

Article

Description of *Bisbalus*, a New Genus for the Gray Brocket, *Mazama cita* Osgood, 1912 (Mammalia, Cervidae), as a Step to Solve the Neotropical Deer Puzzle [†]

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Abstract: The Neotropical deer genus *Mazama* is characterized by homoplastic morphological characters, a high karyotypic diversity, and a polyphyletic condition. The species of the genus have been recovered into two multigeneric lineages, the subtribes Odocoileina and Blastocerina, of the tribe Odocoileini (New World deer) in the family Cervidae. Within the Blastocerina, gray brockets include two non-sister species, *Subulo gouazoubira*, occurring south of the Amazon region, and *Pasralites nemorivagus*, occurring in the Guianas and in the Amazon region. We clarify the taxonomic status and phylogenetic position of *Mazama americana citus* Osgood, 1912 (referred to as either *S. gouazoubira* or *P. nemorivagus* by other authors). We collected a topotype of *M. a. citus* from the eastern shore of Lake Maracaibo, Venezuela, characterize it morphologically and cytogenetically (conventional banding and fluorescence in situ hybridization), and carry out a phylogenetic analysis of its whole mitogenome and *Cytb* alongside two additional specimens of *M. a. citus* from northwestern Venezuela. Our analyses reveal the topotype to be a large gray brocket with a cinnamon band above the eyes and $2n = 61$ and $FN = 70$ karyotype. Using cattle whole chromosome painting and bacterial artificial chromosome X probes, we determined its karyotype to differ in at least 10 rearrangements from that of *S. gouazoubira*. Bayesian inference recovers *M. a. citus* within the Blastocerina subtribe, separated phylogenetically from other gray brockets (100% branch value), revealing the Osgood's gray brocket to be a valid species that should be assigned to a new genus. We propose the generic name *Bisbalus*, with *Bisbalus citus* (Osgood, 1912) as the type species.

Keywords: animal cytogenetics; mitochondrial phylogeny; subtribe Blastocerina; taxonomy; Venezuela

1. Introduction

Neotropical brockets are small to medium-sized (13 to 40 kg) deer, with short and unbranched antlers in males, inhabiting forests from southeastern Mexico to northern Argentina [1,2]. Traditional taxonomic reviews of the Neotropical deer possessing this morphotype have allocated them to the genus *Mazama* Rafinesque, 1817 [3], thought to

include from 4 to 17 species and subspecies [4–6]. With the advance of genetic techniques, several authors have shown the genus *Mazama* to be polyphyletic and its species to be karyotypically diverse. These findings indicate that, owing to high levels of homoplasy, external and cranial morphologies alone are not sufficient to distinguish species and to establish phylogenetic relationships in these deer [7–9]. Phylogenies based on mitochondrial genes have shown species traditionally allocated to *Mazama* to represent at least three genera distributed in the two subtribes, *Odocoileina* and *Blastocerina*, of the tribe *Odocoileini* (New World deer) in the family *Cervidae* [7–10].

The taxonomy of deer traditionally assigned to the genus *Mazama* is in a state of rapid flux. Within the subtribe *Odocoileina*, red brockets have been deemed to represent the true *Mazama*, with *Mazama americana* (Erxleben, 1777) [11] as the type species [7]. Also, within this subtribe, *Mazama bricenii* Thomas, 1908 [12] was synonymized with *Mazama rufina* Pucheran, 1851 [13,14]; the Yucatan brown brocket deer, formerly *Mazama pandora* Merriam, 1901 [15], was transferred to the genus *Odocoileus* Rafinesque, 1832 [7,16]; *Mazama rufa* (Illiger, 1815) [17] was validated [18]; and *Mazama bororo* Duarte, 1996 [19] was synonymized with *Mazama jucunda* Thomas, 1913 [20,21]. Within the subtribe *Blastocerina*, the monophyletic Azara's gray brocket, *Mazama gouazoubira* (Fischer, 1814) [22], has been proposed to belong to the resurrected genus *Subulo* Smith, 1827 [23], thus its updated name would be *Subulo gouazoubira* [24]; and the Amazonian gray brocket, formerly *Mazama nemorivaga* (Cuvier, 1817) [25] and recently updated to *Passalites nemorivagus* (Cuvier, 1817) [25], has been proposed to represent another genus within the *Blastocerina* given that it does not share a common ancestor with *S. gouazoubira* [7,8,26]). Much is yet to be learned regarding the alpha and beta taxonomy of the genus *Mazama*, thus the analysis of taxa described from the type localities across the Neotropics needs to be continued.

One species needing a clarification of its phylogenetic relationships is *Mazama americana citus* Osgood, 1912 [27], whose type locality is “El Panorama, Rio Aurare, eastern shore of Lake Maracaibo, Venezuela”. The specific allocation of this putative subspecies is based on the assumption that *Cervus nemorivagus* Cuvier, 1817 [25] is a synonym of *Cervus americanus* Erxleben, 1777 [5,20–27]. The description of specimens characterizes them as paler and more grayish than those of typical “*M. americana*”, with upperparts grizzled cinnamon that gradually become paler on the sides and underparts, white spots on the tarsal glands and on each side of the rhinarium and above each eye, a slightly larger body, notably larger cheek teeth, and differences in other minor cranial features [27].

Contrary to Osgood [27], Allen [4] deemed *M. nemorivaga* (Cuvier, 1817) [25] a valid species distinct from *M. americana*. Moreover, he deemed *M. americana citus* a valid species on its own, for which he used the name *Mazama cita*. He divided this species into two subspecies, *M. cita cita*, the form described by Osgood [27], and *M. cita sanctaemartae*, which he described. Allen [4] referred to *M. nemorivaga* and *M. cita* as 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 Allen [4], compared with *M. nemorivaga* from the Guianas, *M. cita* differs in being slightly larger, in possessing a heavier dentition, and in showing a “very considerably” distinct coloration. Osgood [27] did not provide etymologies. However, in the same paper he described nine additional species and subspecies of mammals, for which he proposed epithets clearly based on Latin. If *citus* is also based on Latin, it represents the perfect passive participle (verbal adjective) of the verb *cieō*, and means “put in motion”, or “swift”. In Latin, this tense must be masculine, feminine, or neuter. This explains why most subsequent authors have used *cita* instead of *citus* to match gender with the feminine name *Mazama*.

Tate [28] grouped *Mazama* into two “Divisions”, the first of which (“A”) he distinguished from the second (“B”) based on the possession of elongated and narrow (as opposed to short and thick) antlers and large (as opposed to small) teeth and on geographic distribution, with members of Division “A” (*M. americana* group, large brockets) occurring in South America and eastern Panama, and members of Division “B” (*M. simplicicornis* (Illiger, 1815) group, small brockets) occurring in South and Central America and Mexico.

Tate [28] included *M. cita* in his Division “A”. Previously, Lydekker [29] deemed *M. cita* a subspecies of Azara’s gray brocket, namely *M. simplicicornis citus*; whereas, subsequently, others [5,30,31] deemed it a subspecies of the southern gray brocket, namely *M. gouazoubira cita*. In his review of the genus *Mazama* in Venezuela, Bisbal [32,33] applied the name *Mazama gouazoubira* (Fischer, 1814) to all the gray or brown brockets from Venezuela, and recognized two subspecies: *M. g. nemorivaga*, occurring south of the Orinoco (Amazonas and Bolívar states), and *M. g. cita*, occurring in northern Venezuela (Zulia and Falcón states, central coastal region of the country).

Based on novel morphological, classical cytogenetic, molecular cytogenetic, and mitogenomic data, the present study aims to resolve the species level and generic status within the subtribe Blastocerina of Osgood’s gray brocket *Mazama cita*.

2. Materials and Methods

2.1. Specimens and Samples

To search in the field for deer specimens in northwestern Venezuela, where the type locality of *Mazama cita* is located, we used a collecting permit (SISTRA No. 20356) issued by the Ministerio del Poder Popular para el Ecosocialismo. We secured an adult male topotype that had been killed by a local hunter near El Consejo de Ciruma, Zulia state, ca. 35 km ESE from the type locality. The topotype was assigned the identification number T410 of the NUPECCE (Museu do Núcleo de Pesquisa e Conservação de Cervídeos, São Paulo, Brazil) and the catalog number CVULA 9109 (Colección de Vertebrados de la Universidad de Los Andes, Mérida, Venezuela). In addition, muscle tissue samples (VEN04 and VEN05) of two gray brockets were obtained for molecular analysis in nearby Falcon state. We provisionally adopted the binomial “*Mazama cita*” to refer to Osgood’s gray brocket.

2.2. Morphological Description

The freshly collected topotype was photographed, and its external body measurements (cm) and body mass (kg) were determined using measuring tape, a digital caliper, and a pendulum scale. The following measurements were taken: body length, head length and width, ear length, between-the-eyes width, mandible length, neck circumference, thorax circumference, shoulder height, metacarpus length, tail length, metatarsus length, and body mass (Table S1). The skin was removed and treated with a tanning solution. Aspects of the general coat color, chromogenetic fields of the body (neck, dorsal line of the body, ventral region of the body, tail, and front and hind feet), pigmentation patterns, and length of the hair in different regions of the body were assessed. The tarsal regions were examined for the presence of strips of anteverted hair and rounded hair tufts. Additionally, the chromogenetic fields of the head [34] were analyzed. A total of 38 standard cranial measurements for cervids [35] (see Table S2) were taken. The available skull was photographed at different angles to complement the documentation and description of the specimens. The body and cranial measurements of the collected topotype were compared to those of adult specimens of the gray brockets *P. nemorivagaus* and *S. gouazoubira* in the NUPECCE database.

2.3. Cytogenetic Analysis

Skin fragments (5 × 2 cm) from the inguinal region of the topotype were collected and preserved in liquid nitrogen, as described by Duarte et al. [36]. Then, the chromosomes were obtained by fibroblast in vitro culture following the procedure of Verma and Babu [37].

The chromosomal preparations were subjected to conventional Giemsa staining, G-banding [38] (modified), C-banding [39], and Ag-NOR [40]. The chromosomes were classified as acrocentric, submetacentric, or metacentric according to their arm ratio [41], separated into groups based on their relative lengths [9], and numbered as in the case of the *S. gouazoubira* (SGO) karyotype [24].

2.4. Fluorescence In Situ Hybridization

Fluorescence in situ hybridization was performed by applying cattle whole chromosome painting (WCP) probes to the karyotype of the *Mazama cita* topotype. We determined the homologies between the karyotype of *M. cita* topotype and that of *S. gouazoubira*, which retains the ancestral karyotype of the Cervidae [24]. For the preparation of probes, whole cattle chromosomes were isolated either by flow cytometry, using the MoFlo XDP Cell Sorter (Beckman Coulter, Brea, CA, USA), or by microdissection, using the PALM Micro-laser system (Carl Zeiss MicroImaging GmbH, Munich, Germany), as described in Frohlich et al. [42]. Both techniques were employed to overcome the challenges associated with flow cytometry, particularly in distinguishing certain acrocentric autosomes during FISH probe preparation, as they tend to form groups in the flow karyogram. To address this issue, laser microdissection, an alternative and reliable method, was used to prepare probes specific for these chromosomes. The amplification was performed using the degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) [43] and probe labeling during the second PCR with Green-dUTP or Orange-dUTP (Abbott Park, IL, USA) [44]. Five BAC clones were selected from the CHORI-240 library based on the NCBI ARS-UCD1.2 assembly data obtained from BACPAC Genomics (Emeryville, CA, USA) (Table S3). For DNA extraction, we used a protocol adapted from the method outlined for the Wizard[®] Plus SV Minipreps DNA Purification Systems. BAC DNA was labeled with Green-DdUTP (Abbott, IL, USA), biotin 16-dUTP, or digoxigenin-11-dUTP (Roche, Mannheim, Germany) using BioPrime[®] Array CGH Genomic Labeling (Invitrogen, Carlsbad, CA, USA). The selected BAC clones were selected to be distributed along p- and q-arms of the submetacentric X chromosome of cattle. For the pseudo-autosomal region (PAR) probe, two BAC clones were selected and used together as a single probe. FISH was performed as described in Vozdova et al. [45]. A Zeiss Axio Image Z2 (Carl Zeiss Microimaging GmbH, Jena, Germany) fluorescence microscope equipped with appropriate fluorescence filters for the visualization of FISH results was used.

2.5. DNA Extraction and Sequencing

Genomic DNA extraction from tissue samples of *Mazama cita* topotype was performed using the Qiagen DNeasy[®] Blood Qiamp Tissue kit following the manufacturer's instructions. Approximately 1 µg of DNA from each specimen was sent to NGS Soluções Genômicas (Piracicaba, São Paulo, Brazil for massive parallel DNA sequencing. First, the DNA samples were fragmented by Covaris sonication to an average size of 300 base pairs. Then, these fragmented DNA samples were used as input for genomic library preparation using the TruSeq Nano DNA sample preparation kit (Illumina, USA), following the manufacturer's instructions. All genomic libraries were pooled and sequenced on the Illumina HiSeq 2500 Platform (Illumina, San Diego, CA, USA) using 100 bp paired-end sequencing. Unmerged reads were aligned to the *S. gouazoubira* mitogenome (GenBank accession number KJ772514) as a reference by using Bowtie2 v2.4.1 [46] on the Galaxy online server [47]. Annotation was performed on the MITOS [48] web-based platform (<http://mitos.bioinf.uni-leipzig.de/index.py>, accessed on 1 July 2022) and manually adjusted based on comparisons to the reference mitogenome using GENEIOUS (Biomatters; <http://www.geneious.com/>, accessed on 5 July 2022). To carry out phylogenetic analyses, we downloaded from the GenBank mitogenome sequences of *S. gouazoubira*, *P. nemorivagus*, *Mazama americana*, *M. jucunda*, *M. nana*, *M. rufa*, *M. temama*, *Blastocercus dichotomus*, *Hippocamelus antisensis*, *Odocoileus virginianus*, *Ozotoceros bezoarticus*, and *Pudu puda*, and used the sequence of *Alces alces* as the outgroup (see Table 1 for details).

Table 1. Mitochondrial gene sequences used for phylogenetic analysis.

Species	Specimen Voucher	Mitogenome	Cytb	Origin	Description
<i>Mazama cita</i>	T410 topotype	OQ198443	OQ198443	El Consejo de Ciruma, Zulia, Venezuela	This study
<i>Mazama cita</i>	VEN04 (JMA 4031)		OQ658775	Paraguana, Falcon, Venezuela	This study
<i>Mazama cita</i>	VEN05 (JMA 4035)		OQ658776	Paraguana, Falcon, Venezuela	This study
<i>Subulo gouazoubira</i>	T377	MZ350858	MZ350858	Puerto Galileo, Paraguay	Bernegossi et al. [24]
<i>Subulo gouazoubira</i>	T082	MZ350862	MZ350862	Camobi, Rio Grande do Sul, Brazil	Bernegossi et al. [24]
<i>Subulo gouazoubira</i>	T314	KJ772514	KJ772514	Pantanal, Brazil	Caparroz et al. [49]
<i>Subulo gouazoubira</i>	MRGsp2	JN632658	JN632658	Colombia	Hassanin et al. [50]
<i>Passalites nemorivagus</i>	T24	MZ350861	MZ350861	Porto Velho, Rondônia, Brazil	Hassanin et al. [50]
<i>Passalites nemorivagus</i>		JN632659	JN632659	Peru	Hassanin et al. [50]
<i>Passalites nemorivagus</i>		JN632660	JN632660	French Guiana	Hassanin et al. [50]
<i>Passalites nemorivagus</i>			KY928656	Guyana	Gutiérrez et al. [14]
<i>Passalites nemorivagus</i>			KY928858	Bolivar, Venezuela	Gutiérrez et al. [14]
<i>Mazama americana</i>	MAZ9472	JN632656	JN632656	French Guiana	Hassanin et al., [50]
<i>Mazama americana</i>	T358	MZ350857	MZ350857	Cayenne, French Guiana	Bernegossi et al. [24]
<i>Mazama americana</i>	T253	MZ350856	MZ350856	Juina, Mato Grosso, Brazil	Bernegossi et al. [24]
<i>Mazama americana</i>			KY928654	Yaraguay, Venezuela	Gutiérrez et al. [7]
<i>Mazama rufa</i>	T385	OQ198444		Foz de Iguacú, Paraná, Brazil	This study
<i>Mazama nana</i>	T107	MZ350863	MZ350863	Paraguay	Bernegossi et al. [24]
<i>Mazama jucunda</i>	T071		DQ789231	Paraná, Brazil	Cifuentes-Rincón et al. [9]
<i>Mazama jucunda</i>	T215	MZ350859	MZ350859	Parque Estadual Intervalles, São Paulo, Brazil	Bernegossi et al. [24]
<i>Mazama temama</i>	T366	MZ350864	MZ350864	Veracruz, Mexico	Bernegossi et al. [24]
<i>Mazama temama</i>	T362	MZ362858	MZ362858	Campeche, Mexico	Bernegossi et al. [24]
<i>Odocoileus virginianus</i>		KM612278		USA	Ambriz et al. [51]
<i>Odocoileus virginianus</i>			HQ332445	USA	Seabury et al. [52]
<i>Ozotoceros bezoarticus</i>		JN632681		Brazil	Hassanin et al. [50]

We also extracted genomic DNA from hair and muscle samples of two gray brocket deer from northwestern Venezuela identified as *M. cita* using a modified protocol based on the methodology described by Sambrook et al. [53]. The partial cytochrome *b* (Cytb 1040 bp) fragment was amplified, and the PCR products were purified with the Wizard SV gel and PCR Clean-Up System kit (Promega TM, Madison, WI, USA). This was followed by sequencing using the ABI BigDye Terminator kit (Applied Biosystems, San Francisco, CA, USA) in an ABI 3130xl automatic sequencer (Applied Biosystems). Thereafter, we aligned with other sequences for Neotropical deer available in the GenBank (Table 1).

2.6. Alignment and Bayesian Phylogenetic Inference

Both datasets, complete mitogenome and Cytb gene, were aligned separately using the Clustal X program [54]. For the mitochondrion genome, we excluded the control

region from the alignment because of the tandem repeats located inside them and the high incidence of potential homoplasy. The optimal partitioning scheme was selected by means of the Partitionfinder 2.0 tool [55] on the CIPRES Science Gateway [56] based on the Bayesian information criterion. For the *Cytb* data set, we selected the best molecular evolution model using the jModelTest v. 0.1.1 [57], following the corrected Akaike information criterion, AICc [58].

Bayesian inference (BI) was performed using the multiple four chain Metropolis-coupled analysis (with default heating) available in MRBAYES 3 [59], with 10,000,000 generations and sampling every 1000 generations, until a variance of <0.01 was obtained. The first 25% of the sampled trees was discarded as burn-in, and the remaining trees were used to construct a 50% majority-rule consensus tree. The consensus tree was edited using FigTree v.1.4.0 [60].

3. Results

3.1. Morphological Description

The topotype of *Mazama cita* Osgood, 1912 has a general grayish-brown coloration (light brown laterally and whitish ventrally). The dorsal region of the body, head, and neck are dark brown, gradually paler, and with yellowish hairs in the submandibular region. There is a white-yellowish nasal patch, a dark lateral rostral band, an inferior orbital band with yellowish hairs, and a superior orbital band with a pale yellowish coloration that extends in the direction of the rostral band. The base of the ears and the anterior auricular region are whitish. The posterior auricular border is dark brown. Long white hairs are present on the inner auricular surface. The outer auricular surface is cinnamon brown. There is a pale brown mental patch, a grayish mandibular band, and a yellowish buccal region and gular patch. A tuft with dark brown and yellowish hairs is observed in the frontal region of the head. The opening of the preorbital pouch is pale brown. The front legs are grayish-brown in the proximal lateral region, pale brown in the distal lateral region, and whitish in the ventral region. Proximally, the hind legs are grayish-brown in the lateral region and whitish in the ventral region. Distally, the hind legs are dark brown both in the lateral and ventral region. A white spot on the tarsal gland is absent. The tail is white below and pale cinnamon above. The inguinal region is whitish. Short fine hairs cover the whole body. The auditory bulla is small and flattened. Two separate lacrimal holes are present in the orbit. The supraorbital foramen is short and deep. The lacrimal fossa is deep. The preorbital fossa has a triangular and elongated shape (Figures 1 and 2).

Locality: El Consejo de Ciruma, eastern shore of Lake Maracaibo, Zulia state, Venezuela.

Coordinates: 10°29'33.3" N, 71°08'46.6" W.

Specimen housed in: Colección de Vertebrados de la Universidad de Los Andes, Mérida, Venezuela (CVULA).

Catalog number: CVULA 9109 (full skeleton and skull, taxidermized skin).

Tissue sample deposited in: NUPECCE Tissue and Cell banking. TESE 410.

DNA sequence Deposit Number: OQ198443 (complete mitochondrion).

Karyotype: 2n = 61/FN = 70.

The topotype of *M. cita* had a body length of 850 mm, a skull length of 189.39 mm, a condylobasal length of 185.60 mm, a basal length of 166.29 mm, a zygomatic breadth of 81.27 mm, and a cheek tooth row length of 58.16 mm.

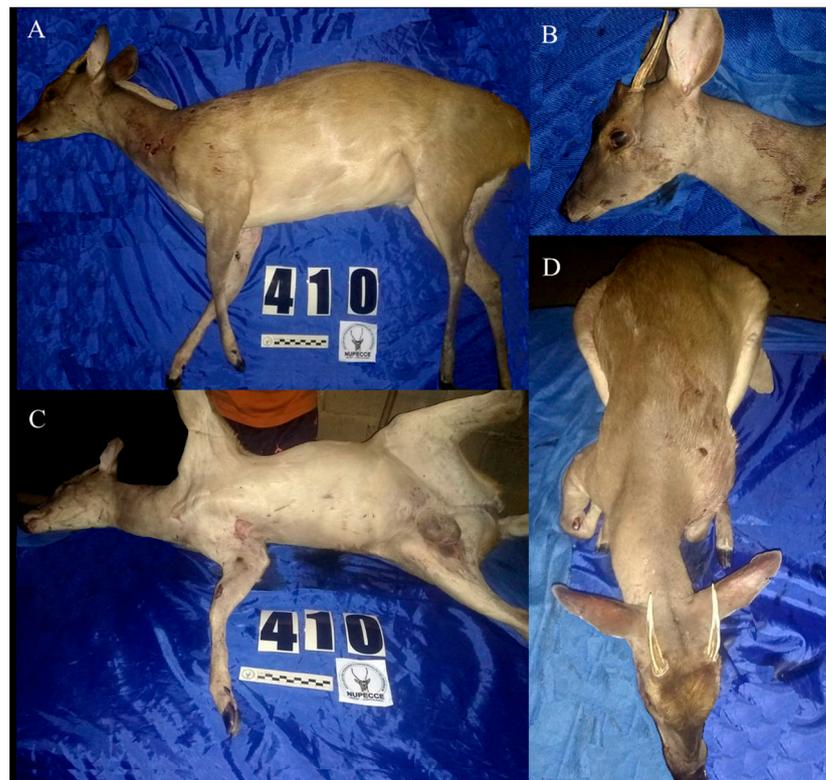


Figure 1. Male topotype of *Mazama cita* (Osgood, 1912) collected in El Consejo de Ciruma, Zulia, Venezuela. (A) Lateral view of the body. (B) Close up of the head. (C) Ventral view of the body. (D) Dorsal view of the body.



Figure 2. Skull of the male topotype of *Mazama cita* (Osgood, 1912) collected in El Consejo de Ciruma, Zulia, Venezuela (A) Dorsal view. (B) Ventral view. (C) Right lateral view. (D) Left lateral view.

3.2. Cytogenetic Description

The Giemsa-stained karyotype of the *M. cita* (MCI) topotype showed a diploid number ($2n$) of 61, a fundamental number (FN) of 70, 30 pairs of autosomal chromosomes, and a simple XY sexual system (Figure 3). The X chromosome was submetacentric, and the Y chromosome was a small metacentric. The chromosomal pair MCI1 was included in group A (large biarmed autosome), the chromosomal pairs MCI2 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 in different metaphases.

The Ag-NOR stained karyotype shows nucleolus organizing regions in the telomeric area of the longer arms (q-arm) of the two chromosomes of pair 1 and in the telomeric region of the q-arm of the two chromosomes of pair 7 (Figure 4). C-banding revealed constitutive heterochromatin blocks in the pericentromeric region of all autosomes and in the X chromosome (Figure 4).

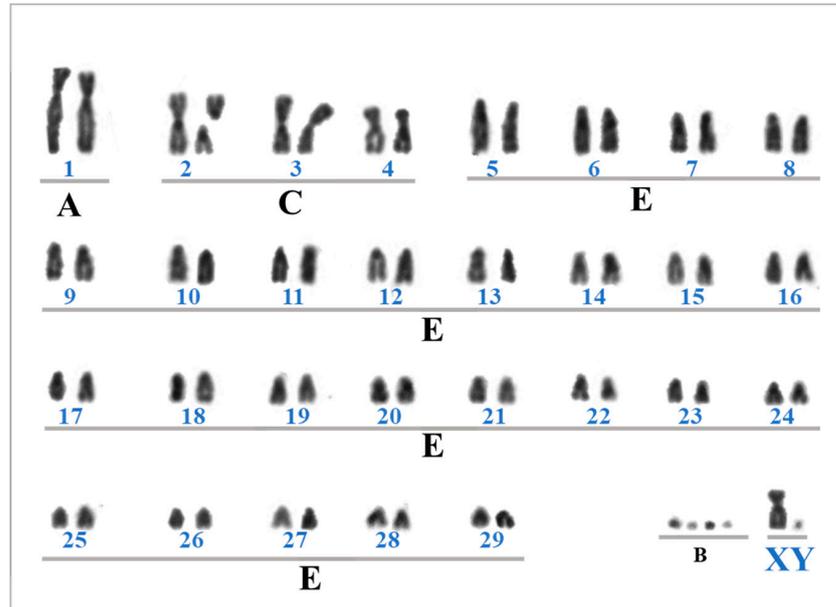


Figure 3. Conventional Giemsa-stained karyotype of the male topotype of *Mazama cita* (Osgood, 1912). Groups of chromosomal relative lengths: A—large biarmed autosome; C—small biarmed autosomes; E—small acrocentric autosome; B—Supernumerary chromosomes; XY—Simple sexual system. Number of each chromosomal pair from 1 to 29.

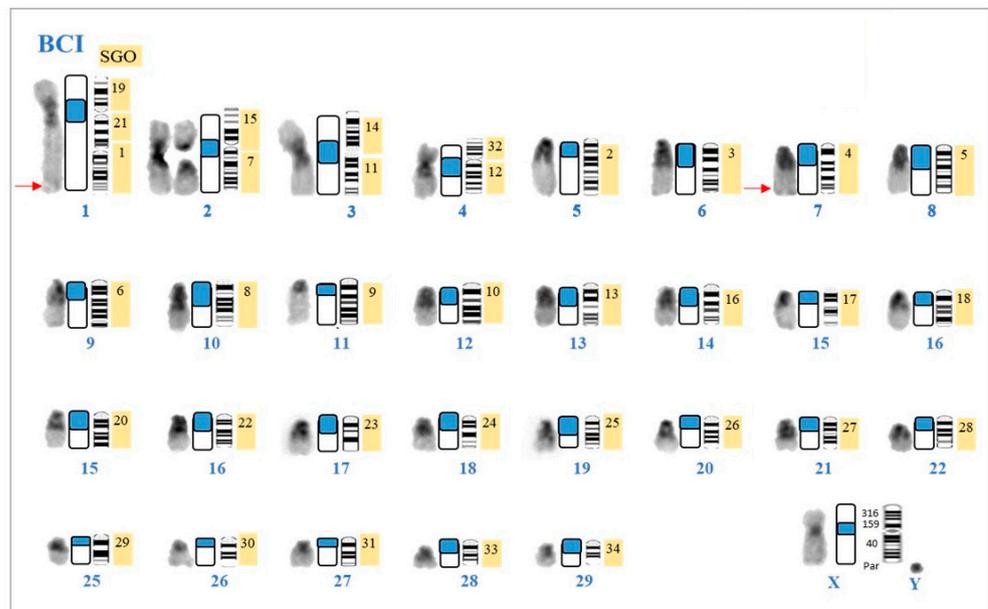


Figure 4. Karyotype of a male topotype of *Mazama cita* (Osgood, 1912) (MCI) and homologies with the karyotype of *Subulo gouazoubira* (SGO). Left to right: C-band, schematic representation of C-band and G-band of MCI, and homology with *Subulo gouazoubira* SGO [24], adapted. Localization of Ag-NOR staining is indicated by arrows. Positions of bovine BAC probes on the MCI X chromosome are shown. PAR corresponds to pseudo-autosomal region probe, comprising clones BAC 453C5 and 326B13. Number of each chromosomal pair from 1 to 29.

The bovine WCP chromosomal painting demonstrated that *M. cita* (MCI) ($2n = 61$ and $FN = 70$) underwent chromosomal rearrangements with respect to the hypothetical ancestral deer karyotype retained by *Subulo gouazoubira* ($2n = 70$ and $FN = 70$) (Figure S1). The q-arm of the large submetacentric MCI1 was the result of tandem fusion of the chromosomes corresponding to SGO21 and SGO1 (BTA5 proximal region and BTA3). The shorter arm (p-arm) of the MCI1 corresponding to SGO19 (BTA17) was fused by centric fusion with the q-arm. The MCI2 was heterozygotic for a centric fusion involving SGO15 (BTA19) and SGO7 (BTA7) (Figure 4). We also observed a homozygotic centric fusion in MCI3 corresponding to SGO 14/11 (BTA21/16) and a homozygotic centric fusion in MCI4 corresponding to SGO 32/12 (BTA25/13), which formed the largest chromosomes in group C (Figure 4). The X chromosome of the *M. cita* topotype showed a submetacentric morphology with a centromeric shift when compared to the acrocentric X chromosome of *S. gouazoubira*, in which the proximal region of SGO X was inverted in the p-arm of the MCI X, as shown by the hybridization pattern of BAC clones. The q-arm preserved the same hybridization pattern as those shown for the distal region of the X chromosome in SGO (Figure 4).

3.3. Phylogenetic Analysis

The BI tree of the mitogenomes (Figure 5A) shows *M. americana*, *M. rufa*, *M. nana*, *M. jucunda*, *M. temama*, and *O. virginianus* as members of the large clade known as the Odocoileina subtribe, with a 100% posterior probability. The topotype of *M. cita* was recovered within the Blastocerina subtribe (100% pp) and was the first lineage that diverged in the tree, followed by *P. puda*. Also, in the Blastocerina clade, *P. nemorivagus* was recovered as a monophyletic species having *B. dichotomus* as its sister taxon. The monophyletic *O. bezoarticus* formed a separate clade together with *H. antisensis* and *S. gouazoubira* as sister taxa. A similar result (two main clades) was obtained with the partial *Cytb* BI tree (Figure 5B). The first corresponded to the Odocoileina subtribe (100% pp) and included *M. americana*, *M. jucunda*, *M. nana*, *O. virginianus*, *M. rufa*, and *M. temama*. The other corresponded to the Blastocerina subtribe, including *M. cita*, *P. nemorivagus*, *S. gouazoubira*, *B. dichotomus*, and *O. bezoarticus*. This clade was divided into three subclades, the *M. cita* subclade (100% pp), the monophyletic *P. nemorivagus* subclade (100% pp), and the monophyletic *S. gouazoubira* subclade (97% pp), which also included *B. dichotomus* and *O. bezoarticus* as successive sister taxa. The topotype of *M. cita* and the two specimens from the northwestern Venezuelan state of Falcon were the first branch that diverged within the Blastocerina subtribe, forming a separate monophyletic clade (100% pp). Within the *M. cita* clade, the individuals from Paraguana Pensinsula (Falcon state) were more closely related to each other than to the topotype from Zulia state. The *P. nemorivagus* clade was split into two clades with strong support, one of them formed by gray brockets from Bolivar state in Venezuela south of Orinoco, Guyana, and French Guiana (98% pp), and separated from the other clade, including brockets from Peru and Brazil (100% pp).

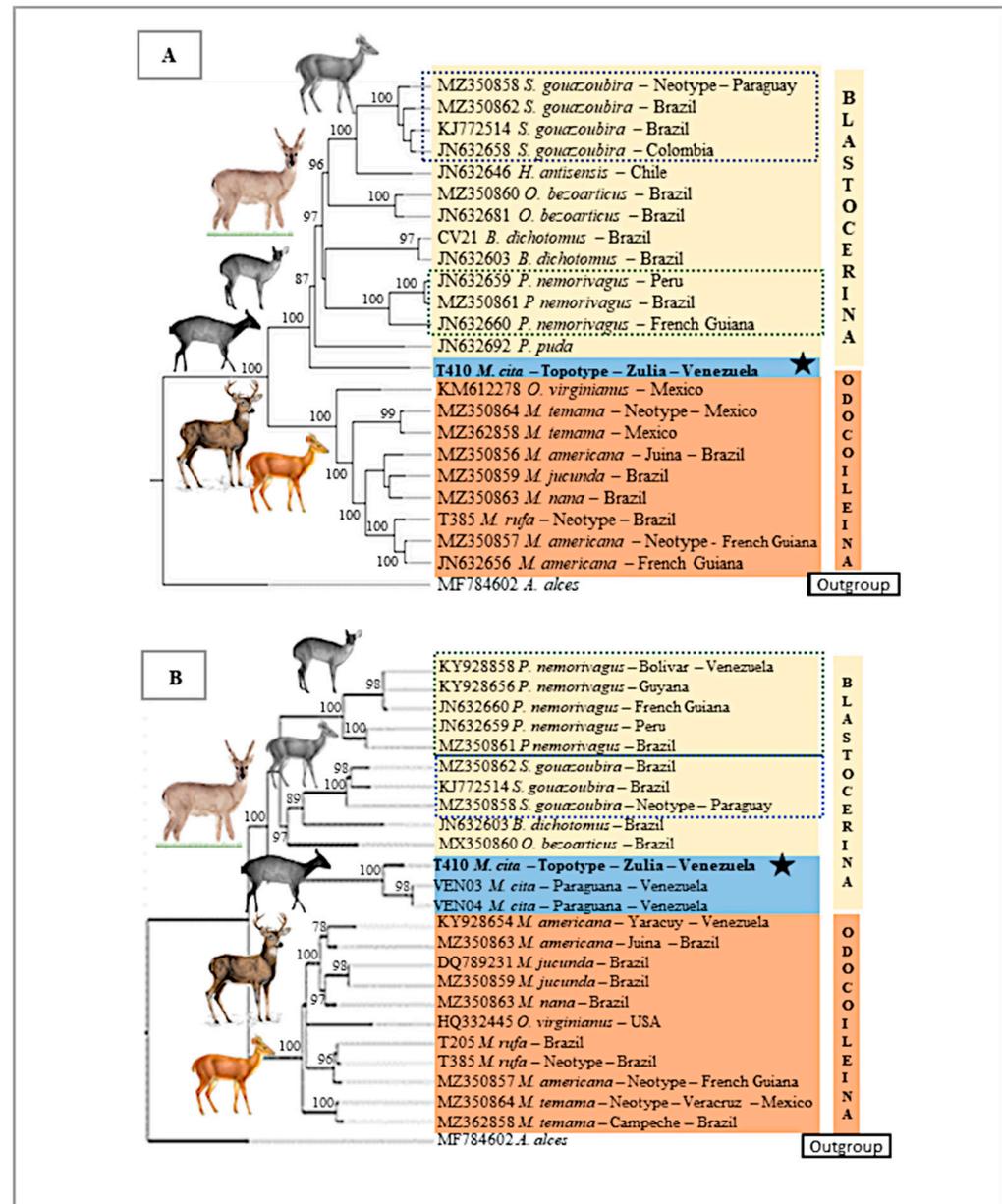


Figure 5. Bayesian Inference (BI) of the mtDNA of several species of Neotropical deer. (A) Complete mitochondrion. (B) Partial *Cytb* 1040 bp. The values above clade nodes represent posterior probabilities. Blastocerina subtribe (light yellow square). *Mazama cita* (Osgood, 1912) clade (blue), topotype (black star). Odocoileina subtribe (melon red square). *S. gouazoubira* (blue dashed lines). *P. nemorivagus* (green dashed line). Outgroup, *A. alces*.

4. Discussion

4.1. Morphological Characterization

The description of the gray brocket deer from northwestern Venezuela, *Mazama cita*, is based on morphological features deemed unique of the type series [27] in comparison with other gray brockets. The pale brownish and yellowish-gray coloration of the topotype matches the previous description of *M. cita*, with the only exception of the absence of a white spot on the tarsal gland, which is present in the holotype. However, this character has been reported to be variable in other brocket species, such as *S. gouazoubira* [24,61]. The general coloration of *M. cita*, together with the yellowish spot over its eyes and the white color around the muzzle, have been proposed to be useful characters to distinguish the species from other gray brockets [4]. However, other authors have considered these characters

to be variable, arguing that body and skull size are more important than coloration to differentiate the species [32,62]. The body and skull measurements of the topotype are close to those published for the gray brockets from northern Venezuela [4,27,32]. This is especially true of the greatest length of the skull, greatest nasal length, maxillary toothrow, zygomatic breadth, width of head, height at shoulder, and chest circumference.

Most measurements are greater in the *M. cita* topotype than reported [24,26] for other gray brockets. Owing to the availability for morphometric analyses of a single specimen of *M. cita*, we could not statistically test the difference. However, supporting this result, Bisbal [32], despite deeming both taxa morphometrically indistinguishable, provided measurements showing that, cranially, "*M. gouazoubira cita*" is larger than "*M. gouazoubira nemorivaga*". Our measurements, considered together with those of Osgood [27] and Bisbal [32], suggest that *M. cita* is indeed larger than *P. nemorivagus* and *S. gouazoubira*, both in body and skull size. Tate [28] already assumed that this is the case by assigning *M. cita* to his large-brocket deer group. A descriptive study of specimens from localities across northern Venezuela is needed for a better morphometric diagnosis of *M. cita*.

4.2. Cytogenetic Description

The cytogenetic characteristics of the gray brockets *S. gouazoubira* and *P. nemorivagus* have been reported by several authors [24–26]. Neitzel [63] was the first to describe the karyotype of *S. gouazoubira* as possessing a $2n$ and an FN both equal to 70, which was recently corroborated based on topotypes found to possess a $2n = 69$ and an FN = 70 owing to the presence of a centric fusion in a heterozygous state [24]. Karyotypical studies of *P. nemorivagus* from Brazil showed a $2n$ range from 67 to 69 and an FN = 70, with a submetacentric X chromosome unlike the acrocentric X chromosome of *S. gouazoubira* [26]. A *P. nemorivagus* collected in the type locality in French Guiana had a $2n = 69$ and an FN = 72 karyotype, with a submetacentric X chromosome [26]. In contrast, our cytogenetic results for the gray brocket topotype collected in northwestern Venezuela show a $2n = 61$ and an FN = 70 karyotype, with 2 submetacentric pairs, 1 metacentric pair, 1 heterozygotic fusion, and 25 acrocentric pairs. We uncover a simple sexual system, with submetacentric X and metacentric Y chromosomes.

Subulo gouazoubira retains the hypothetical ancestral karyotype of the Cervidae [64]. Fluorescence in situ hybridization (FISH) shows the karyotype of the *M. cita* topotype to share the fusion in heterozygosis between autosomes 7 and 15 reported for *S. gouazoubira* [24]. However, such a karyotype differs from that of the latter species by showing tree-centric fusions, one tandem fusion in the autosomal chromosomes, and an X chromosome with a submetacentric morphology as opposed to an acrocentric morphology. Interestingly, the X chromosome of the *M. cita* topotype is similar to the X chromosome of *Capreolus capreolus* and *P. nemorivagus*, with the same order and position of each X probe used in the proximal region and in the qproximal and qdistal regions [26,42]. The karyotypes of the topotype of *M. cita* and the individual of *P. nemorivagus* from French Guiana [26] show the same difference in fusions with respect to the karyotype of *S. gouazoubira*, except for the absence of the fusion in heterozygosis corresponding to BTA7 and BTA19 absent in *P. nemorivagus*. Conversely, the individual from French Guiana has a heterozygous fusion corresponding to BTA7 and BTA23 not observed in *M. cita* and *S. gouazoubira*.

The substantial karyotypic differences between *M. cita* and the other gray brockets *S. gouazoubira* and *P. nemorivagus* could be deemed efficient reproductive barriers should their populations be sympatric [65–67], which they are not. Therefore, based on the biological species concept, we confirm that *M. cita* is a distinct species from *S. gouazoubira* [24] and *P. nemorivagus* [26]. Moreover, we note that the differentiated diploid number of *M. cita* is highly unique for the taxon in the light of the findings of chromosomal studies involving other brockets, such as *M. temama*, *M. rufa*, and *M. americana* [9,18,68].

4.3. Phylogenetic Analysis

Mitochondrial DNA has been widely used to reconstruct the phylogenetic relationships of Neotropical deer [49,50,69,70]. Studies based on cytochrome *b* and the complete mitogenome have proven to be efficient in recovering relationships and enhancing the understanding of the evolutionary history of this complex group [7,8,10]. Our BI analyses of mtDNA confirm the findings of previous studies with respect to the polyphyly of *Mazama* and support the conclusion that different or new generic names are needed for some members of the Blastocerina subtribe [7,24].

Notwithstanding the limited number of mitogenome sequences available for Neotropical deer, based both on the *Cytb* and complete mitogenome trees, the Osgood's gray brocket topotype is recovered as a separate monophyletic lineage with respect to the rest of the Blastocerina, thus *M. cita* can be inferred to have diverged early in the course of the evolutionary radiation of the subtribe. The *Cytb* tree, including sequences of additional *M. cita* individuals, confirms this relationship by showing the gray brocket specimens from northwestern Venezuela forming a monophyletic clade apart from all other Blastocerina. Clearly, *M. cita* does not share exclusive common ancestors with the other gray brockets (*P. nemorivagus* (type locality Cayenne, French Guiana) and *Subulo gouazoubira* (type locality Asunción, Paraguay)) [7,24]. Moreover, our results not only indicate that *M. cita* is a distinct species but also that it belongs to a separate undescribed genus.

In the *Cytb* analysis, the two *M. cita* from Paraguana Peninsula are closely related to the topotype from Zulia state in Venezuela. Based on the morphological peculiarities of one specimen of *M. cita* from Paraguana Peninsula when compared with samples of *M. cita* and *P. nemorivagus* from the Venezuelan mainland, Bisbal [32] raises the possibility of speciation in this population. However, he also recognizes the insufficiency of the sample size to discuss this hypothesis. As noted in other Neotropical deer studies [18,24], only an integrative review (ideally including multi-loci analyses with more samples from Paraguana and other areas of occurrence in Venezuela) may confirm this hypothesis.

4.4. Species-Level Validation of Osgood's Gray Brocket

Taxonomic reviews based on integrative approaches have been advocated in the literature as an efficient method for taxa designation [71]. Our *M. cita* topotype was assessed based on the description of Osgood [27] of specimens collected near the Aurare river on the eastern shore of Lake Maracaibo, Venezuela. The morphology of the topotype agreed with the descriptions of Osgood [27] and Allen [4], which refer to a large gray brocket with a paler brown and more grayish coloration than those of the gray brockets previously described as *P. nemorivagus* (Cuvier, 1817) [25] and *S. gouazoubira* (Fischer, 1814) [22]. The cytogenetic assessment of *M. cita* revealed a unique karyotype when compared with other brockets, with $2n = 61$ and $FN = 70$, a simple sexual system with a submetacentric X chromosome, and exclusive chromosomal rearrangements in the chromosome pairs 1 to 4. A mitochondrial phylogeny allocated the topotype to the Blastocerina subtribe, separated from the *P. nemorivagus* and *S. gouazoubira* clades, and also from the other Neotropical deer, including the genera *Hippocamelus*, *Pudu*, *Ozotoceros*, *Blastocerus*, and *Odocoileus*. A partial *Cytb* phylogeny with more *M. cita* samples from Paraguana Peninsula in Falcón state, also in northwestern Venezuela, confirmed a monophyletic clade with a high posterior probability, confirming the evolutionary separation of *M. cita* from other gray deer species. Thus, the morphological, cytogenetic, and phylogenetic results complement the description of the Osgood's gray brocket and support the recognition of *M. cita* as a valid species within the Blastocerina.

4.5. A New Genus of Neotropical Deer

Mitochondrial phylogenies indicate that the genus *Mazama*, as traditionally defined, is polyphyletic since it includes at least three unrelated clades [7,8,10]. This has led to a revised taxonomy, according to which the application of this generic name is restricted to the brocket deer of the clade that includes the type species *M. americana*, a member of

the subtribe Odocoileina [7,24]. As a consequence of this new taxonomic paradigm, it has become necessary to reassign members of the subtribe Blastocerina to other genera, as has been amply discussed by several authors [7,24]. In this context, with the support of genetic analyses, *M. gouazoubira* has been transferred to the resurrected genus *Subulo* Smith, 1827 [23,24]. Because the Amazonian brown brocket *Mazama nemorivaga* does not share a common ancestor with *Subulo*, it has recently been transferred to the revalidated genus *Passalites* Gloger, 1941 [7,8,24,26,72]. By monotypy, Jasper et al. [73] and Morales-Donoso et al. [26] list *Cervus nemorivagus* Cuvier, 1817 [25] as the type species of *Passalites*.

The subtribe Blastocerina also includes the genera *Hippocamelus*, *Blastoceros*, *Ozotoceros*, and *Pudu*. The recovery in our phylogeny of *M. cita* as the first lineage to have diverged within the Blastocerina clade indicates that this species cannot be placed in the brocket deer genus *Subulo* or in any other genera mentioned above. Four of the existing generic names available for *Mazama* (*Homelaphus* Gray, 1872 [74]; *Doryceros* Fitzinger, 1873 [75]; *Nanelaphus* Fitzinger, 1873 [75]; *Coassus* Gray, 1843 [76]) could not have been applied to the unique lineage represented by the gray brockets from northern Venezuela because they were proposed long before any material of *M. cita* was known to science. Only two names postdate the discovery of *M. cita*: *Doratoceros* Lydekker, 1915 [62], attributed to Fitzinger, which is a *lapsus evidente* for *Doryceros* Fitzinger, 1873 [73,75], which as we have just noted is too old a name to have been applied to *M. cita*; and *Azarina* Larrañaga, 1923 [77], type species *Azarina fusca* Larrañaga, 1923 [77] (based on Azara 1802 [78]), type locality Paraguay (far away from northwestern Venezuela). The genus name *Mazama* and *Homelaphus* are associated with *M. americana* [73]. As we have noted, *M. cita* has been deemed a synonym or subspecies of *P. nemorivagus* or *S. gouazoubira* [5,30,79]. The genus names *Doryceros* (= *Doratoceros*), *Nanelaphus*, and *Coassus* are associated with the latter two species [24,26], none of which share an exclusive common ancestor with *M. cita* in our phylogeny. Therefore, *M. cita* needs to be reallocated to a new monotypic genus, which we name:

***Bisbalus* gen. nov.**

† zoobank: urn:lsid:zoobank.org:pub:59C68712-C4CA-49FB-B936-44D2243EB504

Type species by designation: *Bisbalus citus* Osgood, 1912.

Etymology: The new genus, a masculine noun in the genitive case, is named in honor of Francisco Javier Bisbal Enrich, 28 January 1953–13 July 2020, a Venezuelan biologist and naturalist, in recognition for his studies on the distribution, taxonomy, and biology of brocket deer (“matacán”), including the Osgood’s gray brocket, for his contributions to the knowledge of Venezuelan vertebrates, and for his lifelong dedication to the development and care of the largest zoological collection in Venezuela, which he led, housed at the Museo de la Estación Biológica Rancho Grande in Maracay.

Diagnosis: Because *Bisbalus citus* (Osgood, 1912), new name combination, is the only known member of the earliest diverging clade within the subtribe Blastocerina, this diagnosis is valid both for the genus and the species. *B. citus* possesses a convergent brocket deer morphotype. It is a large brocket, with body length greater than 850 mm and skull length greater than 180 mm. Its post-canine cheek teeth are big (total length greater than 58 mm). Its general pelage coloration is pale brownish-gray, with a yellowish spot over the eyes and white around the muzzle. Its antlers are relatively long and simple. Its karyotype is unique, with a diploid number of 60 to 62, a fundamental number of 70, a submetacentric X chromosome, and a metacentric Y chromosome.

Comparative description: The general gray body coloration differentiates *Bisbalus citus* from *Mazama* species. The yellowish spot over the eyes and white color around the muzzle distinguish the species from other gray brockets. In addition, the triangular and elongated preorbital fossa shape is a differential characteristic of *B. citus* compared with the inverted triangular and rectangular shapes described for *Subulo gouazoubira* and *Passalites nemorivagus*, respectively.

Included species: *Bisbalus citus* (Osgood, 1912). New combination.

Synonymy. *Mazama americana citus* refers to *Cervus nemorivaga* (Cuvier, 1817) (Osgood, 1912:43). Type locality eastern shore of Lake Maracaibo near to Aurare River in Venezuela. *Mazama cita cita* Allen, 1915:550. Name combination [4]; *Mazama simplicicornis citus* Lydekker, 1915:212. Name combination [62]; *Mazama gouazoubira citus* Whitehead, 1994:493. Name combination [30]; *Mazama nemorivaga cita* Hummelink, 1940:135. Name combination [79]; *Mazama gouazoubira cita* Cabrera, 1961:338. Name combination [5]; *Mazama gouazoubira* Handley, 1976:62. Name combination [80]; *Mazama gouazoubira cita* Bisbal, 1991:90. Name combination [32]; *Mazama cita* Groves and Grubb 2011:79. Name combination [81].

5. Conclusions

In the present study, we provide morphological, cytogenetic, and phylogenetic evidence supporting *Mazama americana citus*, Osgood, 1912 to be a monophyletic lineage within the Blastocerina subtribe separated from other Neotropical deer, especially from other brocket deer species. Thus, we recognize the validity of this species and propose the new genus name *Bisbalus*, with *Bisbalus citus* (Osgood, 1912) as the type species. The generic description and the nomenclature rearrangement proposed here contribute to disentangling the complex taxonomic puzzle of Neotropical deer. Our description should be followed by a more complete review of the species applying the criteria of Peres et al. [18]. In this context, it is necessary to carry out a comprehensive molecular (including nuclear markers and a population approach) and cytogenetic characterization of more *B. citus* specimens from throughout the species' range in Venezuela (including the Paraguaná Peninsula) and Colombia.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/taxonomy4010002/s1>, Figure S1: Whole chromosomes probes (WCP). (A) Homologies of chromosomes 1 to 4 between *Mazama cita* (MCI) and *B. taurus* (BTA). (B) Chromosomal correspondence between *Mazama cita* (BCI), *Subulo gouazoubira* (SGO) and *B. taurus* (BTA); Table S1: Body measurements of a male *Mazama cita* (Osgood, 1912), in centimeters (cm) following Driesch (1976); Table S2: Skull measurements of a male *Mazama cita* (Osgood, 1912), in millimeters (mm) following Driesch (1976); Table S3: Bovine BAC clones used for the chromosome X analysis in *Mazama cita* (Osgood, 1912), and their position in the cattle genome (ARS-UCD1.2 Assembly).

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Data Availability Statement: The data presented in this study are available in GenBank. Sequences obtained from tissue samples were recorded with the accession numbers OQ198443, OQ198444, OQ658775 and OQ658776.

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Conflicts of Interest: The authors declare that there are no competing interests.

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