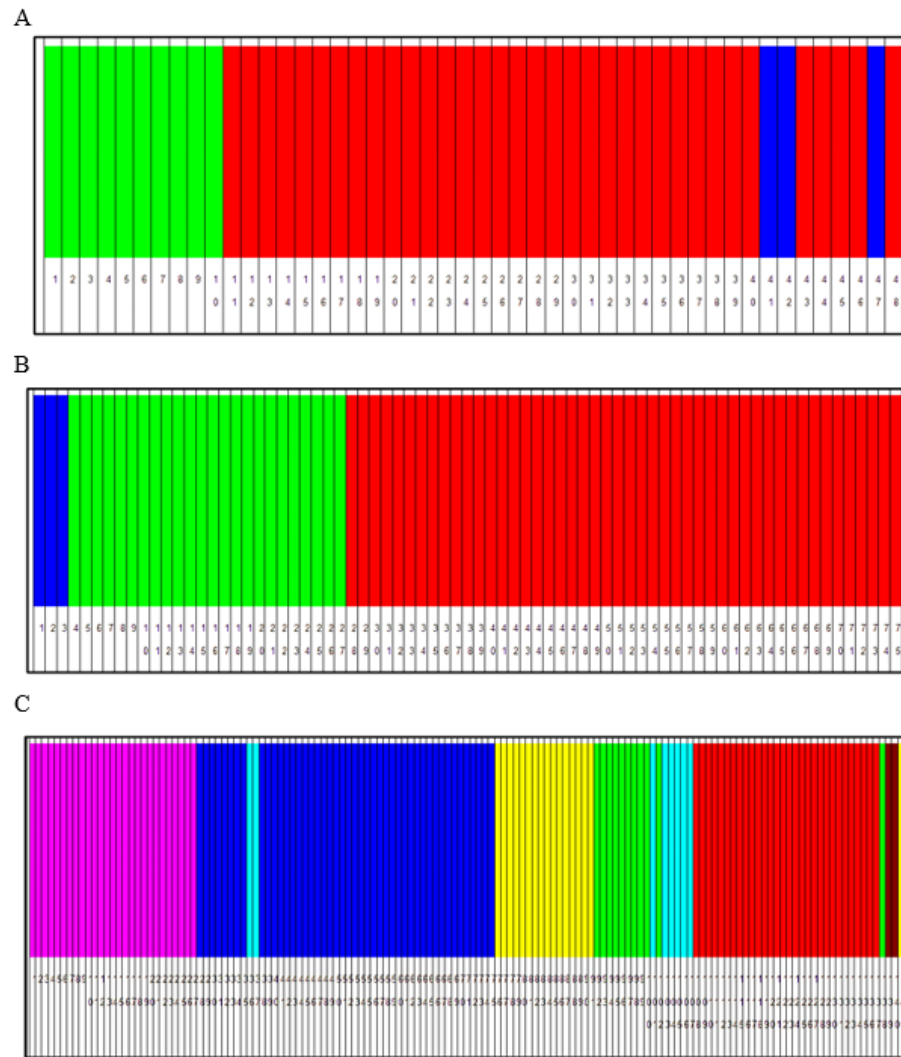


Figure 3: Different populations or taxa of Neotropical deer detected by means of BAPS at the mitochondrial *ND5* and *12S rRNA* genes (BD1). Each different color indicates a different population or taxa. (A) A sample of 48 specimens from *Mazama temama* and the *Mazama americana* complex. In red, specimens from all the grouping of the *M. americana* complex I, II-III, IV, V and VI; in green, specimens of *M. temama* and *M. americana* VIII; in blue, specimens of *M. americana* VII. (B) A sample of 75 specimens from *M. temama*, the *M. americana* complex, *Odocoileus virginianus*, and *Mazama pandora*. In red, all the specimens of *M. temama* and the *M. americana* complex; in green, all the specimens of *O. virginianus*; in blue, the three specimens of *M. pandora*. (C) A sample of 142 from of all the different taxa of brocket deer plus *O. virginianus*. In red, specimens of *M. rufina*; in blue, the specimens all the specimens of *M. temama* and the *M. americana* complex, excluding *M. americana* VIII; in light blue, all the specimens of *Bisbalus citus* (less the specimen from San Jacinto, Bolivar, Colombia), plus *M. americana* VIII; in pink, all the specimens of *O. virginianus* and *M. pandora*; in yellow, all the specimens of *Subulo gouazoubira* plus an specimen of *Mazama* sp from the Bolivian Andes; in brown, two specimens of *Subulo chunyi*; in green, the specimens of *Passalites nemorivagus*, the possible new taxa related to *P. nemorivagus*, one specimen of *B. citus* from San Jacinto, Bolivar (Colombia), and the “rare” specimen from Exaltación, Beni (Bolivia).

Phylogenetic Analysis of Brocket Deer with BD2

The ML tree for BD2 (Figure 4) yielded the following results. The clade formed by *S. gouzaoubira* and *S. chunyi* was robust, as in BD1 (99%). The *S. chunyi* grouping (sequences

from two specimens extracted from GenBank, different from the two used in the previous analysis since we were unable to amplify the mtCyt-*b* gene sequence of the two specimens used in BD1) was highly significant (100%). In this case, this gene could not be amplified for the Bolivian Andean specimen,

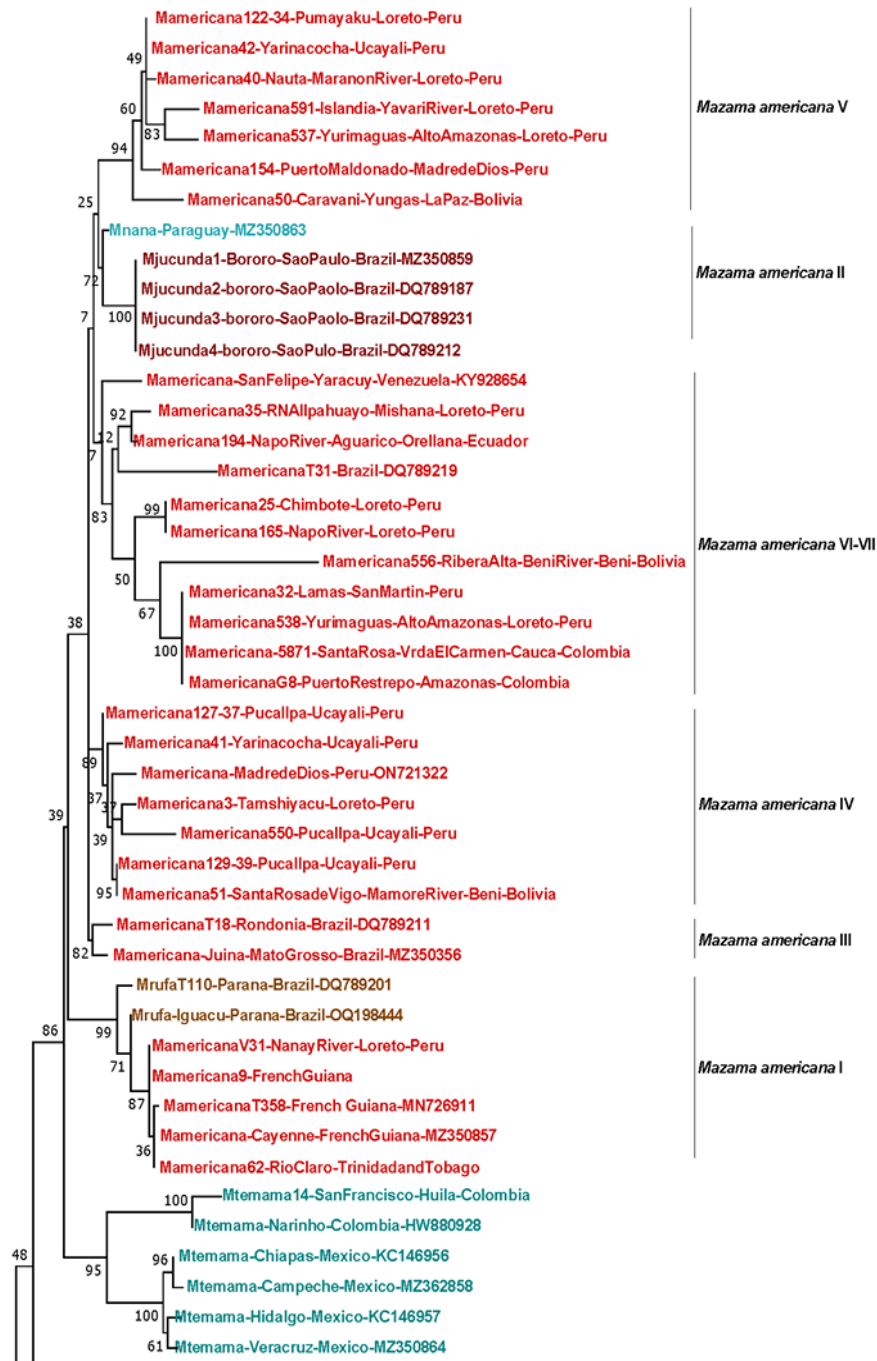
which was interpreted as a new taxon related to but distinct from *S. chunyi*. The clade containing the *S. gouzaoubira* specimens was also robust (86%). Of note within this taxon was the persistence of the Colombian specimen from the department of Huila (Colombia) (the second Colombian specimen that appeared in BD1 could not be sequenced for this gene), the presence of a specimen of this taxon in the Bolivian Andes (Yungas, La Paz, Bolivia) highly related to a specimen from the Pantanal (Brazil), and two specimens also from the Andean area of Argentina (Tucumán) (98%), all of them related to the Colombian Andean specimen from Huila (88%). This subgroup of *S. gouzaoubira* was found in western South America, with most specimens associated with the Andes, while other subgroups within *S. gouzaoubira* related individuals from areas further east in southern South America (Bolivian Amazon, Paraguay, Uruguay, and Rio Grande do Sul in southern Brazil). It is interesting to note that the “rare” brocket deer from Exaltación, Mamoré River (Bolivia), in this analysis, behaved like a specimen of *S. gouzaoubira*. Associated with this group of gray brocket deer, although with low bootstrap percentages, the presence of *O. bezoarticus* (100%) and a cluster of *H. bisulcus* (not analyzed in BD1) (100%) and a subgroup of *H. antisensis* from Bolivia and northern Argentina (Salta Province) (98%) was observed, separated from other *H. antisensis* specimens from southern Peru. More distinct from this group was a clade containing specimens of *B. dichotomus* (100%) and two specimens of *H. antisensis* from southern Peru (Arequipa department), as mentioned previously (100%). This significant finding will be discussed later. The next clade (73%) containing brocket deer specimens included specimens of *B. citus* (100%) (Venezuela and northern Colombia) and specimens of *P. puda* (100%) from Chile. Significantly separated from this clade (100%) was a swarm of *Pudella* specimens, comprising three distinct groups: *P. carlae* (100%) and two groups of *P. mephistophila*. One group included two Ecuadorian specimens (95%), while the other consisted of a specimen from 1896 from Papallacta (Napo, Ecuador) and a captive specimen from Berlin (89%). The next significant group was *P. nemorivagus* (99%). Unfortunately, the two specimens externally associated with the central nucleus of *P. nemorivagus* in BD1, which could constitute new taxa, could not be amplified for this gene. Within *P. nemorivagus*, several groupings were observed, with specimens from the western Amazon being the first whose ancestors differentiated (Peruvian and Colombian Amazon), while specimens from the central Brazilian Amazon and the Guianas region appeared more derived. Up to this point, all the described groupings were part of Blastocerina. The next grouping to differentiate would, in principle, belong to Odocoileina, as occurred with BD1. We are referring to *M. rufina* (100%). As in BD1, the specimen whose ancestor appeared first corresponded to an Ecuadorian specimen. Within *M. rufina*, several highly significant groupings were observed that had a geographical

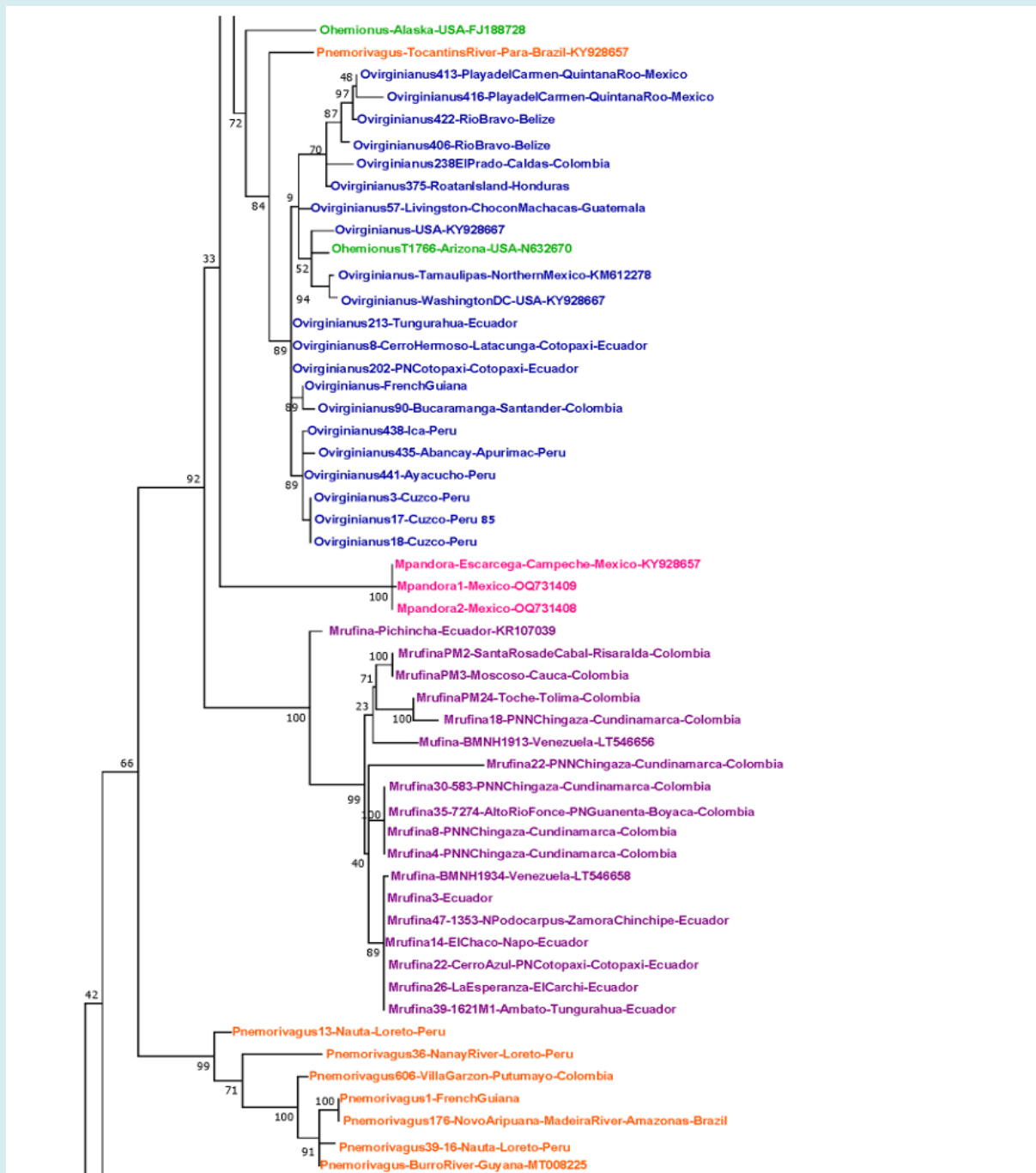
significance. One group consisted of representatives from the departments of Cauca, Cundinamarca, Tolima, and Risaralda in Colombia (71%), another group included specimens from Cundinamarca and Boyacá in Colombia (100%), and a third group comprised Ecuadorian specimens (with the exception of one Venezuelan specimen) (89%). Subsequently, the divergence of *M. pandora* (100%) was evident, as it did not constitute the sister group to *Odocoileus*, as was the case in most of the analyses with BD1.

Subsequently, as in the ML tree of BD1, the common ancestor of *Odocoileus* and the remaining red brocket deer diverged (86%). One specimen of *O. hemionus* and one “rare” specimen of *P. nemorivagus* (already discussed in Ruiz-García, et al. [116]) were the sister groups to *O. virginianus* (89%). The first group of red brocket deer related to *Odocoileus* was that of *M. temama* specimens (95%). In this group, the Colombian (100%) and Mexican (100%) specimens were clearly distinguishable. Next, *M. americana* I diverged (99%), which, like the first database, included *M. americana* “sensu stricto” and *M. rufa*. The next group to diverge (82%) corresponded to the *M. americana* specimen from Juina (Mato Grosso, Brazil), which was also analyzed in BD1 along with another *M. americana* specimen from Vilhena (Rondonia, Brazil) (not studied in the previous BD1). We strictly referred to this group as *M. americana* III. Subsequently, the ancestor of *M. americana* V diverged (89%), followed by *M. americana* VI (83%). Three observations regarding this group: A brocket deer specimen from San Felipe (Yaracuy, Venezuela), which was not analyzed in BD1, was added to it with a very low-magnitude bootstrap. This specimen could constitute a distinct taxon within the *M. americana* complex. Unfortunately, the two “rare” specimens from the previous database analysis, which in some analyses constituted what we called *M. americana* VIII, could not be sequenced for the mtCyt-b gene, and we do not know if they might be related to this specimen from Yaracuy (Venezuela). A second observation was the inclusion of the Bolivian specimen of *M. americana* VII in this *M. americana* VI. The other two specimens that in BD1 constituted *M. americana* VII could not be sequenced for this gene. Therefore, *M. americana* VII was not present in BD2. The third observation referred to the trans-Andean red brocket deer specimen from Cauca (Colombia). In this case, it is grouped with the same Amazonian specimen from BD1 plus two other Amazonian specimens from northern Peru (Lamas and Yurimaguas in the departments of San Martín and Loreto) (100%). A karyotype study of specimens from this subgroup would be a priority. Next, the ancestor of two of the three species appearing in *M. americana* II-III from the BD1 analysis diverged. This is the case for *M. nana* from Paraguay and four specimens of *M. jucunda* (100%) from southern Brazil (72%). With the current database, we named this grouping *M. americana* IIa (*M. nana* from Paraguay) and *M. americana*

Ilb (*M. jucunda*). Finally, *M. americana* IV appeared, which, in addition to expanding throughout the Peruvian Amazon as revealed by BD1, also reached northern Bolivia (94%). Therefore, the only differences in the groupings observed in BD2 compared to BD1 were the splitting, by introducing more GenBank specimens sequenced for the mtCyt-*b* gene, of *M. americana* II-III into *M. americana* IIa, *M. americana* IIb,

and *M. americana* III (including three different species of red brocket deer, *M. nana*, *M. jucunda*, and a presumed taxon of *M. americana* from Rondonia and Mato Grosso, Brazil) and the absorption of the single sequenced specimen of *M. americana* VII into *M. americana* VI. The remaining groupings were the same as in BD1.





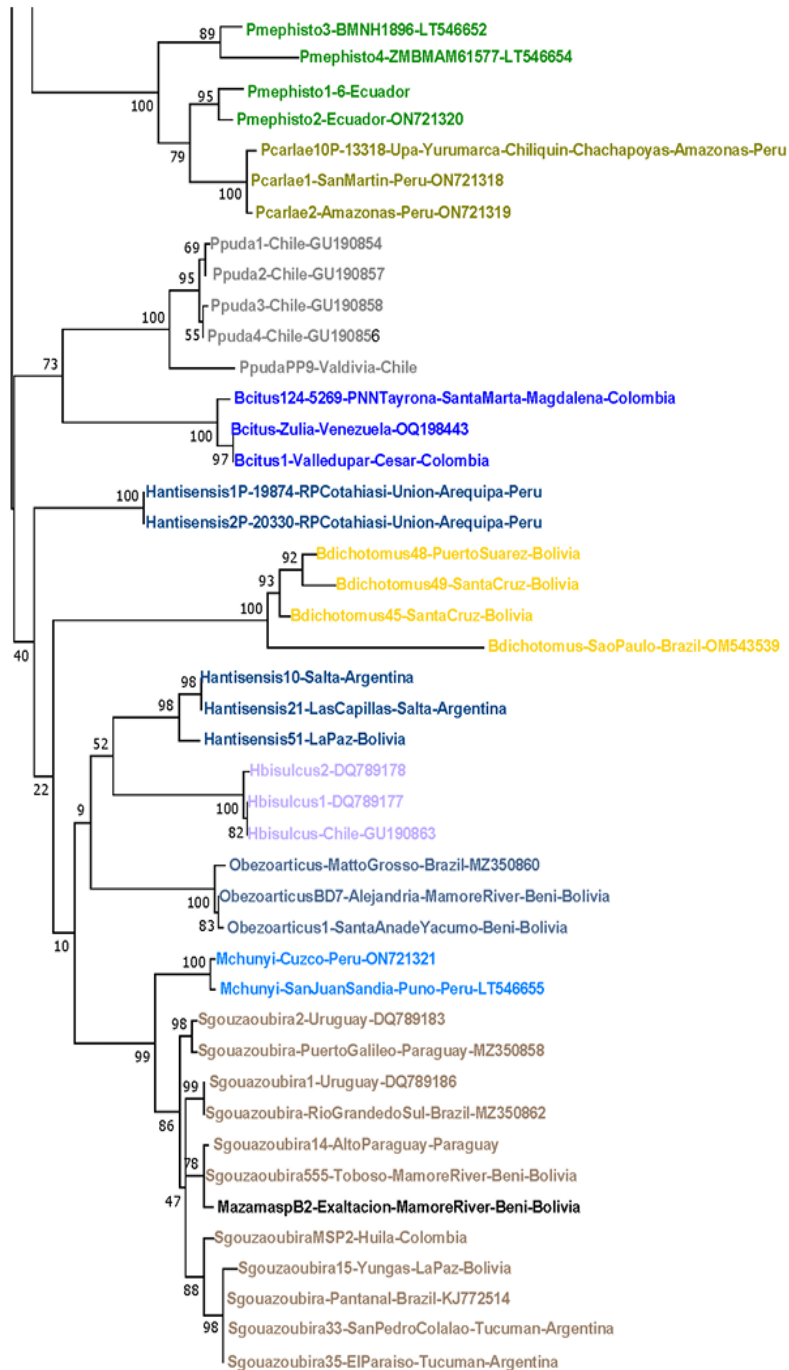
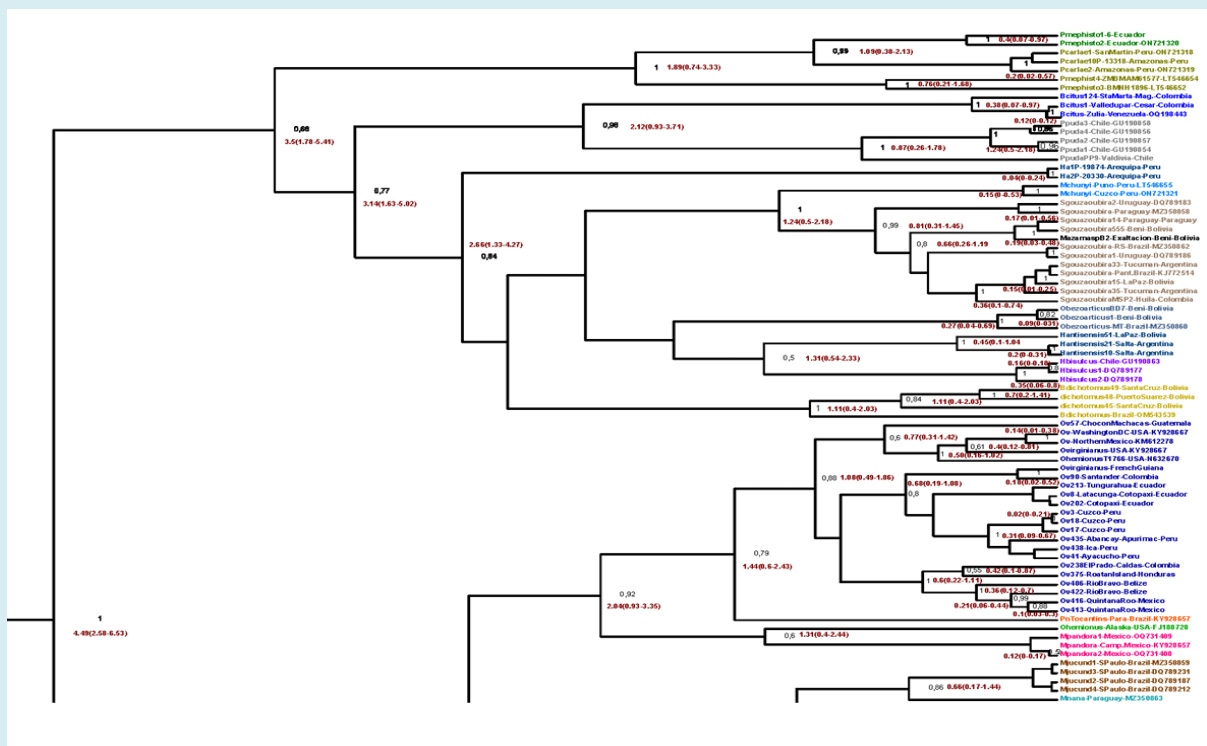


Figure 4: Maximum Likelihood (ML) tree for a total of 137 specimens of different species of Neotropical deer sequenced at the mitochondrial *Cyt-b* gene (BD2). Each species was indicated with different colors. A total of 91 brocket deer specimens were analyzed (*Subulo gouazoubira*, *Subulo chunyi*, *Passalites nemorivagus*, *Bisbalus citus*, *Mazama* (= *Andinocervus*) *rufina*, *Mazama pandora*, *Mazama temama*, *Mazama rufa*, *Mazama nana*, *Mazama jucunda*, and different groups of *Mazama americana*). The different groupings within the *Mazama americana* complex were marked. As outgroups, 21 *Odocoileus virginianus*, two *Odocoileus hemionus*, three *Ozotoceros bezoarticus*, seven *Pudella*, four *Blastoceros dichotomus*, five *Pudu*, five *Hippocamelus antisensis* and three *Hippocamelus bisulcus* specimens were used. Specimens with numerical codes, their sequences were obtained in GenBank. In nodes, bootstrap percentages.

BI tree (Figure 5) showed the separation of two large, distinct clades ($pp = 1$) corresponding to Blastocerina and Odocoileina, with one exception: *P. nemorivagus* was grouped with the latter, although with little significance ($pp = 0.44$). Within Blastocerina, the first ancestor to diverge was *Pudella* ($pp = 1$), with the same three subgroups found in the previous tree (*P. carlae* and two of *P. mephistophila*). Subsequently, with a strong association ($pp = 0.98$), the *B. citus* group ($pp = 1$) and the *Pudu puda* group ($pp = 1$) appeared, just as in the previous ML tree. Also, identically to what was observed in the previous tree, two very distinct clades were found on branches far removed from *Hippocamelus*. In one of them, the two specimens of *H. antisensis* from southern Peru (Arequipa) were found ($pp = 1$), and in another, the three specimens of *H. antisensis* from Bolivia and northern Argentina ($pp = 1$) and the three specimens of *H. bisulcus* ($pp = 1$) were found together, although the statistical value was not very high ($pp = 0.5$). Related to this second group of *Hippocamelus* was the clade with *O. bezoarticus* ($pp = 1$), although weakly ($pp = 0.29$). The association of the gray brocket deer *S. gouazoubira* ($pp = 0.99$) and *S. chunyi* ($pp = 1$) was very strong, as evidenced in all the other analyses ($pp = 1$). Within *S. gouazoubira*, exactly the same groupings were found as in the previous tree. The sister group to the latter grouping, comprised of polyphyletic *Hippocamelus*, *S. gouazoubira* + *S. chunyi*, and *O. bezoarticus*, was *B. dichotomus* ($pp = 1$). Within the Odocoileina clade, as previously mentioned, *P. nemorivagus* ($pp = 1$) appeared as the most divergent clade. It contained more subgroups than

the previous tree. The next grouping to diverge was *M. rufina* ($pp = 1$), exhibiting the same internal structure as the previous tree. The next major clade to diverge ($pp = 0.92$) showed two large groupings. On one hand ($pp = 0.92$), *O. virginianus* and a strange sequence of *P. nemorivagus*, previously discussed in another work ($pp = 0.79$), plus *O. hemionus* along with *M. pandora* ($pp = 0.6$). Although this association was not very strong, this was the second analysis in which we detected a relationship between *pandora* and *Odocoileus*. On the other hand, we found *M. temama* and the *M. americana* complex ($pp = 0.99$). The findings within *M. temama* were identical to those previously reported ($pp = 1$). The clade most closely related to *M. temama*, albeit with limited intensity ($pp = 0.27$), was *M. americana* I ($pp = 1$). Related to each other, but with moderate intensity ($pp = 0.5$), were *M. americana* III ($pp = 0.94$) and *M. americana* V ($pp = 1$). Next, we observed the divergence of *M. americana* VI ($pp = 0.7$), which contained two subgroups. One of these was highly significant ($pp = 1$), containing the trans-Andean specimen from Cauca (Colombia) along with some Amazonian specimens ($pp = 1$) and the specimen from the Bolivian Amazon ($pp = 1$) that originated from *M. americana* VII in BD1. Two other groupings, related to each other but weakly ($pp = 0.4$), were *M. americana* IV ($pp = 1$) and *M. americana* IIa (*M. nana* from Paraguay) ($pp = 0.86$) and *M. americana* IIb (*M. jucunda*) ($pp = 1$). Therefore, all the groupings found with the ML tree were exactly the same as those in the BI tree.



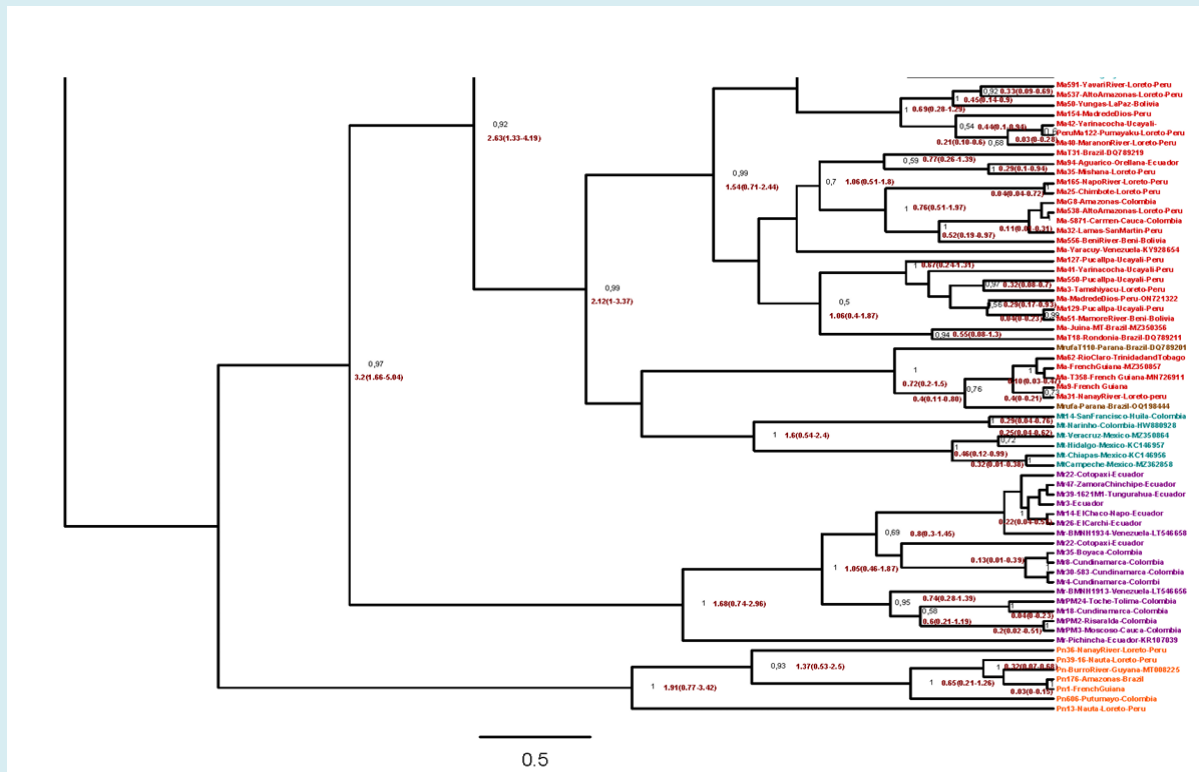


Figure 5: Bayesian inference tree for a total of 137 specimens of different species of Neotropical deer sequenced at the mitochondrial *Cyt-b* gene (BD2). Each species was indicated with different colors. A total of 91 brocket deer specimens were analyzed (*Subulo gouazoubira*, *Subulo chunyi*, *Passalites nemorivagus*, *Bisbalus citus*, *Mazama* (= *Andinocervus*) *rufina*, *Mazama pandora*, *Mazama temama*, *Mazama rufa*, *Mazama nana*, *Mazama jucunda*, and different groups of *Mazama americana*). The different groupings within the *Mazama americana* complex were marked. As outgroups, 21 *Odocoileus virginianus*, two *Odocoileus hemionus*, three *Ozotoceros bezoarticus*, seven *Pudella*, four *Blastoceros dichotomus*, five *Pudu*, five *Hippocamelus antisensis* and three *Hippocamelus bisulcus* specimens were used. Specimens with numerical codes, their sequences were obtained in GenBank. In nodes, posterior probabilities in black and, in red, temporal split estimations (and into parentheses, 95% High Posterior Density, HPD, as confidence intervals; values in millions of years). Psp = *Pudella carlae*; Pm = *Pudella mephistophelica*; Pp = *Pudu puda*; Bd = *Blastoceros dichotomus*; Ha = *Hippocamelus antisensis*; Pn = *Passalites nemorivagus*; Sg = *Subulo gouazoubira*; Sch = *Subulo chunyi*; Mr = *Mazama rufina*; Mt = *Mazama temama*; Ma = *Mazama americana*; Mnana = *Mazama nana*; Mrufa = *Mazama rufa*; Mjucunda = *Mazama jucunda*; Mpandora = *Mazama pandora*; Ov = *Odocoileus virginianus*; Ob = *Ozotoceros bezoarticus*; Bcitus = *Bisbalus citus*.

BAPS analyses (Figure 6) using BD2 yielded the following results. BAPS applied exclusively to specimens comprising *M. temama* and the *M. americana* complex (Figure 6a) showed that the optimal number of groupings was four [Log (marginal likelihood) of the optimal partition = -2,039.336, $p = 1$]. The first grouping to differentiate consisted of a single specimen, the red brocket deer from the northern Bolivian Amazon (*M. americana* VII in BD1 and included in *M. americana* VI in BD2); the second grouping corresponded to the specimens of *M. americana* I (*M. americana* "sensu stricto" and *M. rufa*); the third grouping included all the specimens of *M. temama*, while the fourth grouping comprised the remaining groups of the *M. americana* complex (*M. americana* IIa, IIb, III, IV, V, VI). As with BD1, BAPS tends to detect a smaller number of

significantly different groupings. The BAPS analysis applied to the aforementioned brocket deer species plus *Odocoileus* and *M. pandora* (Figure 6b) showed that the optimal number of groupings was five [Log (marginal likelihood) of the optimal partition = -3,534.316, $p = 1$]. The first grouping to differentiate consisted of all groups of the *M. americana* complex, except *M. americana* I; the second grouping was made up of all specimens of *O. virginianus* and *O. hemionus*; the third was composed of specimens of *M. americana* I; the fourth grouping included all specimens of *M. temama* (regardless of whether they were Colombian or Mexican); and the fifth grouping included *M. pandora*. In this case, this analysis did not associate *M. pandora* with *Odocoileus*. Finally, a third BAPS analysis investigated all brocket deer

species with *Odocoileus* (Figure 6c). This analysis showed that the optimal number of groupings was seven [Log (marginal likelihood) of the optimal partition = -6,898.053, $p = 0.999$]. The first grouping to differentiate consisted of *O. virginianus*, *O. hemionus*, and the unusual form of *P. nemorivagus* already discussed in another work; the second grouping was composed of *M. rufina* specimens; the third grouping consisted of all individuals from all groupings of

the *M. americana* complex; the fourth grouping included *P. nemorivagus* specimens; the fifth grouping was made up of *M. pandora* specimens (again, not associated with *Odocoileus*); the sixth grouping included *S. gouazoubira* and *S. chunyi* specimens (not differentiating them as in most other analyses); and the seventh grouping added *B. citus* specimens.

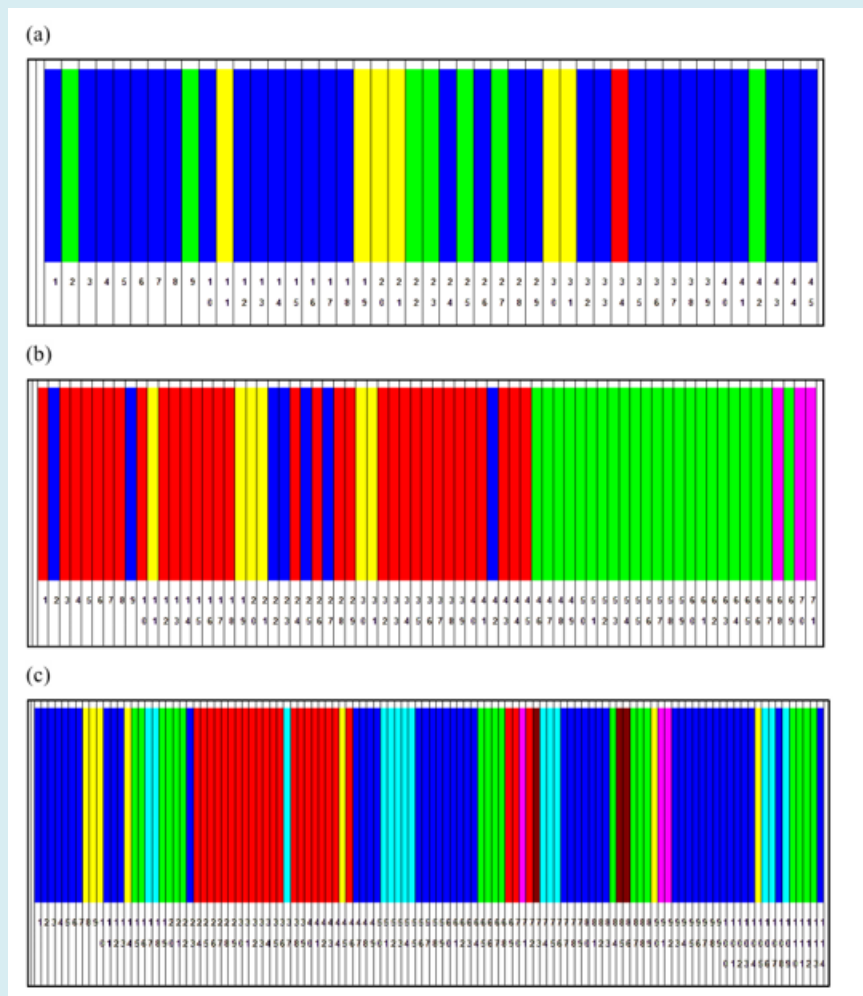
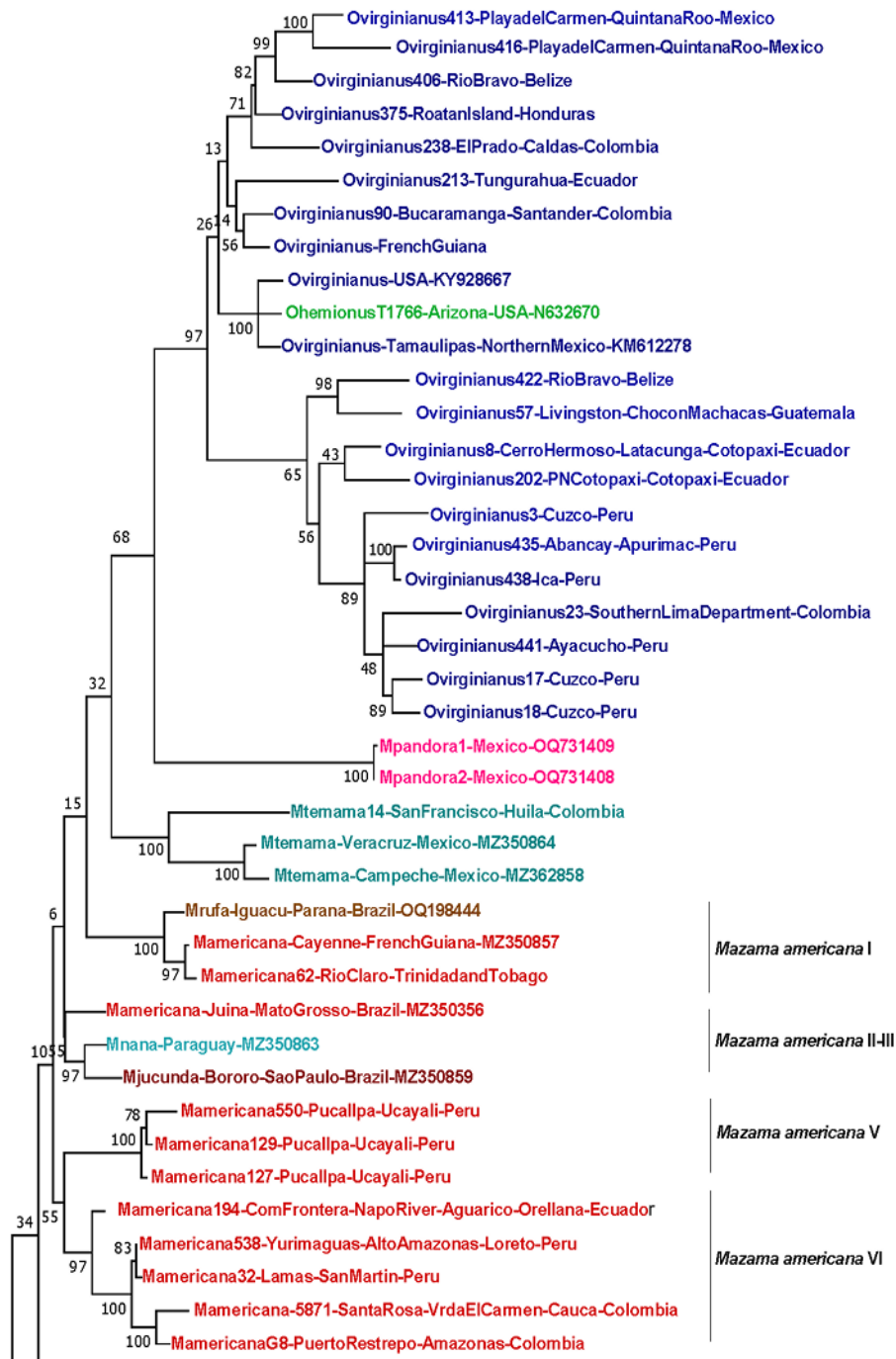


Figure 6: Different populations or taxa of Neotropical deer detected by means of BAPS at the mitochondrial *Cyt-b* gene (BD2). Each different color indicates a different population or taxa. (A) A sample of 45 specimens from *Mazama temama* and the *Mazama americana* complex. In red, one specimen of red brocket deer from northern Bolivian Amazon (*M. americana* VII in BD1 and *M. americana* VI in BD2); in blue, specimens of *M. americana* IIa, IIb, III, IV, V, and VI; in green, specimens of *M. temama*; in yellow, specimens of *M. americana* I (including *M. americana* I sensu stricto and *Mazama rufa*). (B) A sample of 71 specimens from *M. temama*, the *M. americana* complex, *Odocoileus virginianus*, and *Mazama pandora*. In pink, all the specimens of *M. pandora*; in yellow, specimens of *M. temama*; in blue, the specimens of *M. americana* I (including *M. americana* I sensu stricto and *Mazama rufa*); in green, all the specimens of *O. virginianus* and *O. hemionus*; in red, all the specimens of *M. americana* IIa, IIb, III, IV, V, and VI. (C) A sample of 114 specimens from of all the different taxa of brocket deer plus *O. virginianus*. In brown, specimens of *M. pandora*; in pink, specimens of *B. citus*; in blue, all the specimens of *M. temama* and the *M. americana* complex; in green, specimens of *M. rufina*; in yellow, specimens of *P. nemorivagus*; in red, specimens of *O. virginianus* and *O. hemionus*; in light, specimens of *S. gouazoubira* and *S. chunyi*.

Phylogenetic Analysis of Brocket Deer Using BD3

The ML tree (Figure 7) differentiated *Blastocerina* and *Odocoileina* but included *M. rufina* as a taxon within the *Blastocerina*. *S. chunyi* was absent from this tree because we were unable to generate a mitogenome for this species, nor could we find one in the genetic databases. *S. gouazoubira* (100%) proved to be a well-characterized group, containing

the same two subgroups observed in BD2. Specimens from the western South American geographic area, mostly related to the Andes Mountains, including the specimen from the Huila department (Colombia) already mentioned in the other databases (73%), formed their own group. Other specimens from the more central and eastern areas of southern South America formed their own group (53%).



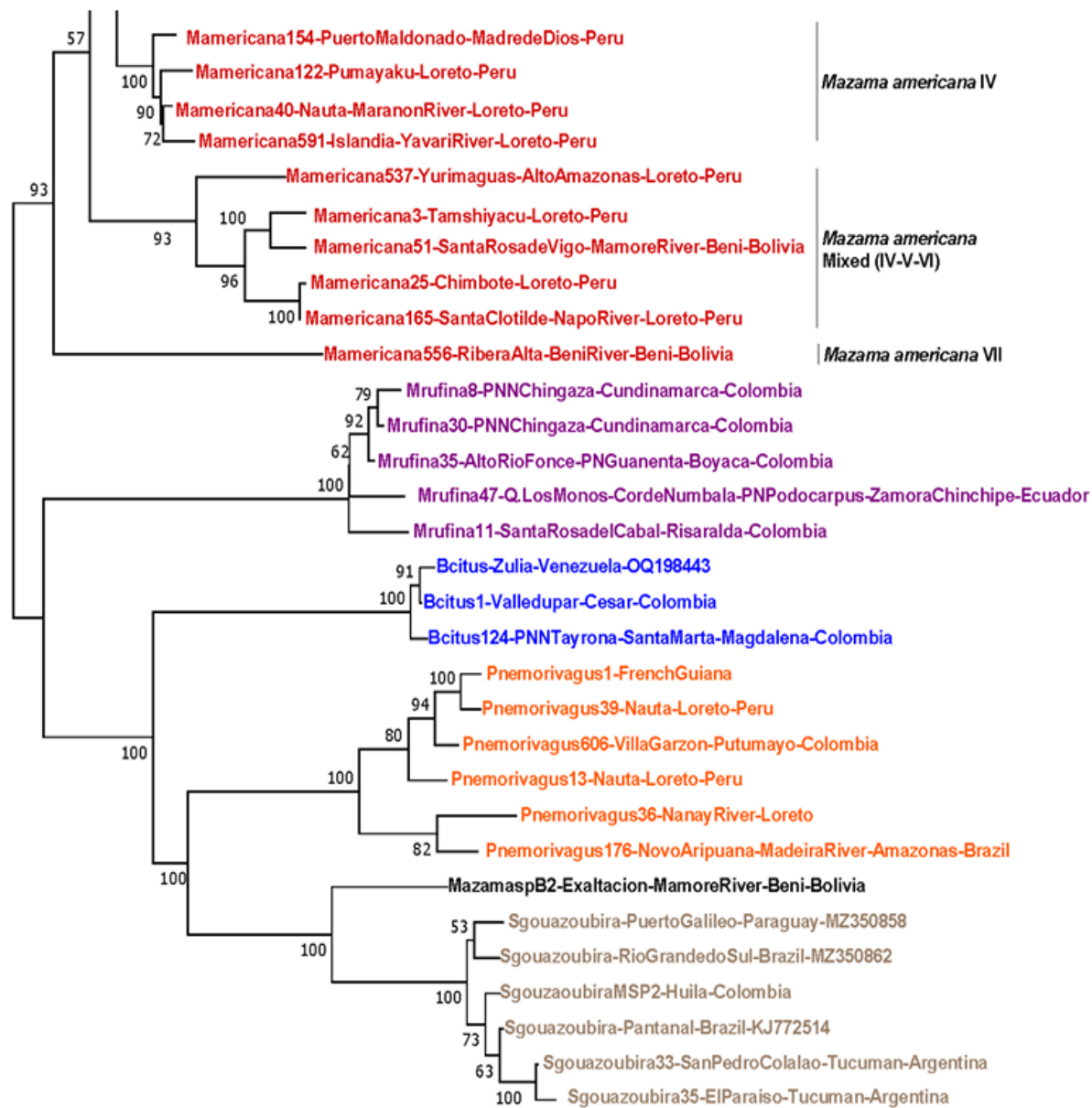


Figure 7: Maximum Likelihood (ML) tree for a total of 72 specimens of different species of Neotropical deer sequenced at their whole mitogenomes (BD3). Each species was indicated with different colors. A total of 50 brocket deer specimens were analyzed (*Subulo gouazoubira*, *Passalites nemorivagus*, *Bisbalus citus*, *Mazama* (= *Andinocervus*) *rufina*, *Mazama pandora*, *Mazama temama*, *Mazama rufa*, *Mazama nana*, *Mazama jucunda*, and different groups of *Mazama americana*). The different groupings within the *Mazama americana* complex were marked. As outgroups, 21 *Odocoileus virginianus* and one *Odocoileus hemionus*. Specimens with numerical codes, their sequences were obtained in GenBank. In nodes, bootstrap percentages.

In this instance, the sister group to *S. gouazoubira* was the “rare” specimen from Exaltación, Mamoré River (Bolivia). For BD1 and BD2, we observed its different position depending on the procedures used. Here it appears as a distinct taxon,

although we were unable to obtain a complete mitogenome from an *O. bezoarticus* specimen for comparison. The next grouping of gray brocket deer was that corresponding to *P. nemorivagus* (100%). Within it, two subgroups were

observed, as already seen with the other two databases, but it was not as clear that these were two geographically distinct subgroups as in the preceding cases. One subgroup contained specimens from the western Amazon (Colombia, Peru) and French Guiana (80%), and the other also from the western Amazon (Peru) and central Brazilian Amazon (82%). The next brocket deer taxon was *B. citus* (100%) and the last taxon associated with Blastocerina, as already mentioned a few lines above, was *M. rufina* (100%). On this occasion, due to the smaller number of specimens of this taxon that could be analyzed, the initial divergence of the ancestors of the Ecuadorian specimens could not be observed. Notwithstanding, a strong association of specimens from Cundinamarca and Boyacá in the Colombian eastern mountain range was observed (92%). Within the Odocoileina, the first to diverge was the ancestor of the specimen from the northern Bolivian Amazon, which in BD1 was included in *M. americana* VII and which in BD2, due to the absence of the other *M. americana* VII specimens, was integrated into *M. americana* VI (although the BAPS analysis continued to show its differentiation) (93%). Therefore, this mitogenomic tree continued to show the presence of *M. americana* VII despite the substantially smaller number of specimens of the *M. americana* complex analyzed. Next, a grouping (93%) appeared that mixed some specimens of *M. americana* VI (100%) with some specimens of *M. americana* V (100%) and one specimen of *M. americana* IV. Subsequently, the remaining *M. americana* IV (100%), the remaining *M. americana* VI (97%) appeared clearly delineated, including the strong association of a specimen from the Colombian Amazon with the trans-Andean specimen from Cauca (100%), and the remaining *M. americana* V (100%). Later, we observed the emergence, again as in BD1, of *M. americana* II-III (55%; we already observed in BD2 how, by including more specimens of the three species contained in this grouping, it separated into more significant groupings) and *M. americana* I (with *M. americana* “sensu stricto” and *M. rufa*) (100%). *M. temama* continued to show its integrity (100%) with internal differentiation between one Colombian specimen and two Mexican specimens (100%). In this analysis, two specimens of *M. pandora* (100%) constituted the sister group to *O. virginianus*, although the bootstrap (68%) was not extremely high. Finally, the clade containing all the *O. virginianus* included in this analysis was observed (97%).

The BI tree (Figure 8) showed a very similar picture to the previous tree, with one important difference. Blastocerina and Odocoileina were clearly differentiated ($pp = 1$), and, as in the ML tree, *M. rufina* ($pp = 1$) was the most divergent clade

within Blastocerina ($pp = 0.95$). Within it, a strong association between individuals from the Colombian Eastern Cordillera was again determined ($pp = 1$). The next taxon to diverge was *B. citus* ($pp = 1$). Subsequently, a common ancestor was observed ($pp = 0.94$) that gave rise to *S. gouazoubira* ($pp = 1$) and *P. nemorivagus* ($pp = 1$). Within *S. gouazoubira*, the same two subgroups were detected as in the previous tree, reaffirming the existence of a subgroup specific to the westernmost part of South America, associated to some extent towards the Andes, which included the Colombian specimen from Huila ($pp = 0.99$), and another association located in central-eastern southern South America ($pp = 0.58$). The “rare” specimen from Exaltación, Mamoré River (Bolivia) continued to behave as a sister taxon to *S. gouazoubira* ($pp = 1$). Subgroups as clearly as those observed in BD1 and BD2 were not observed within the clade of *P. nemorivagus*. In Odocoileina, two major groupings were distinguished from a common ancestor ($pp = 0.89$). On one hand, *Odocoileus* together with *M. pandora*, and on the other, *M. temama* and all but one of the groupings of the *M. americana* complex. In the first grouping, *M. pandora* was the sister group to *Odocoileus*, but with a relatively low significance value ($pp = 0.51$). Within *O. virginianus*, a phenomenon was observed that has not been seen in any other analysis, but which had already been detected by Ruiz-García, et al. [116], and it was the inclusion of a group of *M. americana* specimens (the grouping that in the previous tree was made up of various specimens of *M. americana* IV, V, and VI) ($pp = 1$) related to the *O. virginianus* grouping from the south-central Peruvian Andes ($pp = 1$), although the significance of this union was not extremely high ($pp = 0.6$) but it is highly noteworthy. In the second grouping, *M. temama* ($pp = 1$) was the first to diverge ($pp = 0.9$), separating, as in all previous analyses, the Mexican specimens ($pp = 1$) from the Colombian ones. Next, the ancestor of *M. americana* I (*M. americana* “sensu stricto” plus *M. rufa*) ($pp = 1$) diverged. Unlike the previous tree, in this one, *M. americana* III (a specimen of *M. americana* from Juina, Mato Grosso, Brazil) ($pp = 0.6$) was differentiated from *M. americana* II ($pp = 1$) (the two previously discussed species of *M. nana* from Paraguay and *M. jucunda* from Brazil). Similarly, as in the previous tree and in the earlier analyses, the presence of *M. americana* IV ($pp = 1$) and *M. americana* VI ($pp = 0.88$) was observed, which in this case included the specimen of *M. americana* VII (northern Bolivian Amazon), unlike the previous tree, and continued to show the strong relationship of an Amazonian specimen with a trans-Andean specimen from Cauca ($pp = 1$), and of *M. americana* V ($pp = 1$).

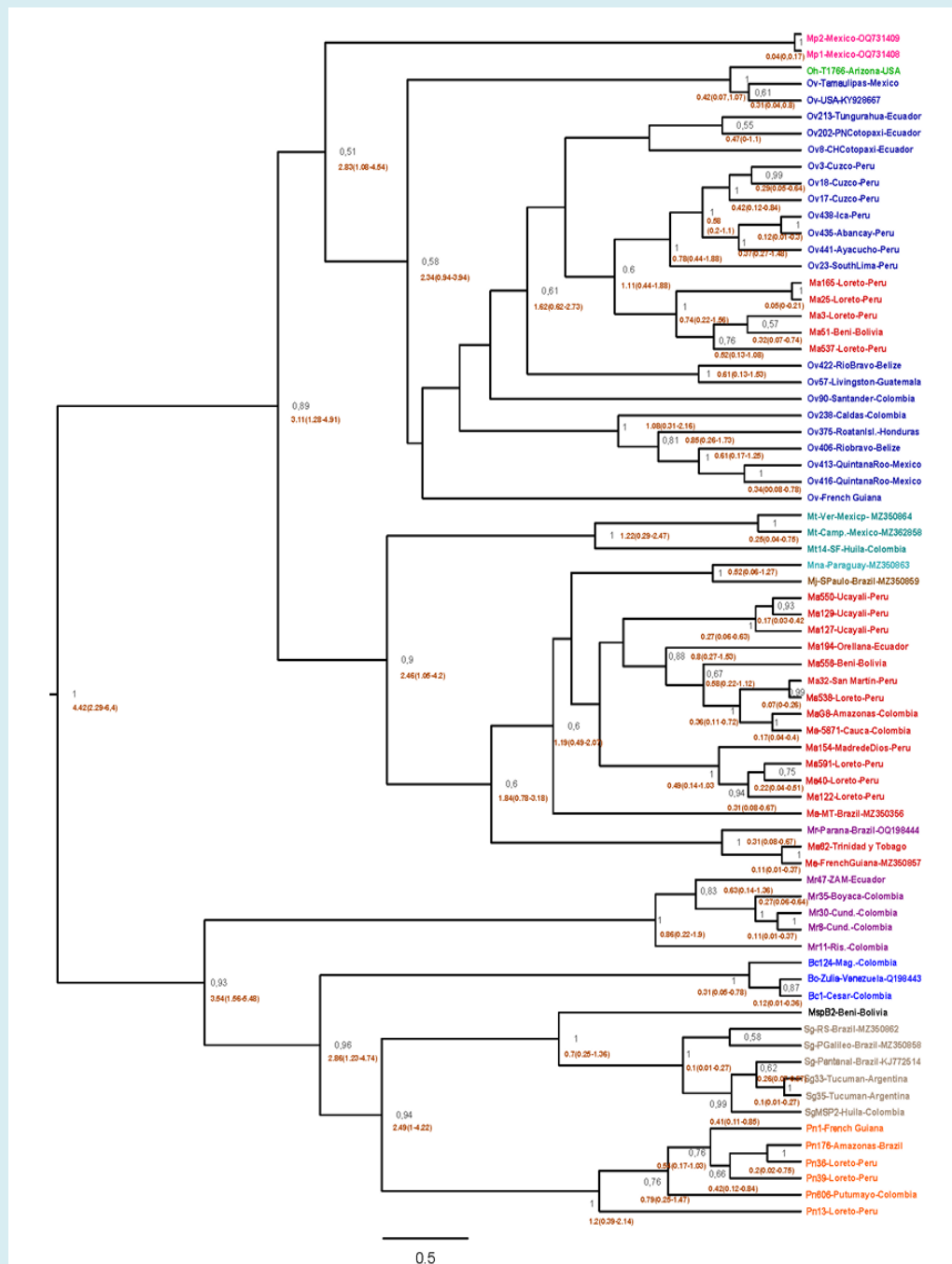


Figure 8: Bayesian inference (BI) tree for a total of 72 specimens of different species of Neotropical deer sequenced at their whole mitogenomes (BD3). Each species was indicated with different colors. A total of 50 brocket deer specimens were analyzed (*Subulo gouazoubira*, *Passalites nemorivagus*, *Bisbalus citus*, *Mazama* (= *Andinocervus*) *rufina*, *Mazama pandora*, *Mazama temama*, *Mazama rufa*, *Mazama nana*, *Mazama jucunda*, and different groups of *Mazama americana*). The different groupings within the *Mazama americana* complex were marked. As outgroups, 21 *Odocoileus virginianus* and one *Odocoileus hemionus*. Specimens with numerical codes, their sequences were obtained in GenBank. In nodes, posterior probabilities in black and, in red, temporal split estimations (and into parentheses, 95% High Posterior Density, HPD, as confidence intervals; values in millions of years). Pn = *Passalites nemorivagus*; Sg = *Subulo gouazoubira*; Mr = *Mazama rufina*; Mt = *Mazama temama*; Ma = *Mazama americana*; Mnana = *Mazama nana*; Mrufa = *Mazama rufa*; Mjucunda = *Mazama jucunda*; Mpandora = *Mazama pandora*; Ov = *Odocoileus virginianus*; Bcitus = *Bisbalus citus*.

Although the number of red brocket deer in the *M. americana* complex was significantly lower in BD3 than in the other two databases, the same groupings were detected, with the exception of the absence of *M. americana* VIII (present only in BD1 and sometimes more closely associated with *B. citus*), and the alternating presence or absence of *M.*

americana VII (which was also observed in BD2). The most significant difference was the appearance of a grouping that simultaneously contained specimens of *M. americana* IV, V, and VI, which could indicate greater diversity within these groups than previously assumed in BD1 and BD2.

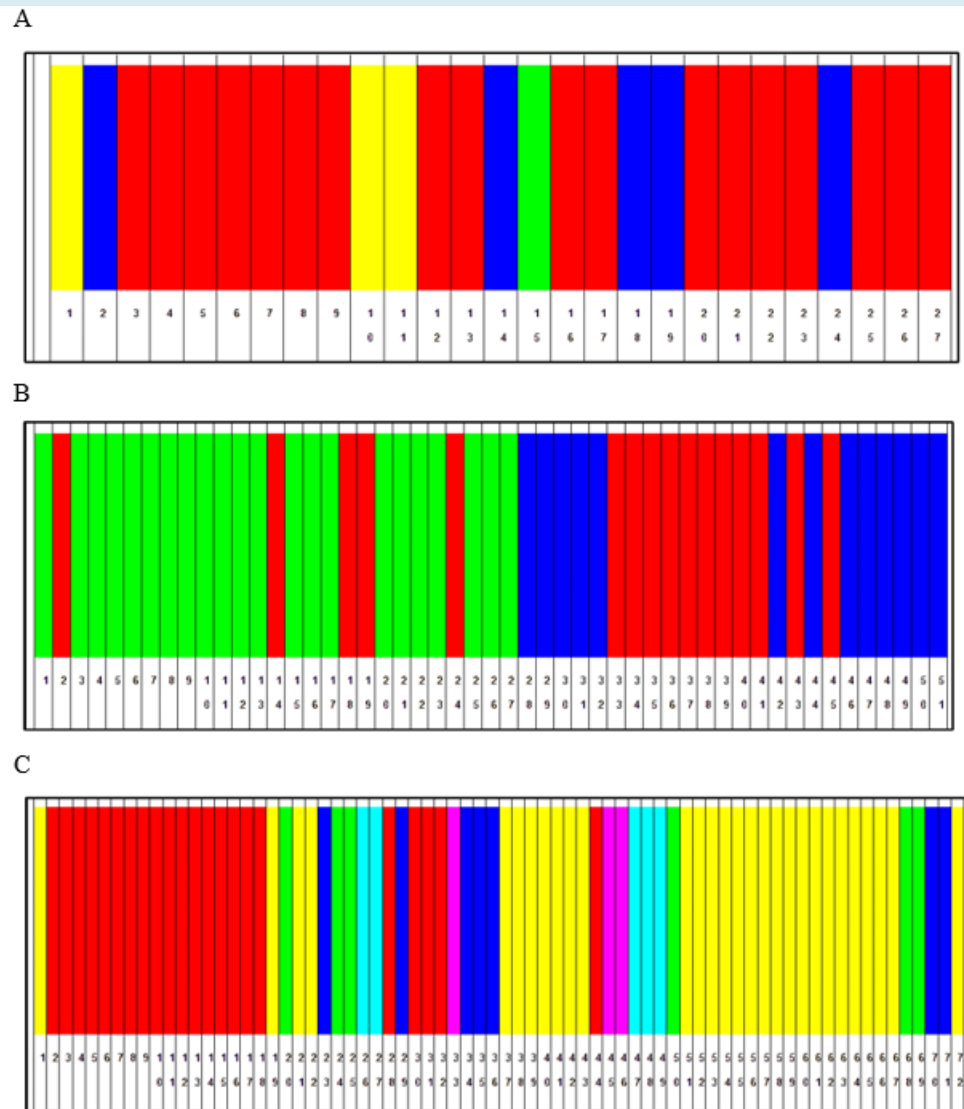


Figure 9: Different populations or taxa of Neotropical deer detected by means of BAPS at the whole mitogenomes (BD3). Each different color indicates a different population or taxa. (A) A sample of 27 specimens from *Mazama temama* and the *Mazama americana* complex. In green, one specimen of red brocket deer from northern Bolivian Amazon (*M. americana* VII); in yellow, specimens of *M. temama*; in blue, specimens mixed from *M. americana* IV, V, and VI; in red, remainder specimens of *M. americana* I, II-III, IV, V, and VI. (B) A sample of 51 specimens from *M. temama*, the *M. americana* complex, *Odocoileus virginianus*, and *Mazama pandora*. In red, the mixed group of *M. americana* IV, V, VI together specimens of *O. virginianus* from Peruvian Andes, Ecuadorian Andes and Central America; in blue, other specimens of *O. virginianus* plus *M. pandora*; in green, all the remainder *M. americana* together *M. temama*. (C) A sample of 72 specimens from of all the different taxa of brocket deer plus *O. virginianus*. In red, specimens of *O. virginianus*; in green, specimens of *P. nemorivagus*; in pink, specimens of *B. citus*; in blue, specimens of *S. gouazoubira* plus the “rare” specimen of Exaltación, Beni (Bolivia); in light blue, specimens of *M. rufina*; in yellow, specimens of all groups of *M. americana*, *M. temama*, and *M. pandora*.

BAPS analyses (Figure 9) using mitogenomes revealed the following. BAPS applied exclusively to specimens comprising *M. temama* and the *M. americana* complex (Figure 9a) showed that the optimal number of groupings was four [Log (marginal likelihood) of the optimal partition = -37,660.123, $p = 1$]. The first grouping consisted of a large portion of the brocket deer from the *M. americana* complex (*M. americana* I, II-III, IV, V, and VI); the second grouping differentiated the specimen from northern Bolivian Amazonia, which was part of *M. americana* VII; the third grouping consisted of intermixed specimens of *M. americana* IV, V, and VI, which is exclusive to BD3; and the fourth grouping was formed by *M. temama*. The BAPS analysis applied to the aforementioned brocket deer plus *Odocoileus* and *M. pandora* (Figure 9b) showed that the optimal number of groupings was three [Log (marginal likelihood) of the optimal partition = -86,230.346, $p = 1$]. The first grouping to differentiate consisted of the brocket deer from the mixed grouping (*M. americana* IV, V, and VI) and a group of *O. virginianus* made up of specimens from the south-central Peruvian Andes, plus two specimens from Ecuador and two Central American specimens; the second grouping consisted of all the red brocket deer, including *M. temama*, except those associated with some *O. virginianus*, and the third grouping was made up of the remaining *O. virginianus*, plus *M. pandora*. The third BAPS analysis investigated all brocket deer forms with *Odocoileus* (Figure 9c). This analysis showed that the most optimal number of groupings was six [Log (marginal likelihood) of the most optimal partition = -142,961.272, $p = 1$]. The first grouping to differentiate consisted of all the *O. virginianus*; the second group included all specimens of *P. nemorivagus*; the third group contained *S. gouazoubira*, plus the “rare” specimen from Exaltación, Mamoré River (Bolivia); the fourth group comprised all the red brocket deer of the *M. americana* complex, plus *M. temama* and *M. pandora*; the fifth group consisted of *B. citus*; and the sixth group of *M. rufina* specimens. It is evident that the groupings differentiated by BAPS were based on which taxa are included in each of the comparative analyses.

Divergence Times among Different Brocket Deer Taxa

The BI tree estimated the onset of haplotype divergence in the ancestor of modern Neotropical deer at approximately 4.47 mya [95% Confidence Interval (95% CI) = 2.37–6.6 mya] for BD1, 4.49 mya (2.58–6.53 mya) for BD2, and 4.42 mya (2.29–6.4 mya) for BD3. Therefore, the three estimates were virtually identical, indicating the early Pliocene phase as the beginning of mitochondrial diversification in modern Neotropical deer. Regarding the gray brocket deer (Blastocercina), the ancestor of *S. gouazoubira* + *S. chunyi* would have begun to diversify around 1.57 mya (0.66–2.77 mya) for BD1, 1.24 mya (0.5–2.18 mya) for BD2, and 1.44 mya (0.5–2.68 mya) for BD3. All estimates were very similar and

indicate the time of diversification in the Middle Pleistocene, coinciding with important climatological changes. The main nucleus of *P. nemorivagus* would have begun its diversification around 0.95 mya (0.39–1.76 mya) for BD1 [although the divergence of the ancestors of two specimens associated with *P. nemorivagus* but which may represent different taxa, one from the Colombian Amazon and the other from the Pacific coast of Ecuador, was older, 1.55 mya (0.65–2.56 mya)], 1.91 mya (0.77–3.42 mya) for BD2, and 1.2 mya (0.39–2.14 mya). The estimates would be relatively similar and would also be chronologically situated in the Middle Pleistocene. In the case of *B. citus*, the analysis with BD1 fragmented this taxon and included two specimens that in other analyses carried out with BD1 constituted *M. americana* VIII. Taking that difficulty into consideration, the divergence related to *B. citus* ranged from 2.09 mya (0.81–3.61 mya) to 1.23 mya (0.42–2.31 mya) for BD1, 2.12 mya (0.93–3.71 mya) for BD2, and 2.86 mya (1.23–4.74 mya) for BD3 in the early Pleistocene phase. The onset of mitochondrial diversification in the ancestors of the most divergent Ecuadorian specimens of *M. rufina* was estimated at around 2.48 mya (1.15–4.29 mya) for BD1, and 1.68 mya (0.74–2.96 mya) for BD2 (it could not be estimated for BD3 because none of the highly divergent Ecuadorian specimens were analyzed). This indicated a divergence in the early Pleistocene. For the core group of most *M. rufina* specimens, the divergence point was established at 1.11 mya (0.52–1.89 mya) for BD1, 1.05 mya (0.46–1.87 mya) for BD2, and 0.86 mya (0.22–1.9 mya) for BD3, all around 1 million years ago (Middle Pleistocene). The diversification point of the ancestor of the present-day *M. pandora* was estimated at around 2.52 mya (1.1–4 mya) for BD1, 1.31 Ma (0.4–2.44 mya) for BD2, and 2.83 mya (1.08–4.54 mya) for BD3. In this case, there would be somewhat more heterogeneity in the estimated origin of the ancestor of *M. pandora*, ranging from the beginning of the Pleistocene to the Middle Pleistocene (the small number of specimens analyzed for this taxon may be the cause of this diversity of estimates). Estimates of temporal divergence for *O. virginianus* were interesting to compare with those of the brocket deer. The mitochondrial divergence of modern white-tailed deer was estimated at 1.64 mya (0.67–2.86 mya) for BD1, 1.44 mya (if we accept that the *P. nemorivagus* specimen from the Tocantins River carries mitochondrial DNA from *O. virginianus*; 0.6–2.43 mya) for BD2, and 2.34 mya (0.94–3.94 mya) for BD3, so the mitochondrial diversification of modern *O. virginianus* would have occurred between the beginning of the Pleistocene and the Middle Pleistocene. In the case of *M. temama*, the oldest ancestors were related to specimens from the Colombian Chocó (Pacific area) and diverged around 1.45 mya (0.55–2.58 mya). Therefore, the ancestors of the Colombian and Ecuadorian specimens diverged earlier than the ancestors of the Mexican specimens for BD1. For BD2, the diversification time was estimated at 1.36 mya (0.54–2.4 mya) and at 1.22 mya (0.29–

2.47 mya) for BD3. Therefore, for this taxon, there was unanimity in determining mitochondrial diversification in the Middle Pleistocene with its origin in the Pacific region of what is now Colombia and subsequent expansion into Central and North America. The diversification of the *M. americana* complex for BD1 would have begun 1.83 mya (0.83–2.98 mya), 1.54 mya (1–3.37 mya) for BD2, and 1.84 mya (0.78–3.18 mya) for BD3. Therefore, all estimates were extremely similar in detecting the initial diversification of the *M. americana* complex shortly before the start of the Middle Pleistocene. Within this complex, *M. americana* IV began its mitochondrial diversification around 0.6 mya (0.13–1.39 mya) for BD1, 0.69 mya (0.28–1.29 mya) for BD2, and 0.49 mya (0.14–1.03 mya) for BD3, with the estimates being extremely similar and coinciding with one of the strongest glacial periods that occurred in the last phase of the Pleistocene. *M. americana* I began its mitochondrial diversification around 0.91 mya (0.3–1.78 mya) for BD1, 0.72 mya (0.2–1.5 mya) for BD2, and 0.47 mya (0.09–1.18 mya) for BD3. Within *M. americana* I, *M. americana* “sensu stricto” and *M. rufa* were distinguished. The latter is thought to have derived from the former, and its mitochondrial diversification was notably more recent for BD1, occurring around 0.16 mya (0.01–0.52 mya), 0.4 mya (0.11–0.89 mya), and 0.47 mya (0.09–1.18 mya), during the late Pleistocene. It was also interesting to note that the diversification of this group of red brocket deer on the Caribbean island of Trinidad was estimated at around 0.18 mya (0–0.62 mya) for BD1, and 0.08 mya (0–0.24 mya) for BD2. A corresponding estimate for BD3 could not be obtained, as the mitogenome could not be obtained from any brocket deer on the island of Trinidad. *M. americana* VI began its mitochondrial diversification at around 1.09 mya (0.43–1.94 mya) for BD1, 1.06 mya (0.51–1.8 mya) for BD2, and 0.8 mya (0.27–1.53 mya) for BD3, with all values being very similar around the end of the Middle Pleistocene. Within *M. americana* VI, an interesting association was detected between an Amazonian specimen and the previously mentioned trans-Andean specimen from Cauca (Colombia). BD1 estimated this relationship between a cis-Andean specimen and the trans-Andean specimen at 0.17 mya (0.02–0.45 mya), while the estimate was 0.02 mya (0–0.13 mya) for BD2, and 0.17 mya (0.04–0.4 mya). Therefore, the appearance of brocket deer from this group in the trans-Andean region occurred during an interstadial period during the last major glaciation of the Pleistocene. The diversification of *M. americana* V began 0.82 mya (0.3–1.53 mya) for BD1, 0.67 mya (0.17–1.1 mya) for BD2, and 0.27 mya (0.06–0.63 mya). In this case, the heterogeneity of values was related to the small number of specimens analyzed for BD3. For the *M. americana* II–III grouping, BD1 yielded an initial diversification value of 0.64 mya (0.12–1.45 mya) for the ancestor of *M. americana* from Juína, Mato Grosso (Brazil), while the split that gave rise to *M. nana* from Paraguay and *M. jucunda* was established around 0.32 mya

(0.03–0.89 mya). In the case of BD2, this group split into *M. americana* IIa, IIb and III. *M. americana* III with BD2 showed an estimated time diversification between *M. americana* from Juína (Matto Grosso, Brazil) and *M. americana* from Rondonia (Brazil) of 0.55 mya (0.08–1.3 mya), while for the time split between *M. americana* IIa (*M. nana* from Paraguay) and *M. americana* IIb (*M. jucunda*) it was estimated at 0.66 mya (0.17–1.44 mya) and the diversification within *M. americana* IIb was 0.11 mya (0.01–0.35 mya). For BD3, *M. americana* III and *M. americana* II diverged, with the origin of the ancestor of the former estimated at 1.47 mya (0.62–2.61 mya), meanwhile the divergence of *M. nana* from Paraguay and *M. jucunda* into *M. americana* II was estimated at 0.52 mya (0.06–1.27 mya). Finally, for BD3, a grouping was formed that presented an intermingling of specimens that in other analyses were associated with *M. americana* IV, V, and VI. Likewise, this brocket deer assemblage was associated with a specific group of *O. virginianus* from the central and southern Peruvian Andes. The common ancestor (or point in time when genetic introgression occurred from one taxon to another) of this association was estimated to be around 1.11 mya (0.44–1.88 mya), while mitochondrial diversification within this brocket deer group was established 0.74 mya (0.22–1.56 mya).

Therefore, it is clear that mitochondrial diversification was older in gray brocket deer, *M. rufina*, *M. pandora*, and *Odocoileus* than in brocket deer of the *M. americana* complex.

Genetic Heterogeneity Analysis among the Brocket Deer Analyzed

In the case of the genetic heterogeneity analyses, these were only carried out with BD1 and BD2 because they contained the largest number of specimens for each of the species and taxa of brocket deer used. Tables 2 & 3 show the overall genetic heterogeneity for the gray brocket deer (the “rare” specimen from Exaltación, Bolivia, *S. gouazoubira*, *S. chunyi*, *B. citus*, *P. nemorivagus*) for BD1 and BD2, respectively. For BD1, all statistics were highly significant (e.g., $\gamma_{ST} = 0.699$, $p = 0.001$; $F_{ST} = 0.707$, $p = 0.001$, with $N_m = 0.21$, indicating extremely low theoretical gene flow among the taxa involved), as was the case for BD2 (e.g., $\gamma_{ST} = 0.694$, $p = 0.001$; $F_{ST} = 0.793$, $p = 0.001$, with $N_m = 0.11$ and $N_m = 0.07$, respectively). Therefore, both BDs showed very strong genetic heterogeneity among the brocket deer belonging to Blastocercina. The pairwise heterogeneity analysis for both BDs can be seen in Tables 4 and 5. For BD1, all pairs of F_{ST} values were very high and significant. The highest values corresponded to the pairs *B. citus* vs. *S. gouazoubira* ($F_{ST} = 0.835$, $p = 0.0001$), the rare Bolivian brocket deer vs. *B. citus* ($F_{ST} = 0.792$, $p = 0.0001$), and the rare Bolivian brocket deer vs. *S. gouazoubira* ($F_{ST} = 0.778$, $p = 0.0001$). The lowest value corresponded to *S. chunyi* vs. *S. gouazoubira* ($F_{ST} = 0.613$, p

= 0.001). The absolute genetic distance (Da) values for this dataset ranged from 7.38% (*B. citus* vs. *S. chunyi*) to 3.48% (*S. gouazoubira* vs. *P. nemorivagus*). For BD2, the F_{ST} values for species pairs were even higher than for BD1. The highest values corresponded to *B. citus* vs. *S. chunyi* ($F_{ST} = 0.953$, $p = 0.0001$) and *B. citus* vs. *S. gouazoubira* ($F_{ST} = 0.894$, $p = 0.0001$), and the lowest value corresponded to *S. gouazoubira*

vs. *P. nemorivagus* ($F_{ST} = 0.660$, $p = 0.001$). The highest Da values also corresponded to *B. citus* vs. *S. chunyi* (9.3%) and *B. citus* vs. *S. gouazoubira* (8.89%), while the lowest value corresponded to *S. gouazoubira* and *S. chunyi* (2.43%). Therefore, for both BDs, the most differentiated taxon within the gray brocket deer was *B. citus*.

Genetic heterogeneity and gene flow statistics	Values	Probabilities
χ^2	175.000 df = 136	0.032*
H_{ST}	0.023	0.0025**
K_{ST}	0.656	0.00001**
K_{ST*}	0.348	0.00001**
Z_S	111.767	0.00001**
Z_{S*}	4.339	0.00001**
S_{nn}	0.975	0.00001**
γ_{ST}	0.699	0.001**
F_{ST}	0.707	0.001*
Nm (γ_{ST})	0.21	
Nm (F_{ST})	0.21	

Table 2: Overall genetic heterogeneity among gray brocket deer species (*Passalites nemorivagus*, *Subulo chunyi*, *Subulo gouazoubira*, *Bisbalus citus* and a “rare” specimen of gray brocket deer from Mamoré River, Beni, Bolivia) at the BD1 (mitochondrial ND5 and 12S rRNA genes). Nm = gene flow; df = degree of freedom; * $p < 0.05$; ** $p < 0.01$ (highly significant).

Genetic heterogeneity and gene flow statistics	Values	Probabilities
χ^2	86.000 df = 58	0.029*
H_{ST}	0.041	0.0055**
K_{ST}	0.661	0.00001**
K_{ST*}	0.289	0.00001**
Z_S	52.886	0.00001**
Z_{S*}	3.652	0.00001**
S_{nn}	1	0.00001**
γ_{ST}	0.694	0.001**
F_{ST}	0.793	0.001*
Nm (γ_{ST})	0.11	
Nm (F_{ST})	0.07	

Table 3: Overall genetic heterogeneity among gray brocket deer species (*Passalites nemorivagus*, *Subulo chunyi*, *Subulo gouazoubira*, *Bisbalus citus* and a “rare” specimen of gray brocket deer from Mamoré River, Beni, Bolivia) at the BD2 (mitochondrial Cyt-b gene). Nm = gene flow; df = degree of freedom; * $p < 0.05$; ** $p < 0.01$ (highly significant).

In the case of red brocket deer (*Odocoileina*), we analyzed the genetic heterogeneity between *M. temama* and the different groups within the *M. americana* complex. Tables 6 (BD1) and 7 (BD2) show the overall genetic heterogeneity for these red brocket deer taxa. For BD1 (the *M. americana* VII group was annexed to *M. americana* VI), all statistics showed

significant values (e.g., $\gamma_{ST} = 0.434$, $p = 0.01$; $F_{ST} = 0.470$, $p = 0.01$, with Nm = 0.65 and Nm = 0.56, respectively). These values were even higher for BD2 (e.g., $\gamma_{ST} = 0.596$, $p = 0.001$; $F_{ST} = 0.598$, $p = 0.001$, with Nm = 0.34 for both statistics). By pairs of taxa (Table 4), BD1 showed that the highest F_{ST} values were those corresponding to *M. americana* VIII with most of

the other groupings of that complex, *M. americana* I vs. *M. americana* IV ($F_{ST} = 0.633$, $p = 0.001$), and *M. americana* II-III vs. *M. americana* IV ($F_{ST} = 0.607$, $p = 0.001$). The two lowest, but also significant, values corresponded to *M. americana* V vs. *M. americana* II-III ($F_{ST} = 0.298$, $p = 0.043$) and *M. americana* V vs. *M. americana* VI ($F_{ST} = 0.327$, $p = 0.035$). Therefore, BD1 revealed strong genetic heterogeneity among the different groups within the *M. americana* complex, often greater among these groups than between some of them and *M. temama*, a species fully accepted by researchers. The Da values for BD1 were lower than those found among the gray brocket deer taxa. The highest Da values were estimated

between *M. americana* VIII and the remaining groups of the *M. americana* complex (between 2.78% and 2.36%). The Da values between *M. temama* and the remaining *M. americana* groupings (excluding *M. americana* VIII) ranged from 1.58% (*M. temama* vs. *M. americana* VI) to 0.61% (*M. americana* II-III and *M. americana* V). This analysis also compared F_{ST} and Da values between all these taxa and *O. virginianus*, *M. pandora*, and *M. rufina*. *O. virginianus* showed lower genetic distances from *M. temama* and all the *M. americana* groupings (except *M. americana* VIII) (1.86%-1.56%) than from *M. pandora* (2.96%), *M. rufina* (5.44%), and *M. americana* VIII (2.94%).

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–	5.9	3.48	4.32	3.49	4.76	5.84	4.35	4.63	4.84	5.2	4.62	4.8	4.68	4.31
2	0.628* (0.485)*	–	4.4	7.38	6.91	7.06	8.03	6.83	7.11	7.26	7.61	6.67	7.28	6.93	7.66
3	0.663* (0.537)*	0.613* (0.513)*	–	5.54	3.4	5.24	5.91	4.47	4.43	4.35	5.04	4.22	4.72	4.25	4.88
4	0.705* (0.549)*	0.723* (0.686)*	0.835* (0.708)*	–	3.83	5.93	5.53	4.56	4.86	4.73	4.6	5	5.2	2.24	4.29
5	0.674* (0.399)*	0.719* (0.685)*	0.788* (0.506)*	0.792* (0.666)*	–	5.76	5.78	3.35	4.16	3.74	4.44	4.06	4.23	4.24	3.87
6	0.711* (0.566)*	0.705* (0.506)*	0.811* (0.681)*	0.825* (0.629)*	0.836* (0.486)*	–	6.19	5.3	5.59	5.62	5.35	5.51	5.59	6.1	5.44
7	0.782* (0.520)*	0.753* (0.717)*	0.866* (0.636)*	0.853* (0.743)*	0.875* (0.846)*	0.852* (0.504)*	–	3.65	3.43	3.02	3.65	3.62	3.68	4.89	2.96
8	0.681* (0.540)*	0.692* (0.625)*	0.772* (0.645)*	0.770* (0.664)*	0.731* (0.549)*	0.781* (0.603)*	0.756* (0.572)*	–	1.25	1.21	1.41	1.48	1.58	2.57	1.68
9	0.728* (0.573)*	0.722* (0.691)*	0.813* (0.670)*	0.822* (0.735)*	0.818* (0.708)*	0.826* (0.621)*	0.797* (0.673)*	0.495** (0.385)*	–	0.71	1.12	1.14	1.22	2.51	1.74
10	0.742* (0.474)*	0.729* (0.694)*	0.818* (0.564)*	0.825* (0.709)*	0.810* (0.775)*	0.833* (0.480)*	0.786* (0.750)*	0.495** (0.330)**	0.433* (0.338)**	–	0.94	0.61	0.83	2.37	1.58
11	0.787* (0.610)*	0.759* (0.759)*	0.879* (0.717)*	0.863* (0.783)*	0.880* (0.844)*	0.862* (0.608)*	0.869* (0.827)*	0.596* (0.453)*	0.633* (0.513)*	0.607* (0.565)*	–	1.17	1.32	2.42	1.56
12	0.674* (0.541)*	0.674* (0.528)*	0.735* (0.610)*	0.763* (0.606)*	0.740* (0.451)*	0.766* (0.631)*	0.725* (0.424)*	0.454* (0.320)**	0.436* (0.283)*	0.298* (0.137)	0.504** (0.293)**	–	0.83	2.66	1.86
13	0.711* (0.577)*	0.711* (0.642)*	0.792* (0.672)*	0.804* (0.700)*	0.786* (0.605)*	0.800* (0.634)*	0.771* (0.577)*	0.513* (0.399)**	0.505* (0.386)*	0.419* (0.266)*	0.603* (0.443)*	0.328** (0.229)**	–	2.78	1.86
14	0.677* (0.400)*	0.681* (0.641)*	0.737* (0.487)*	0.590* (0.500)*	0.748* (0.758)*	0.784* (0.420)*	0.781* (0.790)*	0.590* (0.436)*	0.629* (0.550)*	0.623* (0.659)*	0.677* (0.709)*	0.574* (0.304)**	0.621* (0.452)*	–	2.94
15	0.673* (0.509)*	0.711* (0.476)*	0.780* (0.631)*	0.752* (0.503)*	0.750* (0.338)**	0.779* (0.650)*	0.706* (0.286)**	0.505* (0.297)**	0.565* (0.302)**	0.551* (0.182)	0.606* (0.270)**	0.503* (0.343)**	0.544* (0.334)**	0.615* (0.231)**	–

Table 4: Genetic heterogeneity statistics [F_{ST} and γ_{ST} (into parenthesis)] among all the brocket deer species, both gray and red ones, and the white-tailed deer (*Passalites nemorivagus* (1), *Subulo chunyi* (2), *Subulo gouazoubira* (3), *Bisbalus citus* (4), the “rare” gray brocket deer in Mamoré River, Beni, Bolivia (5), *Mazama rufina* (6), *Mazama pandora* (7), *Mazama temama* (8), *Mazama americana* I (9), *Mazama americana* II-III (10), *Mazama americana* IV (11), *Mazama americana* V (12), *Mazama americana* VI-VII (13), *Mazama americana* VIII (14) y *Odocoileus virginianus*(15)) at the BD1 (mitochondrial *ND5* and *12S rRNA* genes) below the diagonal; Kimura 2P genetic distances (in %) among all the commented brocket and white-tailed deer taxa at the BD1 (mitochondrial *ND5* and *12S rRNA* genes) above the diagonal. * $p < 0.01$; ** $p < 0.05$.

This result does not support the inclusion of *M. pandora* in *Odocoileus*, or, alternatively, *Odocoileus* should be included in *Mazama*. *M. rufina* differed markedly from *M. pandora* (6.2%), *M. temama* (5.3%), and all the groupings of the *M. americana* complex (6.1%–5.35%). This was consistent with the recent proposal to separate *M. rufina* into a different genus. The D_a values of *M. pandora* versus all the associations of the *M. americana* and *M. temama* complex ranged from 4.9% to 3.03%. When analyzing the genetic distances between some of these *Odocoileina* brocket deer and the *Blastocercina* brocket deer, the following was observed: *M. rufina* differed by 5.93% from *B. citus*, by 4.76% from *P. nemorivagus*, and by 5.24% from *S. gouzaoubira*. Therefore, *M. rufina* had very

similar genetic distances from gray brocket and red brocket deer. On the other hand, *B. citus* showed the following genetic distances from different red brocket deer taxa: 4.29% with *O. virginianus*, 5.53% with *M. pandora*, 2.24% with *M. americana* VIII, 4.56% with *M. temama*, and between 5.2% and 4.6% with the different groups of the *M. americana* complex, excluding *M. americana* VIII. *B. citus* showed genetic distances of the following magnitudes from the other gray brocket deer: 4.32% with *P. nemorivagus*, 5.54% with *S. gouzaoubira*, 3.83% with the “rare” Bolivian brocket deer, and 7.38% with *S. chunyi*. Therefore, *B. citus* also had genetic distances of a similar order with both red and gray brocket deer.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	-	7.38	6.92	8.78	7.07	8.58	7.23	6.8	6.84	6.66	6.75	7.23	6.7	7.74
2	0.813* (0.558)*	-	2.43	9.3	8.69	9.38	9.06	9.16	9.7	9.22	9.16	8.58	9.1	8.9
3	0.751* (0.646)*	0.731* (0.346)**	-	8.89	8.37	9.5	7.86	8.29	8.24	7.58	7.76	7.43	7.73	8.16
4	0.826* (0.659)*	0.953* (0.937)*	0.894* (0.712)*	-	9.56	10.26	9.42	9.39	9.35	8.51	9.1	9.63	7.87	9.72
5	0.729* (0.573)*	0.876* (0.462)*	0.821* (0.697)*	0.873* (0.569)*	-	9.18	6.08	6.67	7.01	6.05	6.21	6.12	6.18	6.78
6	0.843* (0.663)*	0.979* (0.978)*	0.923* (0.735)*	0.966* (0.956)*	0.890* (0.563)*	-	7.49	7.18	7.47	7.24	7.29	7.75	7.55	6.93
7	0.703* (0.596)*	0.845* (0.647)*	0.777* (0.675)*	0.838* (0.705)*	0.701* (0.526)*	0.828* (0.670)*	-	3.53	3.33	2.79	3.09	3.39	3.49	4.19
8	0.783* (0.686)*	0.946* (0.870)*	0.885* (0.785)*	0.936* (0.885)*	0.825* (0.627)*	0.949* (0.878)*	0.657* (0.56)*	-	3.16	2.24	2.43	3.93	3.2	4.19
9	0.792* (0.699)*	0.961* (0.925)*	0.893* (0.763)*	0.945* (0.918)*	0.841* (0.590)*	0.962* (0.929)*	0.665* (0.54)*	0.845* (0.761)*	-	1.25	1.72	1.99	1.68	4.63
10	0.761* (0.531)*	0.931* (0.933)*	0.856* (0.609)*	0.910* (0.903)*	0.789* (0.378)*	0.925* (0.934)*	0.577* (0.38)*	0.720* (0.610)*	0.617* (0.597)*	-	0.62	1.84	1.13	3.89
11	0.757* (0.655)*	0.923* (0.790)*	0.851* (0.746)*	0.908* (0.819)*	0.785* (0.592)*	0.916* (0.803)*	0.592* (0.50)*	0.718* (0.611)*	0.667* (0.537)*	0.352* (0.264)*	-	2.04	1.46	4.15
12	0.753* (0.650)*	0.897* (0.727)*	0.825* (0.719)*	0.894* (0.788)*	0.762* (0.576)*	0.898* (0.903)*	0.592* (0.49)*	0.772* (0.673)*	0.650* (0.514)*	0.575* (0.386)*	0.585* (0.590)*	-	1.89	4.68
13	0.682* -0.548	0.841* (0.499)*	0.770* (0.656)*	0.808* (0.540)*	0.700* (0.561)*	0.824* (0.535)*	0.531* (0.39)*	0.627* (0.451)*	0.481* (0.278)*	0.349* (-0.138)	0.400* (0.272)*	0.441* (0.308)*	-	4.15
14	0.753* (0.586)*	0.887* (0.442)*	0.825* (0.690)*	0.883* (0.550)*	0.776* (0.636)*	0.870* (0.470)*	0.627* (0.43)*	0.760* (0.500)*	0.790* (0.470)*	0.719* (0.266)*	0.721* (0.482)*	0.721* (0.500)*	0.619* (0.467)*	-

Table 5: Genetic heterogeneity statistics [F_{ST} and γ_{ST} (into parenthesis)] among all the brocket deer species, both gray and red ones, and the white-tailed deer (*Passalites nemorivagus* (1), *Subulo chunyi* (2), *Subulo gouzaoubira* (3), *Bisbalus citus* (4), *Mazama rufina* (5), *Mazama pandora* (6), *Mazama temama* (7), *Mazama americana* I (8), *Mazama americana* II (9), *M. americana* III (10), *Mazama americana* IV (11), *Mazama americana* V (12), *Mazama americana* VI-VII (13), and *Odocoileus virginianus*(14)) at the BD2 (mitochondrial *Cyt-b* gene) below the diagonal; Kimura 2P genetic distances (in %) among all the commented brocket and white-tailed deer taxa at the BD2 (mitochondrial *Cyt-b* gene) above the diagonal. * $p < 0.01$; ** $p < 0.05$.

By pairs of taxa (Table 5), BD2 showed that the highest F_{ST} values between *M. temama* and the *M. americana* complex groupings were those corresponding to comparisons involving *M. americana* I with other groupings of the *M. americana* complex. The lowest estimates involved the

pairs *M. americana* III vs. *M. americana* IV ($F_{ST} = 0.352$, $p = 0.033$) and *M. americana* III vs. *M. americana* VI-VII ($F_{ST} = 0.349$, $p = 0.041$). As with BD1, BD2 showed clear evidence of significant genetic heterogeneity among the *M. americana* complex groupings, even with somewhat higher levels of

heterogeneity. Similarly, *M. temama* exhibited significant genetic heterogeneity with respect to all *M. americana* complex groupings, but there were several cases where genetic heterogeneity between pairs of *M. americana* complex groupings was greater than in comparisons involving *M. temama*, as occurred in BD1. Regarding genetic distances, the highest corresponded to *M. americana* I vs. *M. americana* V (3.93%), *M. americana* I vs. *M. temama* (3.53%), and *M. temama* vs. *M. americana* VI-VII. The lowest value recorded corresponded to the pair *M. americana* III vs. *M. americana* IV (0.62%). As with BD1, these red brocket deer taxa were also compared with *O. virginianus*, *M. rufina*, and *M. pandora*. *O. virginianus* showed greater genetic distances from *M. rufina* (6.78%) and *M. pandora* (6.93%) than from *M. temama* (4.19%) or the *M. americana* complex groups (4.68% to 3.89%). Therefore, BD2 also did not confirm that *M. pandora* was the sister group to *Odocoileus*. As with BD1, *M. rufina* showed relatively high genetic distances from the other red brocket deer, with values of 9.18% from *M. pandora*, 6.08% from *M. temama*, and between 7.01% and 6.05% from the *M. americana* complex groups. Regarding gray brocket deer, *M. rufina* showed genetic distances of 7.07% with *P. nemorivagus*, 8.69% with *S. chunyi*, 8.37% with *S. gouazoubira*, and 9.56% with *B. citus*. Therefore, with BD2, *M. rufina* exhibited somewhat higher genetic distances from gray brocket deer than from red brocket deer (except *M. pandora*), unlike what was observed with BD1, where genetic distances were similar with both groups of brocket deer. *M. pandora* showed relatively high genetic distances from the other red brocket deer, with a genetic distance of 7.49% from *M. temama* and between 7.75% and 7.17% from

the groups of the *M. americana* complex. Finally, *B. citus* showed the following genetic distances from red brocket deer: 10.26% with *M. pandora*, 9.42% with *M. temama*, and from 9.62% to 7.87% with the groups of the *M. americana* complex. With other gray brocket deer, these values were 8.89% with *S. gouazoubira*, 9.3% with *S. chunyi*, and 8.78% with *P. nemorivagus*. Therefore, as with BD1, *B. citus* exhibits relatively similar genetic distances from red brocket and gray brocket deer.

Mitochondrial Genetic Diversity Levels in Brocket Deer

Table 8 shows the genetic diversity statistics found for the different brocket deer taxa using BD1. All of them presented high haplotype diversity (H_d) values, greater than 0.80, with the exception of *M. pandora* ($H_d = 0.667 + 0.314$), although the number of specimens analyzed was small (3). The nucleotide diversity (π) values were more interesting and, moreover, were not affected by the sample size. Red brocket deer, such as *M. americana* I (although it included specimens of *M. americana* “sensu stricto” and *M. rufa*, two different species) ($\pi = 0.0097$), *M. americana* II-III (even though it included sequences from three different species of red brocket deer) ($\pi = 0.0088$), *M. americana* IV ($\pi = 0.0033$), and *M. pandora* ($\pi = 0.0077$), showed π values typical of populations of many mammal species. The values for *M. americana* V-VII ($\pi = 0.0198$), *M. americana* VI ($\pi = 0.0141$), *M. americana* VIII ($\pi = 0.0198$), *M. rufina* ($\pi = 0.0158$), and *M. temama* ($\pi = 0.0159$) were higher.

Genetic heterogeneity and gene flow statistics	Values	Probabilities
χ^2	152.000 df = 128	0.0726 NS
H_{ST}	0.035	0.0003**
K_{ST}	0.379	0.00001**
K_{ST}^*	0.234	0.00001**
ZS	215.451	0.00001**
ZS*	4.577	0.00001**
Snn	0.974	0.00001**
Y_{ST}	0.434	0.001**
F_{ST}	0.47	0.001**
Nm (Y_{ST})	0.65	
Nm (F_{ST})	0.56	

Table 6: Overall genetic heterogeneity among red brocket deer species (*Mazama temama*, *Mazama americana* I, *Mazama americana* II-III, *Mazama americana* IV, *Mazama americana* V, *Mazama americana* VI, *Mazama americana* VII, *Mazama americana* VIII) at the BD1 (mitochondrial ND5 and 12S rRNA genes). Nm = gene flow; df = degree of freedom; ** $p < 0.01$ (highly significant). NS = no significant.

These results could indicate two different aspects. On the one hand, it could mean that these taxa or species have greater genetic diversity due to their larger effective sizes, or that these clusters actually contain more than one molecularly distinguishable taxon. If we consider the second possibility, these clusters could contain several distinct taxa within them. In the case of the gray brocket deer, *B. citus* ($\pi = 0.0113$) and *S. gouazoubira* ($\pi = 0.0106$), these values

are within the normal range for most mammal species. Additionally, *S. chunyi* (including one specimen that could represent a new taxon) ($\pi = 0.0451$), and *P. nemorivagus* ($\pi = 0.0249$) showed considerably higher nucleotide diversity, potentially including different taxa within their groups. *O. virginianus* also showed a moderately high value ($\pi = 0.0169$), which may be related to the existence of different subspecies within it.

Genetic heterogeneity and gene flow statistics	Values	Probabilities
χ^2	270.089 df = 198	0.0005**
HST	0.11	0.00001**
KST	0.542	0.00001**
KST*	0.319	0.00001**
ZS	190.426	0.00001**
ZS*	4.419	0.00001**
Snn	0.937	0.00001**
γ ST	0.596	0.001**
FST	0.598	0.001**
Nm (γ ST)	0.34	
Nm (FST)	0.34	

Table 7: Overall genetic heterogeneity among red brocket deer species (*Mazama temama*, *Mazama americana* I, *Mazama americana* II, *Mazama americana* III, *Mazama americana* IV, *Mazama americana* V, *Mazama americana* VI-VII) at the BD2 (mitochondrial *Cyt-b* gene). Nm = gene flow; df = degree of freedom; ** $p < 0.01$ (highly significant).

Species	N	NH	Hd	π	θ per sequence
<i>Passalites nemorivagus</i>	11	9	0.964 $\pm$ 0.051	0.0249 $\pm$ 0.0036	15.705 $\pm$ 6.502
<i>Subulo chunyi</i> (probably included a new species)	3	3	1 $\pm$ 0.272	0.0451 $\pm$ 0.0193	27.333 $\pm$ 16.690
<i>Subulo gouazoubira</i>	16	14	0.983 $\pm$ 0.028	0.0106 $\pm$ 0.0009	7.233 $\pm$ 2.911
<i>Bisbalus citus</i>	7	6	0.952 $\pm$ 0.096	0.0113 $\pm$ 0.0050	8.980 $\pm$ 4.357
<i>Mazama rufina</i>	24	21	0.986 $\pm$ 0.018	0.0138 $\pm$ 0.0026	15.800 $\pm$ 5.433
<i>Mazama pandora</i>	3	2	0.667 $\pm$ 0.314	0.0077 $\pm$ 0.0036	4.667 $\pm$ 3.127
<i>Mazama temama</i>	8	8	1 $\pm$ 0.063	0.0158 $\pm$ 0.0021	10.413 $\pm$ 4.815
<i>Mazama americana</i> I	7	7	1 $\pm$ 0.076	0.0097 $\pm$ 0.0028	6.531 $\pm$ 3.259
<i>Mazama americana</i> II-III	3	3	1 $\pm$ 0.272	0.0088 $\pm$ 0.0028	5.333 $\pm$ 3.528
<i>Mazama americana</i> IV	6	4	0.800 $\pm$ 0.172	0.0033 $\pm$ 0.0014	2.628 $\pm$ 1.553
<i>Mazama americana</i> V-VII	13	10	0.962 $\pm$ 0.041	0.0198 $\pm$ 0.0052	18.046 $\pm$ 7.108
<i>Mazama americana</i> VI	9	9	1 $\pm$ 0.052	0.0141 $\pm$ 0.0029	8.830 $\pm$ 4.007
<i>Mazama americana</i> VIII	2	2	1 $\pm$ 0.500	0.0198 $\pm$ 0.0099	12.000 $\pm$ 8.832
<i>Odocoileus virginianus</i>	25	20	0.977 $\pm$ 0.019	0.0169 $\pm$ 0.0028	15.890 $\pm$ 5.415

Table 8: Genetic diversity for all the gray brocket, red brocket and white-tailed deer taxa analyzed studied at the BD1 (mitochondrial *ND5* and *12S rRNA* genes). N = Sample size; NH = Number of haplotypes; H_d = Haplotype diversity; π = nucleotide diversity; θ per sequence = $N_e \mu$ (female effective number x mutation rate per generation).

The mitochondrial genetic diversity values for BD2 can be seen in Table 9. For the H_d values, most of the brocket deer

taxa have values greater than 0.85, with the exception of *B. citus* ($H_d = 0.667$), *M. americana* II ($H_d = 0.400$), and *M. pandora*

($H_d = 0.667$), all of which have small sample sizes. For the red brocket deer taxa, those with π values among the most frequent among mammalian species were *M. americana* I ($\pi = 0.0067$), *M. americana* II ($\pi = 0.0049$), *M. americana* III ($\pi = 0.0107$), *M. americana* IV ($\pi = 0.0123$), and *M. pandora* ($\pi = 0.0010$; this last value can be considered quite low). Additionally, other red brocket deer taxa showed considerably higher values, such as *M. americana* V ($\pi = 0.0186$), *M. americana* VI-VII ($\pi = 0.0314$), *M. rufina* ($\pi = 0.0216$), and *M. temama* ($\pi = 0.0302$). These latter taxa may contain other molecularly identifiable taxa

within them. For the gray brocket deer, *S. chunyi* ($\pi = 0.0031$) and *B. citus* ($\pi = 0.0097$), the values were low. It is interesting to note that with the disappearance of the specimen that may constitute a new taxon (BD1), in BD2, the value of π for *S. chunyi* declined dramatically. *S. gouazoubira* showed an intermediate value ($\pi = 0.0149$), and *P. nemorivagus* ($\pi = 0.0310$) showed a fairly high value that suggests possible distinct taxa within it. Finally, *O. virginianus* ($\pi = 0.0197$) showed a value high enough to consider the possible existence of distinct taxa within it.

Species	N	NH	Hd	π	θ per sequence
<i>Passalites nemorivagus</i>	7	6	0.952 $\pm$ 0.096	0.0310 $\pm$ 0.0079	21.633 $\pm$ 10.010
<i>Subulo chunyi</i>	2	2	1 $\pm$ 0.500	0.0031 $\pm$ 0.0015	2.000 $\pm$ 1.732
<i>Subulo gouazoubira</i>	12	9	0.939 $\pm$ 0.058	0.0149 $\pm$ 0.0011	9.603 $\pm$ 4.021
<i>Bisbalus citus</i>	3	2	0.667 $\pm$ 0.314	0.0061 $\pm$ 0.0029	4.000 $\pm$ 2.726
<i>Mazama rufina</i>	18	9	0.856 $\pm$ 0.062	0.0216 $\pm$ 0.0044	22.387 $\pm$ 8.065
<i>Mazama pandora</i>	3	2	0.667 $\pm$ 0.314	0.0010 $\pm$ 0.0005	0.667 $\pm$ 0.666
<i>Mazama temama</i>	6	6	1 $\pm$ 0.096	0.0302 $\pm$ 0.0075	17.956 $\pm$ 8.764
<i>Mazama americana I</i>	7	5	0.905 $\pm$ 0.103	0.0067 $\pm$ 0.0024	4.490 $\pm$ 2.341
<i>Mazama americana II</i>	5	2	0.400 $\pm$ 0.237	0.0049 $\pm$ 0.0029	3.840 $\pm$ 2.243
<i>Mazama americana III</i>	2	2	1 $\pm$ 0.500	0.0107 $\pm$ 0.0053	7.000 $\pm$ 5.292
<i>Mazama americana IV</i>	7	6	0.952 $\pm$ 0.096	0.0123 $\pm$ 0.0030	10.204 $\pm$ 4.905
<i>Mazama americana V</i>	7	6	0.952 $\pm$ 0.096	0.0165 $\pm$ 0.0036	13.469 $\pm$ 6.365
<i>Mazama americana VI-VII</i>	11	7	0.873 $\pm$ 0.089	0.0314 $\pm$ 0.0063	23.889 $\pm$ 9.711
<i>Odocoileus virginianus</i>	22	18	0.974 $\pm$ 0.024	0.0197 $\pm$ 0.0019	15.636 $\pm$ 5.485

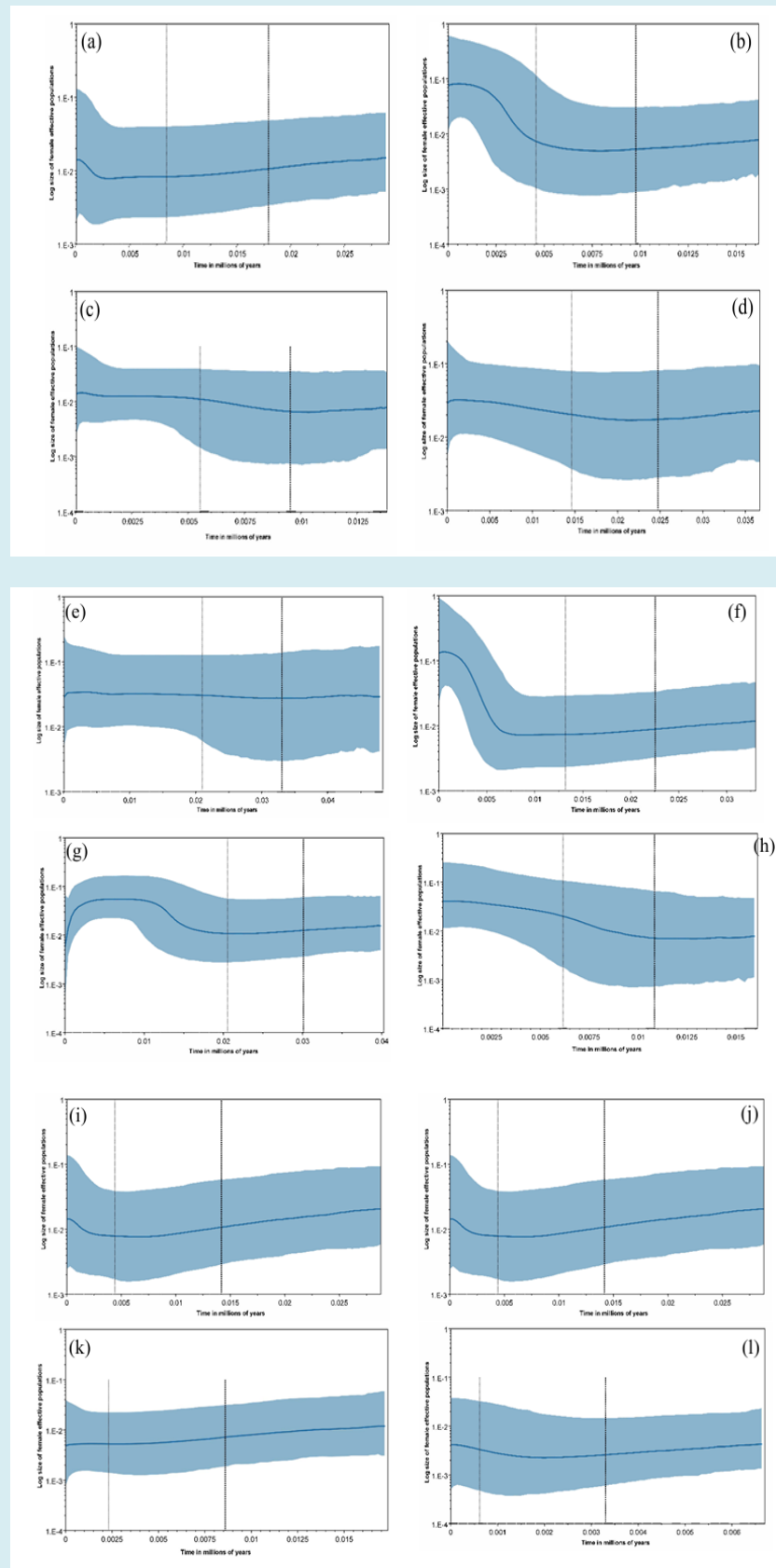
Table 9: Genetic diversity for all the gray brocket, red brocket and white-tailed deer taxa analyzed studied at the BD2 (mitochondrial *Cyt-b* gene). N = Sample size; NH = Number of haplotypes; H_d = Haplotype diversity; π = nucleotide diversity; θ per sequence = $N_e\mu$ (female effective number x mutation rate per generation).

Possible Demographic Changes throughout the Natural History of Brocket Deer

The individualized analysis by taxon and species of brocket deer, regarding the existence of possible demographic changes throughout the evolutionary histories of each, revealed the following picture (Figures 10 & 11).

In the case of the gray brocket deer: 1- *B. citus* showed no evidence of demographic change with BD1 for the different statistics used (it was not analyzed for BD2 due to the limited number of samples). The BSP analysis for BD1 (Figure 10a) showed a slight and steady decline in the population size of female lineages over the last 35,000 years, with some population expansion in the last 2,000 years. The analysis could not be performed for BD2 because only three individuals were analyzed for this database. 2- *S. gouazoubira* showed some evidence of population expansion ($F_s = -6.207$, $p = 0.0041$; $rg = 0.0199$, $p = 0.0326$) for BD1. In fact, the mismatch distribution test was significant (Figure

11a), showing unequivocal signs of population expansion. From this latter test, the start of this population expansion could be estimated at around 643,000 years ago. BD2 showed no evidence of any population change throughout its natural history. For BSP (Figures 10b,10c), with BD1, a very slight gradual decline was observed over the last 16,000 years, with a strong population expansion in the last 6,000 years (Bayesian Factor = 7.3), while with BD2 no noticeable change was detected in the last 14,000 years, as was the case with the previously mentioned statistics. 3- *P. nemorivagus* showed no significant evidence of population changes for BD1 and BD2. The BSP analysis (Figure 10d) for BD1 showed a very slight decline from 40,000 years ago to approximately 17,500 years ago. From then on, a very slight population increase is observed, continuing until practically the present. However, these small changes are not clearly significant. For BD2, the BSP analysis (Figure 10e) showed no clear population changes in the last 50,000 years, consistent with previously estimated statistics.



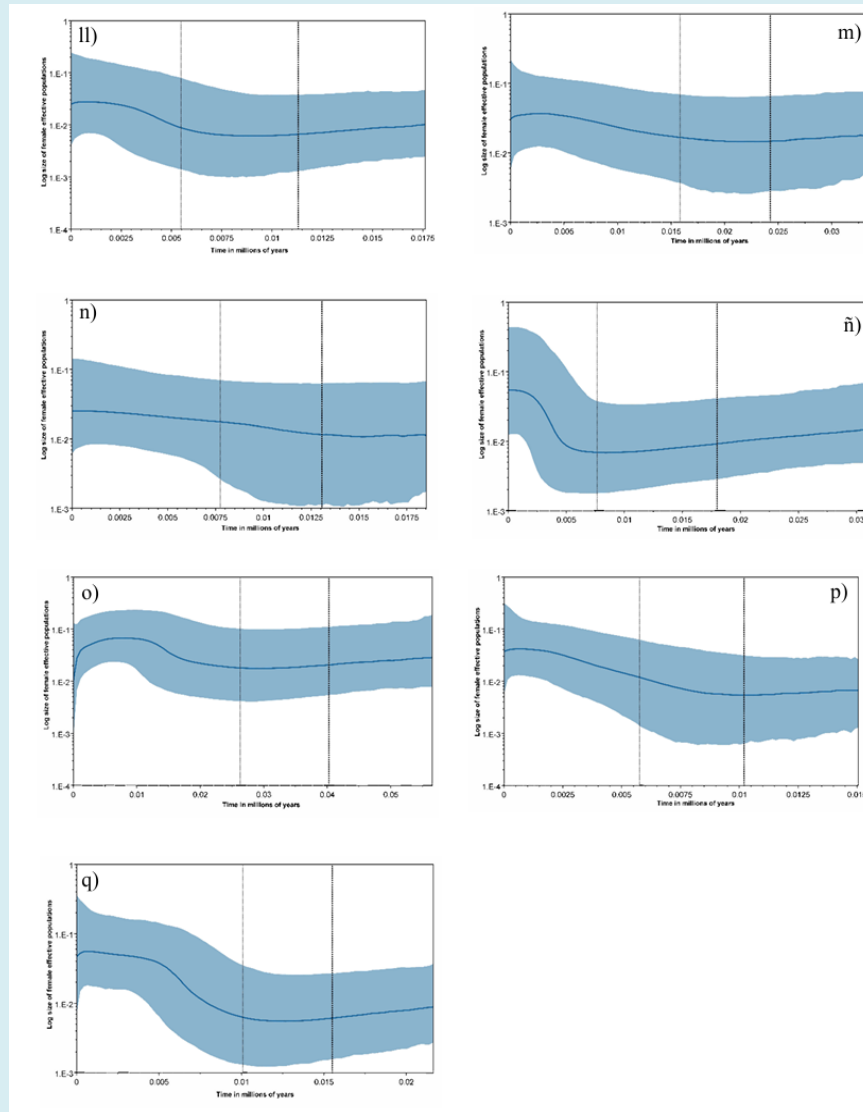


Figure 10: Bayesian skyline plot (BSP) analyses to determine the possible demographic changes across the natural history of different taxa of brocket deer and *Odocoileus virginianus* with mitochondrial *ND5* and *12S rRNA* genes (BD1) and mitochondrial *Cyt-b* gene (BD2): (a) *Bisbalus citus* (BD1), (b-c) *Subulo gouazoubira* (BD1 and BD2), (d-e) *Passalites nemorivagus* (BD1 and BD2), (f-g) *Mazama rufina* (BD1 and BD2), (h-i) *Mazama temama* (BD1 and BD2), (j-k) *Mazama americana I* (BD1 and BD2), (l-ll) *M. americana IV* (BD1 and BD2), (m-n) *M. americana V* (BD1 and BD2), (ñ-o) *M. americana VI-VII* (BD1 and BD2) and (p-q) *Odocoileus virginianus* (BD1 and BD2).

In the case of red brocket deer, different situations were observed: 1- *M. rufina* was the taxon that showed the clearest evidence of population expansion of all the brocket deer analyzed. For BD1, all six tests performed were significant in favor of strong population expansion ($D = -1.836$, $p = 0.0166$; $D^* = -2.223$, $p = 0.0352$; $F^* = -2.270$, $p = 0.0376$; $F_s = -10.474$, $p = 0.0012$; $rg = 0.0141$, $p = 0.0436$; $R_2 = 0.064$, $p = 0.0052$). The mismatch distribution test was also significant (Figure 11b) in favor of a population expansion. This expansion would have begun around 436,000 years ago.

The BSP analysis (Figure 10f), which recorded a period of 35,000 years, showed a very strong population expansion of female lineages in the last 7,500 years (Bayesian Factor = 9.13). For BD2, there was also evidence of strong population expansions, although not as marked as with BD1. Three statistics were significant ($D = -1.600$, $p = 0.041$; $D^* = -1.944$, $p = 0.0467$; $F^* = -1.940$, $p = 0.0490$) in favor of a population expansion. In this case, the mismatch distribution test was not significant. For the BSP analysis (Figure 10g), covering 40,000 years, a strong population expansion was detected,

beginning around 15,000 years ago and lasting until about 2,000-3,000 years ago (Bayesian Factor = 7.12), followed by a population decline that continues to the present (Bayesian Factor = 7.54). 2- In the case of *M. temama*, no evidence of population changes was recorded for either BD1 or BD2. For BD1, the BSP analysis (Figure 10h) showed, over the last 16,000 years, a slight, continuous but gradual increase, which began 11,000 years ago and continuing to the present (Bayesian Factor = 5.9). For BD2, this same analysis (Figure 10i) detected, for the last 55,000 years, a slight, constant, and gradual population decline until about 5,000 years ago, when a slight population expansion occurred up to the present (Bayesian Factor = 6.43). 3- In the case of *M. americana* I, with the statistics used, for BD1, only one statistic was significant for a population expansion ($F_s = -2.621$, $p = 0.037$), while for BD2, no statistic showed significant evidence. Using BSP analysis (Figure 10j), covering the last 32,500 years for BD1, a gradual decline in the population of female lineages was detected until about 7,500 years ago, when a population stabilization began, followed by growth from about 2,500 years ago to the present (Bayesian Factor = 6.56). For BD2, the BSP analysis (Figure 10k), covering the last 17,500 years, detected a slight but gradual decline in the population of female lineages to the present day. 4- In the case of *M. americana* IV, for both BD1 and BD2, significant evidence was found to support possible population expansions. For BD1, three tests were significant ($D = -1.367$, $p = 0.0408$; $D^* = -1.399$, $p = 0.029$; $F^* = -1.398$, $p = 0.047$), while for BD2, four tests were significant ($D = -1.371$, $p = 0.0438$; $D^* = -1.354$, $p = 0.0421$; $F^* = -1.346$, $p = 0.045$; $R_2 = 0.127$, $p = 0.0433$). Nonetheless, in the mismatch distribution test, neither analysis was significant. For BD1, using BSP analysis (Figure 10l), considering the last 7,500 years, a slight but gradual decline was observed until about 2,000 years ago, followed by a slight but gradual increase to the present. Nevertheless, no clearly significant Bayesian factor values were obtained. Using BD2, the BSP analysis (Figure 10ll), covering the last 17,500 years, showed a slight, gradual but continuous decline until about 7,000 years ago. From then on, a relevant population expansion was detected up to the present (Bayesian Factor = 6.88). 5- In the case of *M. americana* V, evidence of population expansion was also detected for BD1 and BD2. In the first case, three tests supported the existence of a significant population expansion ($D = -1.565$, $p = 0.049$; $rg = 0.0192$, $p = 0.0412$; $R_2 = 0.106$, $p = 0.0441$), one of them related to the mismatch distribution test (Figure 11c). From this, an initial period of population expansion was estimated to have begun approximately 395,000 years ago. In the second case, two tests were significant in favor of a

population expansion ($D^* = -1.291$, $p = 0.0454$; $R_2 = 0.128$, $p = 0.0479$). The BSP analysis (Figure 10m), for BD1 containing the last 33,000 years, showed gradual and continuous growth from 15,000 years ago until approximately 2,000 years ago (Bayesian Factor = 6.02), and, from that point onward, a slight decline to the present. For BD2, the same analysis (Figure 10n), containing the last 18,500 years, also detected a slight, but gradual and constant, population growth from approximately 15,000 years ago to the present. The slight decline of the last 2,000 years detected for BD1 was not observed. 6- In the case of *M. americana* VI (VII was included in this group due to its small sample size), little evidence of solid demographic changes was detected. Only for BD1 was significant evidence found in favor of a population expansion ($F_s = -3.215$, $p = 0.0362$), while none was found for BD2. For the BSP analysis (Figure 10ñ), with BD1, a steady and gradual decline in the size of female lineages was evident from 30,000 years ago until about 7,500 years ago, when a strong demographic expansion began that continues to the present (Bayesian Factor = 8.23). Through this analysis (Figure 10o) applied to BD2, a very limited and gradual decline was observed from 60,000 years ago until about 20,000 years ago, when a more or less significant population expansion began (Bayesian Factor 5.88), lasting until about 5,000 years ago when a significant population decline occurred, continuing to the present day (Bayesian Factor = 7.14). 7- Finally, and for comparison, we show the possible demographic changes with the samples used of *O. virginianus*. For BD1 and BD2, this species showed strong evidence of a population expansion. For BD1, three significant tests were obtained ($D = -1.452$, $p = 0.046$; $F_s = -6.156$, $p = 0.0164$; $rg = 0.0103$, $p = 0.0201$), one of them related to the mismatch distribution test (Figure 11d), which allowed us to estimate the time when this population expansion began approximately 495,000 years ago. For BD2, two tests also supported the interpretation of the existence of population expansions in *O. virginianus* ($F_s = -3.948$, $p = 0.0429$; $rg = 0.0124$, $p = 0.0409$), the last one related to the mismatch distribution test (Figure 11e), which allowed us to estimate the time when this population expansion began approximately 555,000 years ago. The BSP analysis (Figure 10p), with BD1 containing the last 15,000 years, showed a strong population expansion in the last 8,500 years to the present (Bayesian Factor = 9.35), while the BSP analysis (Figure 10q), with BD2 and covering the last 22,500 years, showed a gradual and progressive decline until about 10,000 years ago where a strong population expansion was visualized (Bayesian Factor = 9.23) until about 200-300 years ago where a certain decline began to occur.

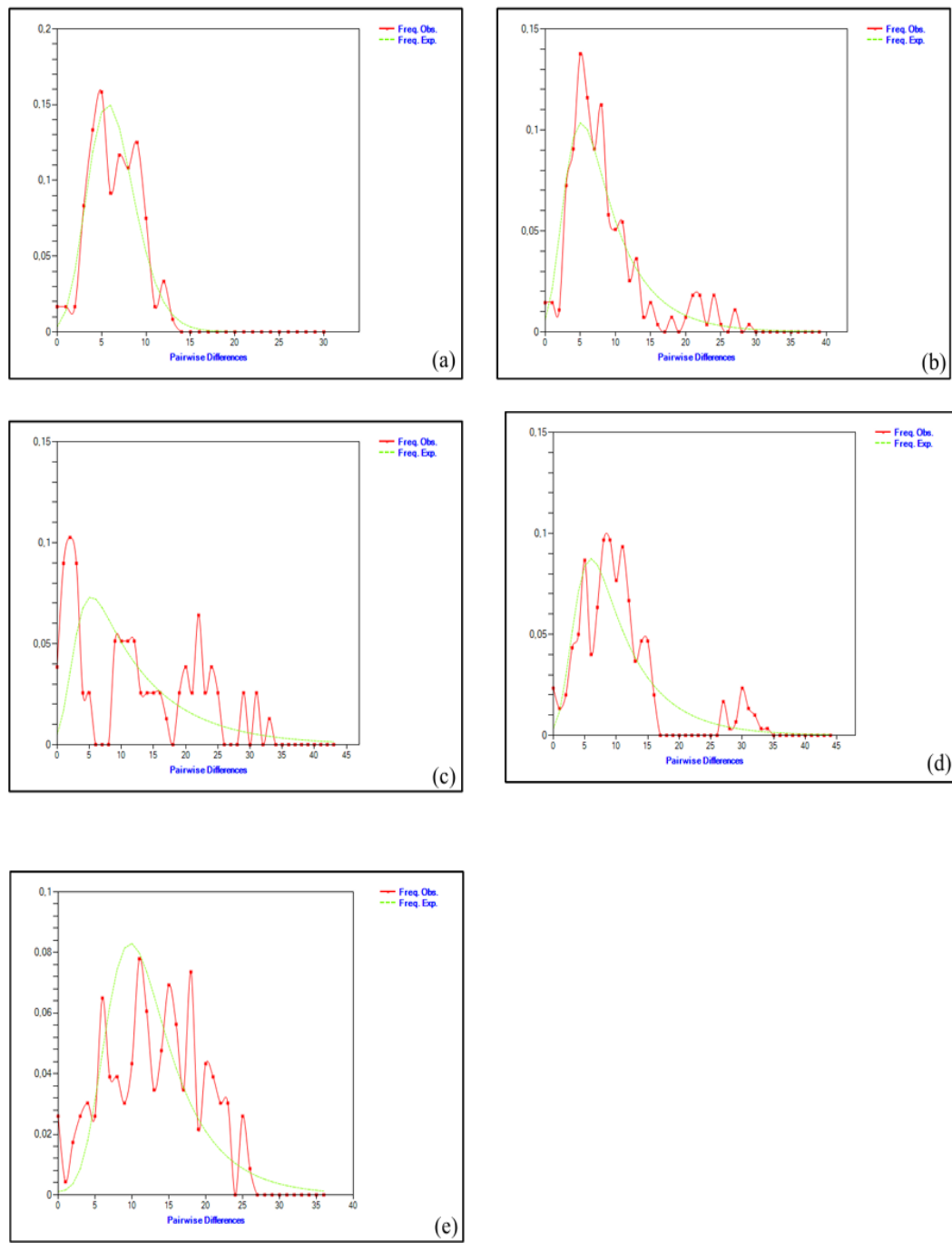


Figure 11: Analysis of significant mismatch distributions (pairwise sequence differences) in some taxa of brocket deer and *Odocoileus virginianus*. (a) *Subulo gouazoubira* (BD1), (b) *Mazama rufina* (BD1), (c) *Mazama americana V* (BD1), (d) *Odocoileus virginianus* (BD1) and (e) *O. virginianus* (BD2).

Therefore, all these analyses to detect possible demographic changes in brocket deer, and, by way of comparison, with white-tailed deer, showed that, especially, *M. rufina* and *O. virginianus* underwent strong and detectable population expansions using databases BD1 and BD2.

Likewise, the *M. americana* IV and *M. americana* V groups showed strong evidence of this type of demographic event for both databases. Nonetheless, for the remaining brocket deer species or taxa, the evidence in favor of demographic expansions is much more limited or nonexistent.

Discussion

This is the study that, to date, has analyzed the most specimens of different species or taxa of brocket deer at the molecular level. Previously, the studies that had analyzed the most specimens at the molecular level of the *M. americana* complex (excluding *M. temama*) were those of Abril, et al. [10] (18 specimens, all from Brazil) and Cifuentes-Rincón, et al. [39] (11 specimens). In the present study, we analyzed 40 specimens of red brocket deer from the *M. americana* complex for BD1 (from Colombia, Venezuela, Ecuador, Peru, Bolivia, Brazil, Trinidad and Tobago, and French Guiana) or 39 specimens from the same complex for BD2. The maximum number of specimens analyzed at the molecular level of *M. rufina*, to date, had been 14 [65]. This report presents the analysis of 30 specimens of *M. rufina* for BD1 and 18 specimens of *M. rufina* for BD2. Molecular data had also been reported for two specimens of *M. temama* from Colombia. In this work, we analyzed six specimens of *M. temama* from Colombia and Ecuador (the first time an Ecuadorian specimen of this taxon has been reported) for BD1. For *B. citus*, Sandoval, et al. [62] reported molecular results for three Venezuelan specimens of this new species. Here, we analyzed seven specimens of *B. citus* for BD1, reporting, for the first time, its presence in northern Colombia. For *P. nemorivagus*, Morales-Donoso, et al. [60] reported five specimens as the maximum number of specimens molecularly analyzed to date. In this study, 11 specimens of *P. nemorivagus* were molecularly analyzed for BD1 and seven for BD2. Similarly, for *S. gouazoubira*, the studies by Bernegossi, et al. [58] and Sandoval, et al. [67] had reported 6 and 7 specimens of this species, respectively, for molecular analysis. In this work, we molecularly analyzed 16 specimens of *S. gouazoubira* for BD1 and 12 specimens for BD2. Nevertheless, there is a local study in Uruguay where 50 specimens of *S. gouazoubira* from that country were analyzed [117]. Therefore, this is the mitochondrial DNA molecular analysis that has studied the most specimens of brocket deer (both red and gray) to date.

Furthermore, the present study showed that, in all cases, all the new taxonomic and systematic changes that have been proposed for brocket deer in recent years were ratified [39,58,60,62,65,67] as well as the discovery of new lineages or taxa within the gray and red brocket deer and that should be ratified, especially, with karyotypic studies to determine if they possess reproductive isolation from other differentiated brocket deer communities. Therefore, with this extensive mitochondrial study (the most extensive carried out to date) we indicate in which geographical areas of South America other groups of researchers should obtain holotypes or neotypes to be chromosomally characterized in order to definitively define the taxonomic aspects of each of the new lineages or taxa reported here.

Evolution and Systematics of the Gray Brocket Deer

The gray brocket deer consistently exhibited the longest branches in all phylogenetic trees obtained (BD1, BD2, BD3) and was also the first to diverge in all analyses performed with respect to the brocket deer. This suggests that their evolution (Blastocercina, in general) is longer and older than that of the various groups of red brocket deer and white-tailed deer studied (Odocoileina), which have substantially shorter branches (indicating that these taxa are more recent). Within Blastocercina, those that appeared to have the longest and densest (and therefore oldest) branches in a Density tree (not shown here) for BD1 were the pudus (both *Pudella* and *Pudu*), *H. antisensis*, and *S. chunyi*. In the ML tree, also for BD1, the ancestor of the first group to appear in Neotropical deer was *S. gouazoubira* + *S. chunyi*. Within the Odocoileina, some of the Ecuadorian specimens of *M. rufina* (whose ancestors appear to have given rise to this taxon) showed the oldest branches for BD1. If we exclude *M. rufina*, it becomes clear that the levels of heterogeneity and genetic distances were consistently higher among the gray brocket deer than among the red brocket deer, which is also related to their greater antiquity.

The Cases of *S. gouazoubira* and *S. chunyi*

The new genera of gray brocket deer (*Passalites*, *Bisbalus*, *Subulo*) proposed by other authors [58,60,62,65,67] are fully confirmed. Starting with *S. gouazoubira*, all our analyses perfectly differentiated this species from any other gray brocket deer at the mitochondrial DNA level. All phylogenetic trees detected the swarm of *S. gouazoubira* individuals as a robust or very robust clade. BAPS also perfectly differentiated *S. gouazoubira*. The genetic heterogeneity analysis showed that all gray brocket deer were highly distinguishable for both BD1 ($F_{ST} = 0.71$) and BD2 ($F_{ST} = 0.79$). The taxon that showed the least degree of differentiation from *S. gouazoubira* was *S. chunyi*, but even so, the differences were significant. For BD1, *S. gouazoubira* and *S. chunyi* presented an F_{ST} value of 0.61 and a Da value of 4.4%, while for BD2, $F_{ST} = 0.73$ and Da = 2.4%. Other characteristics besides molecular ones also differentiate this species from other gray brocket deer. For example, there were craniometric differences between *P. nemorivagus* and *S. gouazoubira*, the latter being on average 5% larger in all craniometric measurements, except for premaxilla-prosthion, basifacial axis, and least breadth between orbits, which were larger in *P. nemorivagus* males and females [118]. This taxon also differs from other gray brocket deer at the karyotypic level. Both *S. gouazoubira* and *P. nemorivagus* have a karyotype with $2n = 70$ (some *S. gouazoubira* has 69), but the FN = 70 in *S. gouazoubira* and FN = 72 in *P. nemorivagus* [119]. The FN divergence is related to a morphological difference of the X sexual chromosome,

which is acrocentric in *S. gouazoubira* and submetacentric in *P. nemorivagus* [119]. Also, based on characteristics of the spermatozoa and ejaculate, both species were differentiated [120]. The characteristics that most clearly differentiated both species were sperm head dimensions and ejaculate color, white for *S. gouazoubira* and reddish for *P. nemorivagus*. Additionally, multivariate analyses of sperm head dimensions accurately discriminated against three groups for both species with a total percentage of misclassified of 0.71. These three groups were constituted as follows. Group 1: included all specimens of *P. nemorivagus*; Group 2: included two specimens of *S. gouazoubira*, and Group 3: had a single specimen of *S. gouazoubira*. Carranza, et al. [121] showed that *P. nemorivagus* and *S. gouazoubira* can interbreed in captivity, although hybrids had serious fertility problems. The results of these authors revealed the existence of facilitating behaviors and copulations significantly more common within each of these two species, evidence of discrimination between species. Nevertheless, these authors also showed that a precopulatory barrier was not strong between both gray brocket deer species. Also, Galindo, et al. [122] ratified different degrees of fertility reduction, from severe subfertility to complete sterility in the hybrids between both gray brocket deer species by evaluating the seminal quality and testicular histology. The temporal differentiation of the common ancestor between *S. gouazoubira* and *P. nemorivagus* ranged from 3.53-2.49 Ma, during the last phase of the Pliocene and the beginning of the Pleistocene.

Within *S. gouazoubira*, we detected two groupings that appear, especially, in all phylogenetic analyses of BD2 and BD3. One grouping contains specimens located in western South America, closer to the Andes, or in the Andes themselves (Tucumán area in Argentina, the Bolivian Andes, and even the Colombian Andes). Another grouping is found more towards the center and east of southern South America (Bolivia Amazon, Paraguay, Uruguay, and southern Brazil). Furthermore, within this latter grouping, for BD2, three subgroups with high significance values can be distinguished. These three associations were detected in central-eastern Paraguay and Uruguay (98%, $pp = 1$), in the Bolivian Amazon and northwestern Paraguay (Alto Paraguay) (78%, $pp = 1$), and in Uruguay and southern Brazil (99%, $pp = 1$).

In the first grouping, initially, two fully Andean specimens were detected (one Bolivian and the other Colombian) separated by 4,000 km but both were Andean specimens. The native Colombian sample, a piece of skin provided by a local farmer, was perfectly identified both in its morphological appearance and in its geographical origin as a brocket deer, although its color was slightly more reddish (but with gray) than the traditional southern *S. gouazoubira*. The first time that this sample was genetically studied [55],

it was identified as *S. gouazoubira*. For the current work, we obtained DNA from a new extraction, which was repeated on several occasions and several PCRs were repeated for the BD1 and BD2 and two times for the mitogenome, and the result was always the same. Thus, the presence of *S. gouazoubira* is confirmed for the central-southern Colombian Andes. Some authors criticized the discovery of a specimen of *S. gouazoubira* in Colombia, suggesting it was likely a case of sample confusion. Nevertheless, for BD1, we analyzed a brocket deer specimen from Toledo, Tamá National Natural Park, in the Eastern Andean Cordillera of Norte de Santander department in northern Colombia. This specimen also showed mitochondrial DNA typical of *S. gouazoubira* in a different area of the Colombian Andes than the first specimen detected further south (Huila department). This specimen had an even more reddish fur color than the specimen from Huila (and for this reason was originally classified as a red brocket deer). Both specimens formed a solid grouping within the clade of *S. gouazoubira*. In fact, the two Colombian specimens were the ones that occupied the outermost position in the ML tree with BD1. Thus, the presence of this species in Colombia is fully confirmed. An alternative hypothesis is that there were forms of brocket deer with a very northern Andean distribution (even with reddish coloration) that carried mitochondrial DNA from some ancient genetic introgression event from ancestors of the current *S. gouazoubira*. Nevertheless, this latter hypothesis seems less plausible since the significant karyotypic differences of *S. gouazoubira* with other gray brocket deer, and even more so with red brocket deer, are substantial and constitute powerful reproductive isolation barriers. Likewise, if the genetic introgression had been ancient, greater genetic divergence would be expected between the sequences of these two Colombian specimens and the sequences of *S. gouazoubira* from the southern part of the continent. It is important to be able to obtain karyotypes of Colombian specimens, molecularly *S. gouazoubira*, and of the groups that are further west in its distribution to determine if there is chromosomal homogeneity among all the specimens classified as *S. gouazoubira* or, on the contrary, there is strong chromosomal heterogeneity among these different geographical groupings in this taxon. If chromosomal differences can be demonstrated among these four clusters within *S. gouazoubira*, each one of them should be considered different subspecies or, even, full species within the genus *Subulo* despite the nucleotide diversity was moderate in the sample analyzed (1.06-1.49 %). In agreement with this, Valeri, et al. [123] analyzed 19 *S. gouazoubira* specimens from the Brazilian Pantanal from a cytogenetically point of view. Among these specimens, nine had B chromosomes and five carried a heterozygous centric fusion ($2n = 69$ and $FN = 70$). Moreover, in three specimens, the fusion occurred between chromosomes X and 16, in one specimen between chromosomes 7 and 21, and in another specimen between

chromosomes 4 and 16 (four karyomorphs). These chromosome polymorphisms could generate two different hypotheses. The first one was that the chromosome rearrangements within *S. gouazoubira* were recent and not fixed in the Pantanal population yet. The second one was that they represent a balanced polymorphism and that heterozygotes have some type of adaptive advantage, but, as we previously commented, these polymorphisms could raise infertility problems, and it can produce reproductive isolation of these populations, and speciation. Also, in Uruguay, *S. gouazoubira* showed differentiated populations. Aristimuño, et al. [117] estimated elevated levels of genetic diversity in the Uruguayan *S. gouazoubira* (24 haplotypes, $H_d = 0.940 \pm 0.016$, and $\pi = 0.049$). They identified two genetic groups in the North of the country, one in the South (that was the most differentiated) and another that comprises all the samples from the East. As they noted in a Median Joining network, the haplotypes of Maldonado and Lavalleya (Southern group) formed a very distinct group from the other ones. The haplotype network showed a star-like structure, indicating a population expansion in the recent past, which was supported by the high diversity and excess of unique or low frequency haplotypes. However, there is one study that did not show any appreciable differentiation between the gray brocket deer of Paraguay and Argentina [62]. Originally, *Mazama simplicicornis argentina* was the name that was given to describe a gray brocket deer collected in the central Chaco region in northern Argentina [124]. It was not clear if this gray brocket deer was the same classified as the holotype of *S. gouazoubira* in Paraguay. Sandoval, et al. [62] analyzed qualitative skull features and cranio-morphometric variables of *M. simplicicornis argentina* showing that were indistinguishable from those of the *S. gouazoubira* neotype. Additionally, the diploid chromosome number of *M. simplicicornis argentina* topotype corresponded with the karyotypical pattern of *S. gouazoubira* with $2n = 70$ and $FN = 70$, revealing clear homologies between karyotypes. Furthermore, the phylogenetic analysis of two mitochondrial genes (mtND5 and mtCyt-b) allocated the *M. simplicicornis argentina* specimens to a monophyletic clade of *S. gouazoubira* (100%). The authors concluded that there was not discontinuity between the Argentinian and Paraguayan gray brockets and *M. simplicicornis argentina* is a synonym of *S. gouazoubira*. Concomitantly, *S. gouazoubira* was shown to possess much more polymorphism at the Transferrin locus (six phenotypes) than other brocket deer species (*M. nana* one phenotype, *M. jucunda* one phenotype, *M. americana* two phenotypes, *P. nemorivagus* two phenotypes). García, et al. [125] interpreted this result by accepting that *S. gouazoubira* is a generalist and has a greater capacity to adapt to diverse environments intervened by humans than the other species. Likewise, these authors observed that *S. gouzaoubira* presented a

band of different electrophoretic mobility at the serum albumin locus compared to the other species mentioned. These authors interpreted this as proof of greater antiquity of *S. gouazoubira* with respect to the other four brocket deer taxa studied.

Additionally, a hunted specimen, morphologically identifiable as *S. gouazoubira*, from the Bolivian Amazon (Exaltación, Mamoré River, Beni department, Bolivia), from which a skin sample was taken, yielded ambiguous molecular results. When analyzed using BD1, despite being morphologically like *S. gouazoubira*, it was strongly associated with an *O. bezoarticus* specimen from Brazil (even though the analysis included a specimen of the same species from the same geographic area). In the BD2 analysis, however, it was strongly associated with the *S. gouazoubira* clade, with a specimen from the same geographic area (Beni department, Bolivian Amazon) and with a specimen from the Upper Paraguay region. For BD3, it behaved as the sister group to *S. gouazoubira* in a highly significant way (100%, $pp = 1$) (no *S. chunyi* genome was included in this analysis). Therefore, we note that in that area of the Mamoré River, in the Bolivian Amazon, there is a taxon phenotypically like *S. gouazoubira* but which appears to exhibit appreciable molecular differentiation (perhaps introgressed in the past by an ancestor of *O. bezoarticus*?). The problem with these specimens, from which samples were opportunistically obtained, is that they have not been deposited in any museum and, therefore, there is no holotype (or neotype) for the taxon that this specimen might represent, nor is there any information on its morphological characteristics, much less chromosomal data. Some denominations of gray brocket deer reported in geographical areas relatively close to where the sample of this specimen was obtained are *Subulo namby* Fitzinger 1873 (type locality: Caicara, Mato Grosso, Brazil) or *Subulo rondoni* Miranda-Ribeiro 1914 (type locality: Vilhena, Mato Grosso, Brazil), because they are the gray brocket deer forms previously defined as subspecies of *S. gouazoubira* in some geographically nearby areas to the Mamoré River in Bolivia, although both forms were from eastern Brazil. Another possibility is that this new molecular finding corresponds to an undescribed form of gray brocket deer. If so, a suggested name should be *Subulo mamorensis*. However, only exhaustive sampling of this remote area of the Bolivian Amazon, by obtaining karyotypes of gray brocket deer from the Mamoré River, would allow for a correct taxonomic designation of this possible new taxon.

For the taxon *chunyi*, the most up-to-date name should be *Subulo chunyi*, since this group is the most closely related to *S. gouazoubira* in all the analyses carried out in this study. In fact, in two analyses (BI tree with BD1 and BAPS with BD2), the *S. chunyi* specimens were placed within the *S. gouazoubira* clade, although most of the analyses performed

placed them as sister groups. Other molecular analyses determined *S. chunyi* to be a sister species of *S. gouazoubira*, corroborating our results [38, 56,58]. Notwithstanding, if the different clades found within *S. gouazoubira* were different species, or if future karyotypic studies, especially in the western population of the continent, were to find profound chromosomal differences, then the taxon *chunyi* might require its own genus. The name of this potential new genus should be created because the original name for this taxon was directly *M. chunyi*, as previously proposed [126]. In BD1, a specimen from the department of La Paz in the Bolivian Andes (3,100 meters above sea level, masl) was analyzed. This specimen was intermediate in size between *S. gouazoubira* and *S. chunyi* and had a strongly grayish coloration. It was included in the phylogenetic trees within the *S. chunyi* clade but was strongly differentiated from it (100%, $pp = 1$). As an example, the nucleotide diversity of the *S. chunyi* clade for BD1, including this specimen, was 4.51% (very high), while in BD2, without this specimen, it decreased to only 0.31% (low). This specimen could potentially be a new species within the genus *Subulo*, or a new species within a new genus to be designated if *chunyi* were to be reclassified if chromosomally differentiated units were found within *gouazoubira*. It is important to obtain chromosomal results from the gray brocket deer found at high altitudes in the Bolivian Andes.

The Cases of *B. citus* and *P. nemorivagus*

The other two well established taxa in the gray brocket deer were *P. nemorivagus* and *B. citus*. It's been a long time since Allen [21] referred to *P. nemorivagus* and *B. citus* as belonging to the *M. nemorivaga* group, which he differentiated from other congeners based on the possession of a short (about 50% of condylobasal length) preorbital portion of the skull. According to the same author, compared to *P. nemorivagus* from the Guianas, *B. citus* is slightly larger, and possesses a heavier dentition, and shows a very considerably distinct coloration. Thus, these taxa differentiated from each other a long time ago.

Recently, Sandoval, et al. [62] showed that the karyotype of *B. citus* topotype showed a $2n = 61$, and $FN = 70$, with 30 pairs of autosomal chromosomes, and a simple XY sexual system. The X chromosome was submetacentric, and the Y chromosome was a small metacentric. Chromosomal pair 1 was included in group A (large biarmed autosome), chromosomal pairs 2 to 4 were included in group C (small biarmed autosomes), and all acrocentric chromosomes were included in group E. The presence of supernumerary chromosomes (Bs) was observed as a variable from 0 to 5. Thus, *B. citus* has its own chromosome characteristics and for this reason, it has been included in its own genus. Sandoval et al. [62] placed *B. citus* on the western coast of

Venezuela. In this study, we showed that this taxon also extends across different departments of the Colombian Caribbean coast (Bolívar, Cesar, and Magdalena), which is an important finding for Colombian fauna statistics. In fact, the position of *B. citus* in the various phylogenetic trees is highly variable. The ML tree of BD1 relates it to *O. bezoarticus*; the BI tree, using the same database, divides it between Blastocerina and Odocoileina, the ML and BI trees of BD2 strongly associate it with *P. puda*, meanwhile the ML and BI trees of BD3 place it as the outermost brocket deer of Blastocerina (if we do not include *M. rufina* within this clade). Therefore, there is no clear resolution regarding the specific phylogenetic position of *B. citus*, although there is more evidence in favor of its inclusion with Blastocerina, but some results group it with Odocoileina. In fact, *B. citus* showed equidistant genetic distances, of the same magnitude, with the gray and red brocket deer for BD1 and BD2.

Nucleotide diversity within *B. citus* was not excessively high (1.13% for BD1 and 0.97% for BD2), so it might not appear that other internally distinguishable taxa exist, at least at the molecular level. Notwithstanding, it should be noted that the BI tree for BD1 associated it with two specimens of red brocket deer (*M. americana* VIII) also from the same geographic area inhabited by *B. citus*, and in that same analysis, the specimen from San Jacinto (Bolívar, Colombia) differed from the other analyzed specimens of *B. citus*. Therefore, the existence of differentiated taxa within *B. citus* cannot be ruled out.

Regarding *P. nemorivagus*, its ancestor appears to be more recent than that of *B. citus* in the analyses with BD1 and BD3, and the situation is less conclusive with BD2, where the ancestor of *P. nemorivagus* seems to have arisen earlier. The fact that the ancestor of *B. citus* may be older than that of *P. nemorivagus* agrees with what was shown by Sandoval, et al. [62]. Nevertheless, it was not ruled out that *P. nemorivagus*, with BD2, to be the first taxon from the gray brocket which diverged. This agrees quite well with the G-banding pattern of *P. nemorivagus* karyotype, which showed the presence of an XX/XY1 Y2 sex chromosome system as a product of an X-autosome tandem fusion, offering $2n = 68$, $FN=70$ in females and $2n = 69$, $FN = 70$ in males. This karyotype only differed from that of *Capreolus capreolus* ($2n = 70$, $FN = 72$) by X-autosome tandem fusion, which may corroborate the basal condition of this gray brocket deer and its proximity to the ancestral karyotype of the American Odocoileina [36]. This could be related to the *P. nemorivagus* specimen from the Tocantins River (Brazil) that appeared related to the ancestors of the current *O. virginianus* [116].

On the other hand, *P. nemorivagus* showed a

considerable heterogeneity of haplotypes. Originally, this taxon was assumed to be limited to the Amazon region. Nonetheless, the existence of two populations of this species has been reported in the Brazilian Atlantic Forest, more than a thousand kilometers away from its assumed distribution limit. Their existence has been proven by the genetic analysis of collected fecal samples [127]. Similarly, its presence appears to have been confirmed on Barú Island (Bolívar, Colombia) [128], although it would not be surprising if this were *B. citus*, which had not been previously reported in Colombia until the current analysis. Related to this wide distribution, the nucleotide diversity found within *P. nemorivagus* (2.49% for BD1 and 3.1% for BD2) was twice as high as that estimated for *S. gouazoubira*. Considering that possible new taxa have been detected in this latter taxon, it is very likely that there are different species or taxa within *Passalites* not yet reported. For example, BD1, which contained the most analyzed specimens of *P. nemorivagus*, showed the possible existence of up to three geographically well-defined subgroups. One of them in the Guyanas and the north of the Brazilian Amazon (Negro River), another typically from the western Amazon in Peru and Colombia, and a third in the central-southern area of the Brazilian Amazon (Madeira River and tributaries) that would also penetrate the western Amazon of Peru. In fact, Gutiérrez, et al. [38] found two reciprocally monophyletic haplogroups within *P. nemorivagus*. One only contained samples from the northern area of this species (Bolívar state in Venezuela, the Guianan region of Potaro-Siparuni, an unknown locality from French Guiana, and in the Rondônia state in Brazil), and the other consisted of samples from two unknown localities from Brazil and Peru, and from the Brazilian states of Pará, Paraná, and Rondônia. This agrees well with the chromosomal finding of Fiorillo, et al. [36] who analyzed the karyotypes of six specimens of *P. nemorivagus* from the eastern Brazilian Amazon. They determined: (1) $2n = 68/69 + 2-7B XX/XY1 Y2 FN = 70$, (2) there were 66 acrocentric autosomes from the E chromosome group, (3) the X chromosome was a medium-sized submetacentric chromosome, and (4) males had one small metacentric Y1 chromosome and one small acrocentric Y2 chromosome. Nevertheless, the analysis of a male from the western Brazilian Amazon had a variant karyotype with $2n = 67 + 7B XY1 Y2 FN = 70$, composed of one pair of large submetacentrics from the A chromosome group and 62 acrocentric autosomes from the E chromosome group. This submetacentric pair was the product of the Robertsonian translocation between the chromosomes 4 and 32 from the karyotype of the eastern *P. nemorivagus*. The fixation of this kind of rearrangement in different populations increases the probability of reproductive isolation and thus of speciation. Furthermore, for BD1, we detected two brocket deer specimens with red

coloration that were only weakly and externally related to the core group of *P. nemorivagus*, although phenotypically they differed. One of these specimens came from Paloma, Infrida River, Guainía (Colombia) (within the distribution range of *P. nemorivagus*), while the other came from the Ecuadorian Pacific coast (San José, Puerto López, Manabí, outside the distribution range of *P. nemorivagus*). Karyotypic analysis of brocket deer from these geographic areas is extremely important, as they likely represent new taxa.

Thus, intensive chromosomal studies within *Passalites* are needed because there may be taxa not yet described within this genus. Several potential new species within *Passalites* could receive their new scientific names from previously proposed subspecies of *S. gouazoubira*, which we now know corresponded to *Passalites*. These taxa are *M. gouazoubira medemi*, *M. murelia*, *M. permira*, *M. rondoni* [60]. Potentially, specimens of *P. nemorivagus* we detected in the Peruvian Amazon should be defined as *P. whitelyi* Gray 1872, if it is not a species yet to be described.

Evolution and Systematics of the Red Brocket Deer

The phylogenetic position (and the taxonomic nomenclature) of the red brocket deer is more complex than that observed in the gray brocket deer.

The case of *M. rufina*

The relationship of *M. rufina* to Blastocerina and Odocoileina is ambivalent depending on the phylogenetic analyses performed. In all analyses using BD1 and BD2, *M. rufina* behaved as the outermost clade of Odocoileina, while with BD3 (mitogenomes) it behaved as the outermost clade of Blastocerina. Genetic distances using BD1 showed that *M. rufina* had equidistant values of the same magnitude as gray and red brocket deer. With BD2, the genetic distances between *M. rufina* and the other red brocket deer tended to be smaller than those with respect to gray brocket deer. What is clear is that, regardless of whether *M. rufina* should be placed in Odocoileina or Blastocerina, this taxon should be placed in a genus different from *Mazama*. The genetic differentiation of *M. rufina* relative to other red brocket deer is of the same order as the genetic differences between *Passalites* and *Subulo*. In this respect, we fully agree with Ramírez-Chaves, et al. [65]. They designated this taxon with the new name *Andinocervus rufinus*. Nevertheless, we are not entirely convinced that this is the correct taxonomic nomenclature for this taxon. For example, Brooke [129] designated the specimen described by Pucheran [16] from Pichincha (Ecuador) as *Cariacus (Coassus) rufinus*. *Cariacus* is used to designate some fossil forms of Cervidae and is also associated with *O. virginianus*. Nevertheless, *Coassus* is

a synonym of *Mazama* but has not been used for any extant brocket deer taxon and has previously been related to *rufina*.

Another interesting aspect is that in BD2, two sequences extracted from GenBank were added, which were attributed to the taxon known as *M. bricenii*, native to Venezuela. The analysis showed that *M. bricenii* was molecularly indistinguishable from *M. rufina*, and is a synonym of the latter, as previously shown [130]. These authors showed that there were no morphological differences between specimens geographically attributable to *M. bricenii* and *M. rufina*, and their phylogenetic analyses supported *M. bricenii* nested within *M. rufina* such as we also found. Diverse analyses revealed the existence of suitable climatic conditions for *M. rufina* in the Táchira Depression during the last glacial maximum and currently, suggesting that gene flow between populations of this species in the eastern Colombian Andean cordillera and in the Mérida Cordillera has existed in the recent past and may continue to exist today. In fact, Allen [21] showed that both forms were so similar in size, color, and pelage character that the two species would be regarded as local forms of the same species if they shared the same geographical range (northern Andes). Initially it was thought that the depth of the lacrima fossa and the shape of the frontal bones could be used to distinguish between *M. rufina* and *M. bricenii* [131]. Notwithstanding, these two characters have proven to be highly variable, and they did not work well for differentiating the alleged taxa [57,130].

M. rufina has traditionally been considered monotypic [132,133]. Nevertheless, with BD1 and BD2, it was observed that the haplotypes related to the most ancestral members of this taxon differ considerably from the haplotypes of the group whose ancestors are more recent. For example, in the ML tree of BD1, the first eight specimens to diverge were Ecuadorian, and two of them were quite divergent from the rest (one specimen from Ambato, Tungurahua province, and one specimen from San José de Sigchos, Cotopaxi province). BD2 also identified an Ecuadorian specimen of unknown origin as the most divergent in that database (BD3 did not include any of these Ecuadorian specimens). Similarly, nucleotide diversity in *M. rufina* was high (1.58% for BD1 and 2.16% for BD2), which is consistent with possible taxonomically distinct groupings within it. The spatial structure within this taxon also appears remarkable. Therefore, it is possible that at least two different taxa exist within *M. rufina* in Ecuador. Obtaining karyotypes from different Ecuadorian specimens of *M. rufina* could be vital for determining possible distinct taxa within this species. It is also evident from these data that the origin of the current *M. rufina* occurred in the present-day Ecuadorian Andes.

The case of *M. pandora*

Recently, it has been suggested that this brocket deer should be included as a third species within the genus *Odocoileus* (*O. pandora*). Gutiérrez, et al. [38] were the first to propose the inclusion of *Odocoileus pandora* in the genus *Odocoileus*. Subsequently, the phylogenetic relationship between *M. pandora* and *Odocoileus* was further confirmed in a more informative analysis based on mitogenomes [60,62], suggesting the adoption of *Odocoileus pandora*. Notwithstanding, our results are ambiguous in this regard. Using the ML and BI trees of BD1, *M. pandora* appeared to be the outermost group of red brocket deer, excluding *M. rufina*, and therefore not the sister group to *Odocoileus*. BAPS analyses with BD1 showed two different results. On the one hand, there was no association between *M. pandora* and *Odocoileus* when only all red brocket deer (excluding *M. rufina*) and *Odocoileus* were considered in the analysis. On the other hand, when all analyzed brocket deer taxa were considered together with *O. virginianus*, *M. pandora* appeared associated with the white-tailed deer. Using BD2, with the ML tree, it was again the outermost group of red brocket deer (excluding *M. rufina*), but with the BI tree, it was indeed established as the sister group to *Odocoileus* and, in fact, was associated with *O. hemionus*, although the *pp* value was not highly significant (*pp* = 0.6). The two BAPS analyses showed no association between *M. pandora* and *Odocoileus*. Using BD3, an association between *M. pandora* and *Odocoileus* was found in all phylogenetic trees. Nevertheless, the significance of these associations in both the ML (68%) and BI (*pp* = 0.51) trees was quite moderate. Using BAPS, with red brocket deer and *O. virginianus*, *M. pandora* was associated with some of the *Odocoileus* specimens (North American specimens), while the remaining *Odocoileus* specimens were associated with a group of *M. americana*. Using all analyzed brocket deer taxa plus *O. virginianus*, however, *M. pandora* was associated with red brocket deer, but not with *O. virginianus*. Although the sample of *M. pandora* we used is very limited, it does not appear that other molecularly well-differentiated taxa exist within it, as the nucleotide diversity was very limited (0.7% for BD1 and 0.1% for BD2). This uncertainty regarding the position of *M. pandora* had already appeared in other studies. It seems vital to perform an analysis with nuclear DNA to definitively resolve the taxonomic position of *M. pandora*.

The case of *M. temama*

Another well-defined taxon within the red brocket deer group was *M. temama*, which was perfectly distinguishable with all the phylogenetic methods used, such as other authors have mentioned [27,63,64, 134].

All our phylogenetic trees identified *M. temama* as a well-characterized group, generally the sister group to the red brocket deer of the *M. americana* complex. The only exceptions were some BAPS analyses. With BD1, using only red brocket deer (excluding *M. rufina* and *M. pandora*)

in the analysis, *M. temama* was distinguished from the other brocket deer of the *M. americana* complex, except for the two “rare” red brocket deer from the Colombian and Venezuelan Caribbean area that remained associated with it (*M. americana* VIII). The other two BAPS analyses with BD1 did not distinguish *M. temama* from the other brocket deer of the *M. americana* complex. The first two analyses with BAPS and BD2 did differentiate *M. temama* from the different groupings of the *M. americana* complex, while the third did not differentiate it from those other red brocket deer. The first BAPS analysis with BD3 also distinguished *M. temama* from the groupings of the *M. americana* complex, but the remaining two BAPS analyses did not. Overall, this shows the existence of an observable molecular differentiation between *M. temama* and the *M. americana* clusters, but within a context of relationship between them.

Some morphometric results separated *M. temama* from *M. americana* but not from the other small *Mazama* species [67]. Nevertheless, Escobedo-Morales, et al. [63] found divergence in skull size between individuals from Mexico and Guatemala (northern group; *M. temama temama*) relative to specimens from Honduras, Nicaragua, Costa Rica, and Panama (southern group; *M. temama reperticia*), the latter being 8% larger than the northern group. Centroid size showed a significant correlation with geographic distance suggesting an isolation-by-distance pattern for the 2D geometric morphometrics in the Mexican and Central American *M. temama* with low geographical overlap between these two taxa, which suggests niche specialization for each one of these *M. temama* forms.

Within *M. temama*, using all available databases, the existence of two groupings with geographical and taxonomic significance was clear. The nucleotide diversity of *M. temama* was high (1.59% for BD1 and 3.02% for BD2). This suggests the presence of different taxa within it. Indeed, all phylogenetic analyses detected two groups of specimens within *M. temama*. The larger sample, that of BD1, showed the differentiation of Colombian specimens and one Ecuadorian specimen from Mexican specimens. This work demonstrates, for the first time, the molecularly confirmed existence of *M. temama* in Ecuador. The most divergent specimen in this grouping came from the Chocó region (Colombia). This suggests that the oldest ancestor of this group may have originated in that Pacific region of what is now Colombia. BD2 and BD3 also showed the difference between the Colombian and Mexican specimens. It is noteworthy to comment that in some analyses we observed that the Mexican sequences of *temama* were derived from the southern sequences from Colombia. While we were obtaining the results that showed a significant molecular differentiation between *M. temama* from Colombia and Mexico, the work of Escobedo-Morales, et

al. [64] appeared, who, using genome-wide ultraconserved elements (UCE) and mitogenomes, confirmed the differentiation between Mexican and Colombian specimens (they only used one of this last nationality, previously analyzed by us), in addition to differentiating within *temama* three groups. These three groupings have been given the status of full species: *Mazama temama sensu stricto* (Mexico to Honduras), *Mazama reperticia* (Nicaragua to Panama), and *Mazama zetta* (Colombia). Following this work, the Colombian and Ecuadorian specimens should be cited as *M. zetta*. Like the findings in this work, Escobedo-Morales, et al. [64] determined that the Mexican *temama* derived from the southern Central American ones. Thus, it is possible that *temama* dispersed from South America to Central America and differences in available resources with subsequent isolation due to climatic barriers, and chromosomal changes, may have promoted differentiation in size (lower size in northern specimens), although without extensive changes in shape. In this context, Escobedo-Morales, et al. [63] suggested that the Motagua-Polochic-Jolotán fault system probably played a key role in promoting morphological differentiation by climatic isolation of *M. temama sensu stricto*.

On the other hand, Escobedo-Morales, et al. [64] did not specify whether they found significant spatial structure within *M. temama sensu stricto* in Mexico. Our analysis with BD2 apparently showed a spatial structure for this species in Mexico, with some differences between the central Mexican area (Hidalgo and Veracruz) (61 %, $pp = 0.72$) and the southern Mexican area (Campeche and Chiapas) (96 %, $pp = 1$). Related with this, Sandoval, et al. [67] showed that the *M. temama* neotype (from Veracruz) had a karyotype with $2n = 44 + 4-5Bs$, $FN = 70$, and a simple sexual system type XY. Chromosomal pairs 1–4 were classified as belonging to group A (large autosomal of two arms), pairs 5–12 in group C (small autosomal of two arms), and all acrocentric (pairs 12–21) belonging to group E (small chromosomes of one arm). The X chromosome appeared as a submetacentric chromosome and the Y as a small metacentric. Nonetheless, specimens from Campeche (southern Mexico) showed a karyotype of $2n = 46/47 + 1-4Bs$, $FN = 70$, with polymorphism in the chromosomes of groups C and E in relation to the neotype from Veracruz, indicating that fusions of chromosomes of group E of the Campeche specimens generated the differences in relation to the chromosomes of group C with the neotype. Nevertheless, when these authors employed the same mt*Cyt-b* gene that we used (our BD2), they did not find a spatial genetic structure for these Mexican *M. temama*, but when employing the mt*D-loop* marker, they did. Finally, with reference to *M. temama*, it should be noted that robust chromosomal studies should be carried out with specimens from the three groups found by Escobedo-Morales, et al. [64]. If these chromosomal studies detected appreciable

differentiation between them, the designation of the three groups as full species would be confirmed, suggesting the certainty of the proposal by these authors, but, in that case, the nomenclature of a new genus for *temama* complex should be used. On the contrary, if chromosomal differences did not exist, or were slight, between the three groups, then they should be treated as subspecies from *M. temama* and this taxon could be maintained in the genus *Mazama* due to its relationship with the *M. americana* complex. One final consideration related to *M. temama*. Recently, Sandoval, et al. [66] reported a supposed new species of brocket deer from La Ramada, Lambayeque department, in the western Peruvian Andes. The taxon was named *Mazama tschudii*. Karyotypic analysis showed that it was very different from all the brocket deer previously analyzed from this perspective, except for one, the Mexican neotype of *M. temama*. Both were similar, although the Peruvian specimen had some different chromosomal characteristics compared to *M. temama*. Notwithstanding, at the mitogenomic level, it remained strongly associated with the mitogenome we generated years ago from a Colombian *M. temama* (San Francisco, Huila, Colombia) [55]. If we accept that the form found in Colombia is *M. zetta* and knowing from this work that it is the same as that found in Ecuador, it is likely that the specimen from northern Peru named *M. tschudii* is an *M. zetta* specimen that has reached the north of the aforementioned country.

The case of the *M. americana* complex

Regarding the specimens of traditional *M. americana*, the systematic question is extremely complex, as other authors have shown using chromosomal and molecular studies in recent decades [10,11,24,25,33, 39,41-44]. This is motivated by the large chromosomal variation found in the traditional *M. americana*, which can be explained by the theory of chromosomal fragility [29,135,136], which would induce the occurrence of breakages and chromosomal exchanges. These chromosomal rearrangements could have led to the formation of new species in just a few generations after geographical isolation, given that chromosomal changes can promote incipient divergence and lead ultimately to species diversification [137]. We used the concept of complex for the specimens we sampled “a priori” (or whose sequences we obtained from GenBank), assuming that they corresponded to traditional *M. americana* due to the constant new reports of chromosomal variation for many of these specimens. We designated each grouping within this complex with Roman numerals, although this does not necessarily imply a taxonomic commitment. We set the BD1 as a reference point because it included more samples than the analyses carried out in BD2 and BD3. With BD1, we distinguished eight different groups within the *M. americana* complex and seven of these groups will also be found, although with fewer specimens, in BD2 and BD3.

The one we referred to as *M. americana* I was the most

divergent with BD2 and BD3 and the closest with these datasets to *M. temama*. We are certain that this cluster contains the “true” *M. americana* because it contains two specimens from French Guiana. One of them was the one used as neotype [39] to define *M. americana* “sensu stricto”. The neotype of *M. americana* found by Cifuentes-Rincón, et al. [39] in French Guiana according to its karyotypic pattern did not fit into any of the red brocket deer cytotypes previously studied in Brazil [10]. It is a molecularly homogeneous and well-constituted group. Some BAPS analyses detected the distinct genetic profile of this group, even though this technique showed lower differentiation among many groups compared to phylogenetic trees. For example, the first two BAPS analyses applied to BD2 perfectly distinguished *M. americana* I from the other *M. americana* groups. Besides French Guiana, it extends across northern Brazil (Pará state), the Caribbean Island of Trinidad, and even, with BD2, we detected an individual in northern Peruvian Amazonia (Loreto department). Notwithstanding, it presents another surprising fact: within *M. americana* I, several *M. rufa* individuals were found. In BD1, two specimens were analyzed, one from Paraná (Brazil) and another obtained directly by us from the Canindeyú department in Paraguay. In BD2, two specimens from Paraná (Brazil) were analyzed. *M. americana* I is an association with medium-low nucleotide diversity (0.97% for BD1 and 0.67% for BD2), but it still includes two chromosomally distinct red brocket deer species. Therefore, *M. rufa* is molecularly very slightly dissimilar from *M. americana* “sensu stricto”. A study concluded that the karyotype of *M. rufa* diverged from that of *M. americana* “sensu stricto” by fifteen rearrangements, which confirmed their substantial chromosomal divergence and probable reproduction isolation [43]. This study also proposed molecular differentiation of *M. rufa* from *M. americana* “sensu stricto”, the first identified as distinct molecular operational taxonomic units by the General Mixed Yule Coalescent species delimitation analysis, although morphological data from craniometry and body biometry indicated an overlap of quantitative measurements between *M. rufa* and *M. americana* [43]. Nevertheless, BD1, BD2, BD3 showed that both taxa share the same grouping when more specimens “a priori” classified as *M. americana* from more diverse origins were included. This introduces additional complexity. Due to the chromosomal fragility in the brocket deer, taxa that have temporally very close ancestors and are therefore molecularly very similar can become new species in a very short time (molecularly undetectable as different species). They do this through reproductive isolation resulting from significant chromosomal rearrangements. Therefore, within this *M. americana* I group we find two well differentiated species according to cytogenetic evidence because differences of more than two chromosomal pairs generate an efficient postzygotic reproductive barrier [11,42]. Additionally, intra-specific hybrids between cytotypes of *M. americana*

with heterozygous tandem chromosome fusion had severe fertility reduction, although Robertsonian translocations did not show a severe effect on the reproductive fitness of these hybrids of *M. americana* [122]. This agrees quite well with what is observed with heterozygous tandem fusions, which are considered highly deleterious chromosomal rearrangements, rapidly removed or fixed at meiosis during speciation [138-141]. In fact, the reproductive fitness of these hybrids is severely aggravated by the chromosomal imbalance observed in sperm. Greater than three chromosome divergence pairs represent a post-zygotic reproductive barrier, and, even, the subfertility observed among red brocket deer with a minor chromosome divergence (one or two chromosome pairs) would already represent some degree of isolation. This leads to the hypothesis that each *M. americana* cytotype could correspond to a distinct species [10,11, 42,122,123]. The same can be said for the other groups in the *M. americana* complex besides *M. americana* I. Consequently, the actual number of red brocket deer taxa in the eight groups we detected within this complex is surely considerably greater than eight.

This same phenomenon—finding associations within the *M. americana* complex containing species that have been previously differentiated chromosomally—also occurs in the *M. americana* II-III group (BD1 and BD3), and in *M. americana* II (a,b) and *M. americana* III (BD2). In BD2, as the number of specimens analyzed from that group increased, *M. americana* II-III split into two distinct groups, just as two different subgroups (IIa and IIb) were identified within *M. americana* II. Within *M. americana* II-III (BD1 and BD3), the sequences of three specimens were found: one corresponding to the species known as *M. jucunda* (previously *M. bororo*) from Brazil [25,44], another corresponding to the species *M. nana* from Paraguay, and another belonging to a specimen of *M. americana* from Juína (Mato Grosso, Brazil). For example, the first two species have been well differentiated chromosomally. Previously, *M. jucunda* was named *M. bororo* [25]. Nevertheless, the study of Mantellato, et al. [44] showed that the type localities of *Mazama americana jucunda* and *M. bororo* were very similar on morphological traits. Additionally, they obtained DNA sequences of the holotype of *M. americana jucunda* and compared it with other species of *Mazama*, including the holotype of *M. bororo*. The phylogenetic tree obtained showed a strong relationship among these taxa. Thus, it was clearly shown that *M. jucunda* and *M. bororo* were the same biological entity. *M. jucunda* was the oldest name available for the small red brocket occurring in the Atlantic Forest from southern Brazil, leaving *M. bororo* as a junior synonym. Although the molecular divergence of both forms (*M. jucunda* and *M. nana* from Paraguay) is somewhat greater than that found between *M. americana* “sensu stricto” and *M.*

rufa, it was again evident that speciation in brocket deer is rapid and abrupt due to significant chromosomal changes, and that molecularly they are not easily distinguishable in some cases because the separation was very recent.

For example, the nucleotide diversity for *M. americana* II-III is rather low (0.88% for BD1 and 0.49% for BD2). All phylogenetic trees detected this association, even though these species are perfectly distinct at the chromosomal level. To further complicate matters, a third sequence, that of an *M. americana* from Juína (Mato Grosso, Brazil), was added to these two. This had already been detected by Cifuentes-Rincón, et al. [39], using the mitochondrial DNA control region, as they found that *M. jucunda* and *M. nana* were associated with *M. americana* specimens from Juína and Rondônia. When more red brocket deer sequences related to these taxa (BD2) were introduced, this grouping split, as previously mentioned, into two distinct groups. In this case, *M. americana* II included *M. nana* from Paraguay (IIa) and *M. jucunda* from Brazil (IIb), while *M. americana* III contained one specimen of *M. americana* from Juína (Mato Grosso, Brazil) and another specimen of *M. americana* from Vilhena (Rondônia, Brazil). It is even possible that differentiated taxa may exist within *M. americana* IIa. In fact, Abril, et al. reported a diploid chromosome number for this species ranging from 36 to 40 and an FN ranging from 50 to 60 and there were seven B chromosomes. Furthermore, Abril, et al. [142] showed the existence of heterozygous karyotypic variants in the Brazilian *M. nana*, which is a proof of recent karyological evolution. This should be the product of translocations continuously generated by selectively neutral recent chromosomal changes and fixed due to related specimens, which could originate different species. This hypothesis is strengthened by the higher chromosome fragility found in the brocket deer. Notwithstanding, different analyzes of body measurements in *M. jucunda* and *M. nana* did not reveal morphometric differences with some specimens of *M. americana* even though both taxa are chromosomally well established [143].

In the present work, we discovered a new group that had previously gone unnoticed by all authors who have studied the genetic evolution of the red brocket deer. This is *M. americana* IV, which extends widely throughout the Peruvian Amazon, encompassing the Amazon River basin, the Ucayali River basin, and the Madre de Dios River basin, reaching at least as far as the Amazonian border between Peru and Brazil at the Yavari River. *M. americana* IV was the *M. americana* complex grouping closest to *M. temama* in BD1. For this grouping, nucleotide diversity is not very high (0.33% for BD1 and 1.23% for BD2), although BD2 included a specimen from the lower Yungas region of the La Paz department (Bolivia), which seems to increase its distribution area and, therefore, it could contain at least two distinct taxa. This

grouping has not yet been chromosomally characterized, so it warrants urgent chromosomal analysis.

We also highlighted another *M. americana* grouping (*M. americana* V), which extends sympatrically with the previous grouping throughout the Peruvian Amazon. In addition to extending throughout the Peruvian Amazon, this group reaches at least as far as the center of the Bolivian Amazon (Mamoré River) and as far as the south-central part of the Brazilian Amazon (Alborada, Aripuana River, a tributary of the Madeira River). It had also gone unnoticed as a genetically distinct grouping, because previously, only a single sequence had been generated from a specimen from the Peruvian department of Madre de Dios. Nevertheless, this sequence was never assigned to any particular group of red brocket deer. *M. americana* V possessed high internal nucleotide diversity (1.98% for BD1 and 1.85% for BD2), so it may possibly contain at least two different taxa within it (one exclusively in the Peruvian Amazon, reaching the border with the Brazilian Amazon at the Yavari River, and others with a broader geographic range throughout the Peruvian Amazon, extending into the Bolivian Amazon and south-central Brazil). Like the previous grouping, it requires extensive chromosomal studies to determine the presence of new red brocket deer species.

We also identified, for the first time, the *M. americana* VI group, whose geographic distribution range (BD1) was found in north-central Peruvian, Ecuadorian and Colombian Amazon. Surprisingly, a specimen from this group was found on the other side of the Andes (trans-Andean) in the Cauca department of Colombia (BD1, BD2, BD3). In BD2, some sequences from GenBank were introduced, and it was observed that a specimen from Manaus (Brazilian Amazon) grouped with *M. americana* VI, thus extending the geographic distribution of this group. Similarly, a specimen of *M. americana* from Yaracuy (Venezuela) appeared weakly associated with this group, which could very likely constitute a different taxon. *M. americana* VI showed high nucleotide diversity (1.41% for BD1 and 3.14% for BD2), so it probably contains more red brocket deer taxa.

BD1 allowed us to detect a new grouping not previously recorded. This is *M. americana* VII, originally composed of only three specimens: two from the Ucayali River area (in its middle section) and one from northern Bolivian Amazon (Ribera Alta, Beni River, Beni department, Bolivia). The first BAPS analysis for BD1, BD2, and BD3 differentiated this grouping from the others within the *M. americana* complex. In some trees with BD2, since only the Bolivian specimen was retained (as the two Peruvian specimens could not be amplified for BD2 and BD3), it was associated with *M. americana* VI (ML and BI trees for BD2, and BI tree for BD3).

Finally, we detected a pair of red brocket deer (although

the body was half red, half gray) that formed *M. americana* VIII. This grouping was only present in BD1 and, despite being composed of a small sample, exhibited high nucleotide diversity (1.98%), suggesting that both individuals could represent distinct taxa. The first BAPS analysis (BD1) clearly differentiated this grouping from the other groupings within the *M. americana* complex. The third BAPS analysis, also for the same database, also differentiated this pair of specimens but associated them with *B. citus* (as did the BI tree). A density tree for BD1 (not shown here) showed that *M. americana* VIII, like *M. americana* VII, were the first branches to differentiate within the *M. americana* complex. This pair of specimens was distributed in the same geographic area of Venezuela and northern Colombia as *B. citus*. Since we were unable to include the specimen from Yaracuy (Venezuela), a geographically close area to the distribution zone of *M. americana* VIII, in BD1 (and, likewise, since we were unable to introduce these two specimens into BD2), we do not know if the specimen from Yaracuy part of *M. americana* VIII could be.

It should also be noted that in BD3, a grouping was detected that had not appeared in either BD1 or BD2, consisting of one specimen of *M. americana* IV, two specimens of *M. americana* V, and two specimens of *M. americana* VI. Remarkably, this grouping showed, for the BI tree and the third analysis with BAPS (for BD3), a strong association with the *O. virginianus* group from the south-central Peruvian Andes. A possible hypothesis is that, starting from ancestral brocket deer forms with ancestral karyotypes similar to those of modern *Odocoileus* (which is considered to have a primitive cervid karyotype), there may have been genetic introgression from those brocket deer to some *O. virginianus* population, or that some remnant of brocket deer, not studied karyotypically, still exists that exhibits karyotypic similarity to *O. virginianus* and could successfully interbreed with them, these brocket deer carrying mtDNA from *O. virginianus*. This could explain the strange, yet strong, phylogenetic relationship between this *M. americana* group from the northern Peruvian and Bolivian Amazon and the *O. virginianus* from the Peruvian Andes. Furthermore, a result like this could have a very considerable impact on the genus *Odocoileus*. Escobedo-Morales, et al. [46], and other authors [1,35,144] advocate for placing *Odocoileus* within the genus *Mazama*.

Likewise, this mixed grouping shows that there are more taxa within the *M. americana* complex than the eight groupings detected. Many of these taxa (both those detected and those not) are probably full species, as they must have significant chromosomal differences that become effective reproductive isolating mechanisms. In fact, some groupings within the *M. americana* complex exhibit more heterogeneity among themselves than with *M. temama*. If we accept that

this latter taxon is a full species (or, more accurately, contains three full species following Escobedo-Morales, et al. [64]), then these taxa within the *M. americana* complex should also be considered full species.

Therefore, we report at least five new groupings within *M. americana*, which are likely to contain several potential new red brocket deer species. Nevertheless, it is important to highlight, for example, that *M. americana* I, IV, V, VI and VII converge in the Peruvian Amazon. However, in the field, it is impossible to distinguish them since they are morphologically practically identical. As Duarte, et al. [9] commented, the red brocket deer is considered one of the most impressive and surprising cases of morphological convergence among mammals. In fact, Rossi [24] reported on the difficulty in detecting any pattern of similarity between *M. americana* samples according to cranial morphological characteristics. Additionally, a tree of morphological distances, generated from the cranial measurements of the different *M. americana* cytotypes, showed that two clades were found. Specimens belonging to the same cytotypes were positioned in either clade indicating a variation of cranial morphometric characteristics across specimens. This variation did not appear to have any geographical relation with the red brocket deer especially those studied in Brazil [39].

Abril, et al. [10] reported two possible species within *M. americana* in Brazil by using *mtD-loop* and *mtCyt-b* genes with high bootstrap values. One was composed of Juína, Rondônia, and one haplotype from Santarém cytotypes, whereas the other was composed of Paraná, Carajás and another haplotype from the Santarém cytotypes. The first group was composed exclusively of specimens from northern Brazil. In the second group, there were three specimens from the north of Brazil (one from Santarém, and two from Carajás) clustering with the Paraná cytotypes (southern Brazil). The first group (including the cytotypes of Juína and Rondônia) showed a low diploid number ranging from 42–45 chromosomes and a FN of 46–48. This northern western Brazilian Amazon group diversified around 1 mya. The other group included Paraná, Carajás and Santarém cytotypes with a higher diploid number ranging from 48–53 chromosomes and a FN of 54–56. This eastern Amazonian through southern Brazil at the Paraná River basin group diversified around 2 mya. The species *M. nana* and *M. jucunda* were more related to the first group than to the second group. Henceforth, with only the inclusion of 35 new specimens of *M. americana* (BD1), we enlarged these two Brazilian groups to eight *M. americana* groups in South America, with multiple subgroupings within some of these groupings. It is easy to suspect that, once molecular and chromosomal results will be obtained from more red brocket deer specimens from other South

American countries such as Colombia, Ecuador, and Bolivia, the number of new species will increase considerably.

Mitogenome analysis (BD3), by analyzing a smaller number of individuals, also omits or obscures the possibility of detecting more consolidated groupings, although when it does detect them, statistical consistency is generally greater. Nonetheless, seven groupings mentioned above were evident in the mitogenomic data, although the relationships between them undergo some important changes.

Finally, a taxonomic note. We believe that the genus *Mazama* should only be used for groupings within the *M. americana* complex. The form *temama* should be given a different generic name to include the three species identified by Escobedo-Morales, et al. [64] (*M. temama*, *M. reperticia*, and *M. zetta*), while the name *M. americana* should be used exclusively for *M. americana* I specimens that do not belong to *M. rufa*. All other groupings within the *M. americana* complex should be given their own specific scientific names (new or existing) within the genus *Mazama*.

Other Striking Systematic Relationships in Blastocerina

Two systematic and phylogenetic relationships attracted attention in Blastocerina. Recently, Barrio, et al. [115], using the *mtCyt-b* gene, demonstrated that the systematics and taxonomy of pudus, which have traditionally been classified into a single genus, *Pudu*, and two species, *Pudu mephistophiles* (northern pudu) and *Pudu puda* (southern pudu), should be modified to two different genera, *Pudella* (new genus) for the northern form (Colombia, Ecuador, and Peru) and *Pudu* for the southern form (Chile and Argentina), since they are not monophyletic. Within the genus *Pudella*, two species have been recognized: *Pudella mephistophila* (the form north of the Huancabamba Depression) and a new species, *Pudella carlae* (the form south of the Huancabamba Depression). In BD1 and BD2, we use various specimens that are associated with the aforementioned species of pudus. With BD1, the ML tree perfectly matched that shown by Barrio, et al. [115]. *Pudella* formed its own clade with the two well-differentiated species (*Pudella mephistophila* and *Pudella carlae*, with two specimens we obtained from the Huancabamba Depression area), while *Pudu puda* was grouped in another clade with *B. dichotomus*. Notwithstanding, also with BD1, the BI tree united *Pudella* and *Pudu* in the same clade. For BD2, both trees (ML and BI) showed *Pudella* forming its own clade, while *Pudu* was associated with another clade with *B. citus*. Within *Pudella*, three highly significant groups appeared: two for *Pudella*

mephistophila (one more associated with *Pudella carlae*) and one for *Pudella carlae*. Unfortunately, we were unable to obtain mitogenomes from these pudu samples. Therefore, it would be advisable to analyze complete mitogenomes, nuclear genes, and karyotypes of these different pudu taxa.

Another result with strong systematic and phylogenetic implications was observed with *H. antisensis*. Only in BD2 were it possible to introduce samples of *H. bisulcus*. Both ML and BI trees showed paraphyly for *H. antisensis*. Two specimens from the Arequipa department in southern Peru showed their own clade, while three *H. antisensis* specimens (Bolivian and Argentinian) formed a separate (monophyletic) clade but were associated with a sister clade made up of three *H. bisulcus* specimens. Since there is no doubt that the specimens from southern Peru, as well as those from Bolivia and Argentina, were phenotypically *H. antisensis*, we can hypothesize that the *H. antisensis* specimens from the southern distribution were genetically introgressed in the distant past by *H. bisulcus*, while the *H. antisensis* from Arequipa (Peru) were never introgressed by *H. bisulcus*. An alternative hypothesis, although we favor the first, would be the existence of two cryptic species within *H. antisensis*, with the southernmost one giving rise to *H. bisulcus*. Nevertheless, in the trees obtained, the southernmost group of *H. antisensis* was not observed to be older than that of *H. bisulcus*. Fortunately, we have generated complete mitogenomes for *H. antisensis* to test these hypotheses.

Times and Epochs of Divergence in the Evolution of Neotropical Deer

The initial divergence of the ancestor that gave rise to the current Neotropical deer was estimated at the end of the Miocene and beginning of the Pliocene (between 4 and 5 mya). This agrees quite well with the estimates of other authors [9]. For example, Escobedo-Morales, et al. [46] estimated the divergence between red and gray brocket deer at 4.45 mya (3.41–5.54 mya 95% HPD) at mt*Cyt-b*, while an earlier estimate of 4.65 mya (3.86–5.65 mya) was obtained with a concatenated three mt marker dataset (*ND2*, *Cyt-b*, *D-loop*). Henceforth, there is a complete agreement between the divergence times obtained for the beginning of the evolution of brocket deer and white-tailed deer obtained in this study and those estimated by other authors.

This was a period in which planet Earth experienced a marked trend toward global cooling, increased aridity, and high climatic seasonality. The cold, dry climate of the Pliocene caused a massive retreat of tropical rainforests, which were replaced by open biomes, such as savannas and shrub steppes. Essentially, it was at this time that

the differentiation event that gave rise to Blastocerina and Odocoileina occurred. If the arrival of deer in South America was due to the definitive closure of the Isthmus of Panama (around 3 mya), then this differentiation between Blastocerina and Odocoileina must have taken place in North America or the northern part of Central America, and the arrival of present-day South American deer (especially brocket deer) must have occurred during the Great American Biotic Interchange (GABI), the beginning of which is dated to 2.8–2.5 mya [145]. The traditional view has been that the GABI occurred during the development of a land bridge across the Panamanian region, and that this corridor ended around 3 mya. Notwithstanding, paleontologists are aware that there was limited exchange of taxa in both directions before this time, and before the development of a well-defined land bridge in what is now Panama, whose precursors were designated as “New Island Hoppers” [146] or “heralds” [147,148]. Geological and tectonic reconstructions [149] have shown that the Panamanian region contained a series of islands around 6 mya, with the Central American Sea largely interrupted by an evolving volcanic arc around 12 mya, when the southwestern margin of the Caribbean Plate collided with the South American continent. Apparently, this facilitated the arrival of the first sloths in North America around 9 mya [150], and the arrival of the first Holarctic mammals in South America (Argentina) approximately 7.3 mya during the Huayquerian (Late Miocene; 9–6.8 mya). These were procyonids of the genera *Cyonasua* and *Chapalmalania* [151, 152]. In fact, four taxa of North American origin (proboscids, tapirs, peccaries, and dromomericines such as *Suramerix*), dating back 9.5 mya, have been found in the Amazon basin, in strata supposedly older than those reported for both procyonids. The next immigrants were endemic genera of sigmodontine rodents and a procyonid genus, *Parahyaenodon*, from the Monte Hermosan Age (6.8–4 mya, Late Miocene–Early Pliocene; [153]). Many of these taxa were linked to forest environments and were not well adapted to island colonization. Therefore, a land bridge was necessary for these colonization events. All these events could be related to the development of the Baudó Bridge around 10 mya, which connected northwestern South America with central Panama through the San Blas and Baudó mountain ranges [154]. They could also be linked to the closure of the Central American Maritime Canal, which occurred around 10 mya [155,156]. Therefore, either as “hopeful islanders”, to use Simpson’s [146] expression, or via the land bridges that existed during the Late Miocene, the ancestors of present-day Neotropical deer were able to reach South America before the final closure of the Isthmus of Panama (3–2.5 mya) and begin their diversification “in situ” in South America.

In fact, our estimates of mitochondrial diversification for Blastocerina at its inception date 3.53 mya, 3.5 mya,

and 3.54 mya for BD1, BD2, and BD3, respectively, and for *Odocoileina* at 3.63 mya, 3.2 mya, and 3.11 mya for BD1, BD2, and BD3, respectively, during the Pliocene, in a period immediately preceding or during the final closure of the Isthmus of Panama.

In the case of gray brocket deer, the ancestor of *B. citus* (assuming it is indeed a *Blastocercina*) began its diversification in the late Pliocene, early Pleistocene (2.86–2.09 mya). For *O. virginianus*, slightly lower estimates were obtained (2.34–1.44 mya), but these could potentially also imply the beginning of the Pleistocene. In the case of *M. rufina* (assuming it is indeed an *Odocoileina*), the ancestors of the most divergent Ecuadorian specimens were also estimated to have originated around the same time as *B. citus* (2.48–1.68 mya). Thus, the origin of this red brocket deer could be older than that of all other red brocket deer groups. Therefore, our new temporal estimate for *M. rufina* was considerably older than that estimated by other authors who considered that all red brocket deer diversified within the last 2.5 million years [11]. One of the estimates obtained for the onset of mitochondrial diversification in the ancestor of *M. pandora* was also close to the beginning of the Pleistocene (2.83 Ma), although a second estimate was substantially lower (1.31 mya; this heterogeneity of estimates is due to the very small sample size used for this species). Previous temporal split estimates for *M. pandora* were carried out by Escobedo-Morales, et al. [46]. Using three concatenated mt genes, the split of *M. pandora* was around 2.66 mya (2.05–3.37 mya) (late Pliocene, beginning of Pleistocene), whereas, with only the mtCyt-*b* gene, this split was dated to have occurred around 1.87 mya (1.15–2.7 mya) (Pleistocene). Therefore, these estimates overlap with those obtained in this study. The same is true regarding the separation of the ancestors of the *M. temama* complex and the *M. americana* complex. Escobedo-Morales, et al. [46] had estimated this split to have occurred around 2.03 mya (1.6–3.35 mya) with mtCyt-*b*, and 1.86 mya (1.4–2.35 mya) with three mt genes. Subsequently, the same authors estimated the separation between the ancestors of these two complexes at 2.3 mya (1.9–2.7 mya). Our current estimates were 2.22 mya (BD1), 2.12 mya (BD2), and 2.46 mya (BD3), perfectly in line with the estimates obtained by Escobedo-Morales, et al. [46,63,64].

The beginning of the Pleistocene (approximately 2.6 mya) was marked by the intensification and definitive establishment of global glacial cycles, alternating severely cold and arid glacial periods with shorter, warmer interglacial periods. In South America, these drastic climatic changes altered atmospheric circulation, lowered sea levels, and caused a massive contraction of tropical rainforests, which were largely replaced by savannas,

grasslands, and cold steppes. This may have positively impacted large deer such as *Odocoileus*, *Hippocamelus*, *Blastoceros*, and *Ozotoceros*. In fact, the cold, dry climate of the early Pleistocene favored the emergence of enormous species, following Bergmann's rule, which predicts larger bodies to retain body heat in cold climates, with gigantic, branched antlers. Some of these genera were *Morenelaphus* and *Antifer*, which adapted in a mixed fashion to consume the abundant grasses and shrub vegetation of these new South American pampas. Likewise, the retreat of the forests created Pleistocene refugia [157–159] that allowed the brocket deer to survive in these different refuges and which, along with their chromosomal fragility, may have been an incredibly important factor in their strong speciation processes.

During the Middle Pleistocene, we found diversification within several brocket deer species. These include the beginning of diversification of the ancestor of *P. nemorivagus* (1.91–1.2 mya), the separation between the ancestor of *S. gouazoubira* and *S. chunyi* (1.57–1.24 mya), mitochondrial diversification within *S. gouazoubira* (1.55–0.81 mya), diversification within the most recent nucleus of *M. rufina* (1.11–0.86 mya), diversification in the *M. temama* complex (1.45–1.22 mya), the beginning of mitochondrial diversification for the *M. americana* complex (1.84–1.54 mya), and the separation between the Colombian and Mexican ancestors of *M. temama* (1.6–0.99 mya) [a value extremely similar to that obtained by Escobedo-Morales, et al. [64] at 1.4 mya (1.1–1.7 mya)]. The diversification of these mitochondrial lineages in Pleistocene refugia is highly conceivable since this period coincided with an extremely cold glacial period corresponding to the beginning of the Middle Pleistocene Transition (MPT), particularly the Marine Isotope Stage 38 (MIS 38) approximately 1.25 mya, and the classic Alpine glaciation known in Europe as the Günz Glaciation. This time interval also coincides with the end of the Bramertonian Interglacial (1.55–1.30 mya) and the beginning of the Pre-Pastonian glacial period (1.30–0.8 mya), the latter being the period with the most extensive glaciations in southern South America. Therefore, this event could have fragmented brocket deer populations in Pleistocene refugia, allowing for the accumulation of significant genetic differences (karyotypic and molecular) among them, which could have led to cryptic speciation processes.

Within the *M. americana* complex, the separation times of ancestors from different lineages or the diversification within each lineage were shorter. Highlights include estimates of the initial mitochondrial diversification of *M. americana* IV (0.69–0.49 mya), *M. americana* I (0.91–0.47 mya), *M. americana* VI (1.09–0.8 mya), *M. americana* V (0.82–0.27 mya), *M. americana* II (0.66–0.32 mya), *M.*

americana III (0.64-0.55 mya), the separation of the ancestors of *M. rufa* and *M. americana* “sensu stricto” within *M. americana* I (0.47-0.16 mya), and the separation of the mixed group of *M. americana* with the Peruvian Andean group of *O. virginianus* (0.74 ma). All these estimates fall within a period encompassing the Aftonian interglacial (0.62–0.45 mya), followed by the Mindel glaciation (Central Europe)/Elster glacial complex (Scandinavia)/Kansas glaciation (North America) (475,000–424,000 ya), which corresponds to Marine Isotope Stage 12 (MIS 12). This was one of the most intense glaciations of the Pleistocene. Following this intense glacial period, a major interglacial event (MIS 11) occurred from 424,000 to 374,000 ya, during which the planet experienced extreme heat for 50,000 years. This caused much of Greenland to melt and sea levels to rise between 6 and 11 meters above current levels. This could have led to a strong dispersal of different haplotype lineages in many groups of brocket deer that had fragmented in Pleistocene refugia in previous periods. Subsequently, the glaciation associated with MIS 10 (374,000-337,000 ya) occurred, with a drop in sea level of approximately 120 meters, followed by the onset of another major glaciation, the Riss (Central Europe)/Saale complex (in Scandinavia), which coincides with Marine Isotope Stage 8 (MIS 8) from 337,000 to about 240,000 ya [160], followed by an interglacial period known as Marine Oxygen Isotope Stage 7 (MIS-7; 240,000-190,000 ya). This succession of very cold periods interspersed with some very warm interglacials caused significant genetic diversity within the aforementioned haplotype lineages, as well as genetic heterogeneity among them. Two other divergence periods were more recent: the separation of the continental *M. americana* sensu stricto, referring to those found on the Caribbean Island of Trinidad (0.18–0.08 mya), and the separation of the ancestor of the Amazonian and trans-Andean forms (Cauca, Colombia) of *M. americana* IV (0.17–0.02 mya). Both events occurred during the fourth major Pleistocene glaciation. This last cold period began approximately 116,000 ya and lasted until about 10,000 ya. There was a very cold period from about 70,000 to 35,000 ya, when large glaciers (Nevados) existed in Colombia [161]. Van der Hammen [162] estimated that the temperature was 7°C lower than today in the Bogotá Savannah. All these climatological events shaped the emergence of many different forms of brocket deer in the Neotropics.

Demographic Changes in the Evolutionary History of the Brocket Deer

For many of the brocket deer taxa analyzed, there is no solid evidence of possible demographic changes throughout their respective evolutionary histories over the last 600,000 years. For example, for *B. citus*, *P. nemorivagus*, *M. temama*,

M. americana I, and *M. americana* VI, there is insufficient evidence to propose clear demographic changes. In some cases, the small sample size may have prevented the detection of such events. For *S. gouazoubira*, little evidence of demographic changes was also provided, but for BD1, a significant population expansion was detected around 643,000 ya, coinciding with the Aftonian interglacial (0.62–0.45 mya). The taxa that showed strong evidence of population expansion were *O. virginianus* (550,000–495,000 ya), also during the Aftonian interglacial period. *M. rufina* (the species that experienced the most evidence of population expansion) showed the beginning of a strong expansion 436,000 ya during the end of Kansas (North America) (475,000–424,000 ya), followed by the powerful interglacial MIS 11. This could have produced significant genetic differentiation in Andean Pleistocene refugia (initially in Ecuador) with strong population expansion during the MIS 11 interglacial. *M. americana* IV also showed significant evidence of population expansion, as did *M. americana* V, which showed the beginning of a strong population expansion 395,000 ya during the MIS 11 interglacial.

Most of the studied brocket deer and *O. virginianus* taxa appeared to have originated from population declines between the last 30,000–10,000 ya (observable in *B. citus*, *S. gouazoubira*, *M. rufina*, *M. temama*, *M. americana* I, *M. americana* IV, *M. americana* V, *M. americana* VI, and *O. virginianus*). The coldest part of the fourth major Pleistocene glaciation corresponded to the period from 18,000 to about 10,000 ya. The last phase of this period (named “El Abra” in the northern Andes, Younger Dryas, or Dryas III, in Scandinavia and northern Europe, 11,800–9,600 ya, [163]; or the central European Late Glacial, [164]) was crucial in the extinction of some large mammals, such as Gomphoteriidae (proboscideans), giant sloths, large notoungulates, and the famous *Smilodon*. Nevertheless, a more benign period preceded the arrival of the El Abra mountain range. From 14,000 to 11,000 ya, precipitation and temperature increased, and glacial retreat occurred. This allowed the formation of a lake (Tauca Lake; 43,000 km²) in the southern Bolivian Altiplano, which lasted until 11,000 ya [1999]. Van der Hammen [162] detected this climatic change in the Colombian Cundinamarca department (named the Guantia period), where there was a rise in lake levels in the Bogotá Savannah coupled with the expansion of oak forests. Some of the studied brocket deer species began to show a degree of population recovery around 12,000–15,000 ya, coinciding with this period. Nonetheless, most of these taxa experienced population expansions (some gradual, others more explosive) during the Holocene, over the last 7,500 years. The Holocene began 10,000 ya. During this period, temperatures increased to a maximum around 6,000 ya. Consequently, there was recent marine introgression depositing shells and marine sediments known as Queradinenses in continental areas

[153]. In the first part of the Holocene (9,500–6,000 ya), there was an increase in temperature and precipitation, with a climate optimum 7,000–3,000 ya. In the Bogotá Savannah, lake levels have increased in the last 9,500–7,000 years, and the Andean forests expanded. At ca. between 5,000 and 4,500 ya, the level of Lake Titicaca rose, and around the 10th century BC, the temperature increased [165]. When the climate became more hospitable and forests expanded, many Neotropical deer taxa also began a population expansion. Some of these taxa, specifically, appear to have declined in recent centuries (even in the last 2,000 years). This is the case for *M. rufina*, *M. americana* I, *M. americana* V, *M. americana* VI, and *O. virginianus*. The Little Ice Age (1400–1850 BC) is the most recent period of glacial advance. The ice sheets in Colombia descended on average to 4,300 meters above sea level (currently the ice front is approximately 5,000 meters above sea level). Coinciding with the cooling of the environment, European colonizers arrived in South America. With their arrival came the introduction of cattle (bovine, ovine, equine, and swine), which competed with Neotropical deer, as has the demographic pressure caused by hunting, which has reduced the populations of these beautiful creatures.

Surely, the systematics and taxonomy of Neotropical deer will still yield more surprises due to their intricate and complex evolution as specimens from more diverse geographical areas are sampled and as a greater number of molecular procedures are employed. For red brocket deer, we have already shown how the analysis of only 39 new specimens has revealed the existence of eight groups within the *M. americana* complex, six of them are new to science. Molecular and chromosomal analyses of red brocket deer in countries such as Colombia, Venezuela, Ecuador, Peru and Bolivia could reveal the existence of additional groups or species not yet detected. In the same manner, a sampling effort is necessary to detect possible taxa related to *S. gouazoubira* in the Andean mountains from Bolivia, Peru, Ecuador, and Colombia to determine exactly the geographic distribution of this taxon.

In order to first determine, and then assign a correct taxonomic nomenclature to, the various brocket deer groups analyzed, it is absolutely essential to be able to identify neotypes that can undergo complete chromosomal studies along with their respective molecular analyses (mitochondrial and nuclear) to identify them with the groups found (and surely many others yet to be discovered). Notwithstanding, this is a complex task because many of the brocket deer analyzed come from remote locations that we were able to sample opportunistically (for example, in the Bolivian and Peruvian Amazon), but we were unable to obtain material to carry out chromosomal studies. On the other hand, in the case of the red brocket deer of the *M.*

americana complex, the extreme phenotypic homogeneity greatly hinders this required task. For example, only in the Peruvian Amazon do we find representative specimens of *M. americana* I, IV, V, VI, and VII that likely lack any distinguishing morphological features. Therefore, being able to chromosomally and molecularly analyze any brocket deer specimen from some of the indicated locations will probably be a significant advance in the taxonomic, systematic, and, above all, evolutionary understanding of this very special case that constitutes the Neotropical brocket deer.

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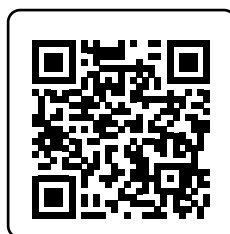
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