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Molecular cytogenetics markers reveal the existence of a cryptic complex of *Mazama temama* species

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Mazama temama, commonly named Central American red brocket deer, was described in Veracruz, east-central México. Cytogenetic studies have characterized differentiated karyotypes observed in captive individuals of the species with a diploid number (2n) = 49 to 50, in a recently collected neotype with a diploid number (2n) = 44 and fundamental number (FN) = 70, and in specimens collected in Campeche in the southeast of México with a diploid number (2n) = 47 and fundamental number (FN) = 70. Then, we used BAC probes derived from cattle genome aiming to describe the chromosomal differences in *M. temama* karyotypic variants. We compared three individuals from Campeche (CAM) and the Veracruz neotype (VER). A total of 38 clones have been mapped by fluorescent *in situ* hybridization onto the chromosomes of both variants and a comparative map has been established. We assessed Cytochrome b (Cytb) gene sequences to perform phylogenetic analyzes including *M. temama* individuals from these localities and other Neotropical deer. The integrated analysis of hybridization results showed the real and surprising differences between the specimens. Besides the morphological similarities between the *M. temama* specimens, the results showed a difference of 10 chromosomes involved in rearrangements that separate their karyotypic composition, associated with tandem and centric fused chromosomes. Bayesian Inference tree evidenced Campeche and Veracruz individuals in two separated subclades within *M. temama* clade. The observed chromosomal and genetic differences are a substantially evidence of a reproductive isolation mechanism between the Veracruz and Campeche individuals suggesting the existence of a cryptic complex of species under *M. temama* nomenclature. Therefore, conservation strategies should be considered separately for each population of central American red brocket deer.

Mazama temama es conocido comúnmente como temazate rojo y fue descrito en Veracruz en el centro-este de México. Estudios citogenéticos han caracterizado cariotipos diferenciados observados en individuos de cautiverio con un número diploide 2n = 49 a 50, en el neotipo recientemente colectado con un número diploide 2n = 44 y número fundamental NF = 70 y en los especímenes colectados en Campeche en el sudeste de México con un número diploide 2n = 47 y FN = 70. De tal forma, utilizamos sondas BAC derivadas del genoma de bovino con el objetivo de describir las diferencias cromosómicas entre las variantes cariotípicas de *M. temama* comparando tres individuos de Campeche (CAM) con el neotipo de Veracruz (VER). Fueron mapeados un total de 38 clones a través de hibridación fluorescente *in situ* en los cromosomas de ambas variantes y de esa forma, un mapa comparativo fue establecido. Se accedió a secuencias de gene Citocromo b (Cytb) para realizar análisis filogenéticos incluyendo individuos *M. temama* de esas localidades y otros cérvidos neotropicales. Los resultados del análisis integrado de hibridación mostraron las diferencias reales y sorprendentes entre los especímenes. A pesar de las semejanzas morfológicas entre los individuos *M. temama*, fue observado que 10 cromosomas diferentes están involucrados en rearreglos que separan la composición cariotípica de ambos grupos. Tales rearreglos se corresponden con una fusión en tándem y fusiones céntricas. El árbol de Inferencia Bayesiana evidenció a los individuos de Campeche y Veracruz en dos subclados separados dentro del clado *M. temama.* Las diferencias observadas son una evidencia sustancial de un mecanismo de aislamiento reproductivo entre los individuos de Veracruz y Campeche revelando la existencia de un complejo de especies crípticas bajo la nomenclatura *M. temama*. Por tal motivo, deben ser consideradas estrategias de conservación para cada población de temazate rojo centroamericano.

Keywords: BAC probe; cervids; fluorescent *in situ* hybridization; karyotype; Central American red brocket deer.

Introduction

Cytogenetic analyzes have been a very important tool for species delimitation of Neotropical deer, especially those belonging to the genus *Mazama*. This is associated with the existence of cryptic species complexes, which are almost exclusively differentiated by chromosomal changes (Duarte *et al.* 2008). Several studies consider the morphological similarities among *Mazama* as adaptative convergence that is not correlated with karyotypic a molecular variation (Duarte *et al.* 2008; Abril *et al.* 2010; Cifuentes-Rincón *et al.* 2020; Peres *et al.* 2021).

The great chromosomal variation in the group was first studied by Duarte and Jorge (1996) revealing the cryptic diversity of *M. americana* species and describing karyotypical variants in Brazil. Thus, it allowed the identification of cytotypes from two distinct chromosomal lineages (Abril *et al.* 2010). The analysis of the *M. americana* neotype showed its karyotypic pattern with considerably chromosomes dif-

ferences from the Brazilian cytotypes and subsequently, recognized that these variants should be considered as different species (Cifuentes-Rincón *et al.* 2020, Peres *et al.* 2021).

Cytogenetic techniques also contributed with the taxonomic differentiation of *Subulo gouazoubira* and *Passalites nemorivagus*, showing a high frequency of B chromosomes and a fused X-autosome that formed the multiple sexual chromosome system in *P. nemorivagus* (Resende *et al.* 2012). Also, Abril and Duarte (2008) considered that centric fusions and quantities of B chromosomes variation are involved in the eight different karyotypes described for *M. nana*. The authors suggested that the species has not reached its optimal karyotypic evolutionary state accumulating rearrangements by selective pressure (Abril and Duarte 2008).

Thus, cytogenetic differences in the genus *Mazama* are a surprising characteristic especially when correlated with its small morphological diversification (Duarte *et al.* 2008; Abril *et al.* 2010). The chromosomal variation is closely related to speciation within the group, since karyotype differences are efficient reproductive barriers and isolate potentially sympatric populations (Cursino *et al.* 2014; Salviano *et al.* 2017). Differences greater than two pairs of chromosomes between individuals have a significant impact on the offspring fertility, that when crossed referenced with each other, producing infertile individuals or with serious fertility problems, although they cannot be morphologically distinguished (Cursino *et al.* 2014; Salviano *et al.* 2017; Galindo *et al.* 2021a, b).

In this context, the recognition of *M. temama* as a valid species was also based on karyotypical analyses. Jorge and Bernishke (1977) described cytogenetics features of three captive specimens with known origin in Tamaulipas, México. In these specimens the observed diploid number was (2n) = 49 to 50 and fundamental number (FN) = 70 contrasting with the 2n = 68 and FN = 74 of *M. americana* (Taylor *et al.* 1969) from whom it was considered subspecies (Hershkovitz 1951). The classical cytogenetic characterization of a recent neotype of *M. temama* from Veracruz, México, allowed the description of a karyotype with 2n = 44 and NF = 70 and detected variant karyotypes with 2n = 46 to 47 and NF = 70 from specimens collected in Campeche, in the southeast of México (Sandoval *et al.* 2022). The authors associated the karyotypical differences with two fused acrocentric pairs in the Veracruz neotype compared with the Campeche specimens, considered as more ancestral karyotype, and with an additional heterozygotic centric fusion in pair 11 of Campeche´s female (Sandoval *et al.* 2022). Understanding this karyotypical divergence could be relevant in a context in which recent studies suggest the morphological and ecological differences between the *M. temama* from Guatemala and México, compared with populations from Nicaragua, Honduras, Costa Rica and Panamá (Escobedo-Morales *et al.* 2023).

To confirm those rearrangements, the association of the G Band with molecular cytogenetics is a promissory technique since the karyotypical study of *Mazama* species requires high quality bandings and, even in this condition, the classical cytogenetic analyzes can lead to misunderstanding (Bernegossi *et al.* 2022). Mapped cattle chromosome probes have been described as a strategy to optimize chromosomal analysis and its efficiency in Neotropical deer species was already confirmed (Galindo *et al.* 2021a). The Whole Chromosome Probes from bovine fluorescently labeled have been employed to characterized inter-chromosomal rearrangements, and the region-specific probes derived from microdissection or DNA clones as in Bacterial Artifical Chromosomes (BAC) provides information on intra-chromosomal rearrangements (Frohlich *et al.* 2017; Romanenko *et al.* 2017). Recently, Bernegossi *et al.* (2022, 2023) characterized both types of probes derived from the entire set of cattle chromosomes in *S. gouazoubira* and demonstrated that this species had the same pattern of rearrangements proposed by Dementyeva *et al.* (2010) and Frohlich *et al.* (2017) for Cervidae. Moreover, mapped cattle chromosomes were used to compare *M. americana* neotype and Paraná cytotype, supporting the description of *M. rufa* as a distinct species with at least 15 rearrangements with *M. americana* species (Peres *et al.* 2021).

Then, we used BAC probes derived from cattle genome aiming to describe the chromosomal differences in *M. temama* karyotypic variants, comparing the male neotype from Veracruz and three individuals from Campeche. We also analyzed the mitochondrial Cyt-b gene phylogeny including individuals for these Mexican populations.

Material and methods

Animal samples and metaphases preparation. The chromosomal preparation was obtained from the cell culture procedures described in Sandoval *et al.* (2022) from *M. temama* samples of specimens collected in México, permission for collection SGPA/DGVS number 06821. The identities and localities of collection of each sample used for molecular cytogenetic also follows Sandoval *et al.* (2022): A) a male neotype (T366) from Veracruz, collected in a location called San José Acualco, coordinates 18º 38′ 53″ N and -96º 56′ 57″ W. B) a male (T363) and a female (T362) adult from Campeche, collected in Zoh Laguna, near the Calakmul Reserve, coordinates 18° 35′ 32.2″ N and -89° 24′ 55.3″ W. C) a male fetus (T364) from Zoh Laguna, Campeche.

Probe acquisition and labeling. Fluorescence *in situ* hybridization (FISH) using BAC probes was performed to characterize the homologies between the karyotype of *M. temama* from Veracruz (VER) and *M. temama* from Campeche (CAM).

Bacterial Artifical Chromosomes clones acquisition followed the procedure described by Bernegossi *et al.* (2022), for each selected probe from the cattle CHORI-240 library based on the NCBI ARS-UCD1.2. Assembly data and obtained from BACPAC Genomics, Emeryville, CA, USA (Table 1). After DNA extraction, probes were purified using

a Wizard® Plus SV Minipreps DNA Purification Systems kit, and BioPrime® Array CGH Genomic Labeling (Invitrogen, Carlsbad, CA, USA) kit was used to labeled BAC DNA with Green-DdUTP (Abbott, IL, USA), biotin 16-dUTP or digoxigenin-11-dUTP (Roche, Mannheim, Germany).

Fluorescence in situ hybridization. FISH was performed using the BAC probes on karyotypes of the *M. temama* specimens studied here. FISH was performed as presented in Vozdova *et al.* (2019), according to this protocol, the slides with metaphase spreads were incubated in 2×SSC at 72 °C, denatured in 0.07M NaOH, and dehydrated in ethanol series. The probe mixtures were denatured separately and applied on the denatured slides. A Zeiss AxioCam MRm camera attached to an Olympus BX60 microscope, equipped with appropriate fluorescence filters for the visualization of FISH results, was used.

Phylogenetic analysis. We assessed the mitochondrial gene Cytochrome B (Cyt-b) sequences for Neotropical deer available in the Genbank, including *M. temama* individuals from Veracruz and Campeche localities in México (Table 2). All sequences were aligned using the Clustal X program (Thompson *et al.* 1997). We followed the Akaike information criterion AICc (Akaike 1973) and selected the best molecular evolution model using the jModelTest v. 0.1.1 (Posada and Crandall 1998). Bayesian inference (BI) analysis was performed using MRBAYES 3 (Huelsenbeck and Ronquist 2001) with 10,000,000 generations, sampling every 1,000 generations and a variance of <0.01. A burn-in of 25 % was used and the consensus tree was constructed. Tree topology was recovered using FigTree v.1.4.0 (Rambaut 2012).

Results

The karyotypes of the male neotype from Veracruz and both adult specimens from Campeche locality are shown in Figure 1. Due to the similarity of diploid and fundamental number between the fetus and adult male from Campeche, we only show the karyotype of the adult specimen. We observed that these *M. temama* male individuals (2n = 46) differ from the female of the same locality (2n = 47) by one chromosomal translocation (Figure 1B, 1C).

The comparative analysis by FISH confirmed a karyotypic distinction among *M. temama* individuals from Campeche. Specifically, both males presented a centric fusion in a homozygous state, forming pair 3, while the

Table 1. List of BAC clones from the CHORI-240 cattle (BTA) library and their correspondence in *Mazama temama* neotype from Veracruz (VER) and *M. temama* individuals from Campeche (CAM) chromosomes.

VER	CAM	BTA chromosome	BAC clone (position Mb)	VER	CAM	BTA chromosome	BAC clone (position Mb)
1p	8p	25	89A17(21.27-21.51)	9p	9p	2	124N14 (96.51-96.71)
							437C7 (135.51-135.71)
1qa	8q	12	68F22 (11.10-11.33)	9q	9q	6	46O3 (61.12-61.34)
1qb	6q	11	98M8 (3.70-3.92)	10p	6p	16	140I17 (80.23-80.49)
2p	1p	19	50L8 (34.35-34.57)	10q	10p	5	78L8 (70.86-71.06)
2qa	1qa	7	57O13 (1.36-1.56)	11p	15	23	77G24 (28.26-28.47)
2qb	1qb	17	63H8 (3.90-4.12)	11q	2p	22	155B17 (59.06-59.29)
3p	3p	18	105L6 (65.58-65.76)	12p	11p	1d	69G2 (57.29-57.48)
3q	3q	3	106P15 (98.75-98.97)	12q	11q	1p	109I18 (116.68-116.91)
			24H18 (4.38-4.62)			1t	273F5 (154.36-154.55)
4p	4p	8d	504A4 (112.10-112.29)	13	12	26/28	47G11(24.54-24.77)/ 108O21 (24.74-24.94)
4q	4q	4	259C9 (119.29-119.48)	14	13	21	377F11 (33.89-33.98)
5p	10q	15	122B6 (5.29-5.51)	15	14	1c	106N15 (2.08-2.25)
			121K12 (41.87-42.09)				
5q	2qb	10	214M18(103.02-103.24)	16	17	9p	78C10 (60.39-60.60)
6p	5p	5	411D6 (5.81-5.99)	17	18	6t	200F18 (117.34-117.56)
			56D20 (55.50-55.75)				
6q	5q	2	110M8 (44.41-44.65)	18	19	8p	71G4 (62.73-62.93)
7p	16	24	205G22 (0.61-0.82)	19	20	29	103L15 (28.99-29.16)
7q	2qa	20	189H8 (5.16-6.89)	20	21	27	126M6 (41.90-42.14)
8p	7p	13	114C2 (42.80-43.05)	21	22	9t	90A6 (103.38-103.59)
8q	7q	14	319C15 (0.87-1.06)				159O16 (23.04-23.22)
							67P21 (33.77-34.00)
				X	X	X	311B9(47.76-47.97)
							316D2 (68.49-68.68)
							40H2 (74.95-75.12)

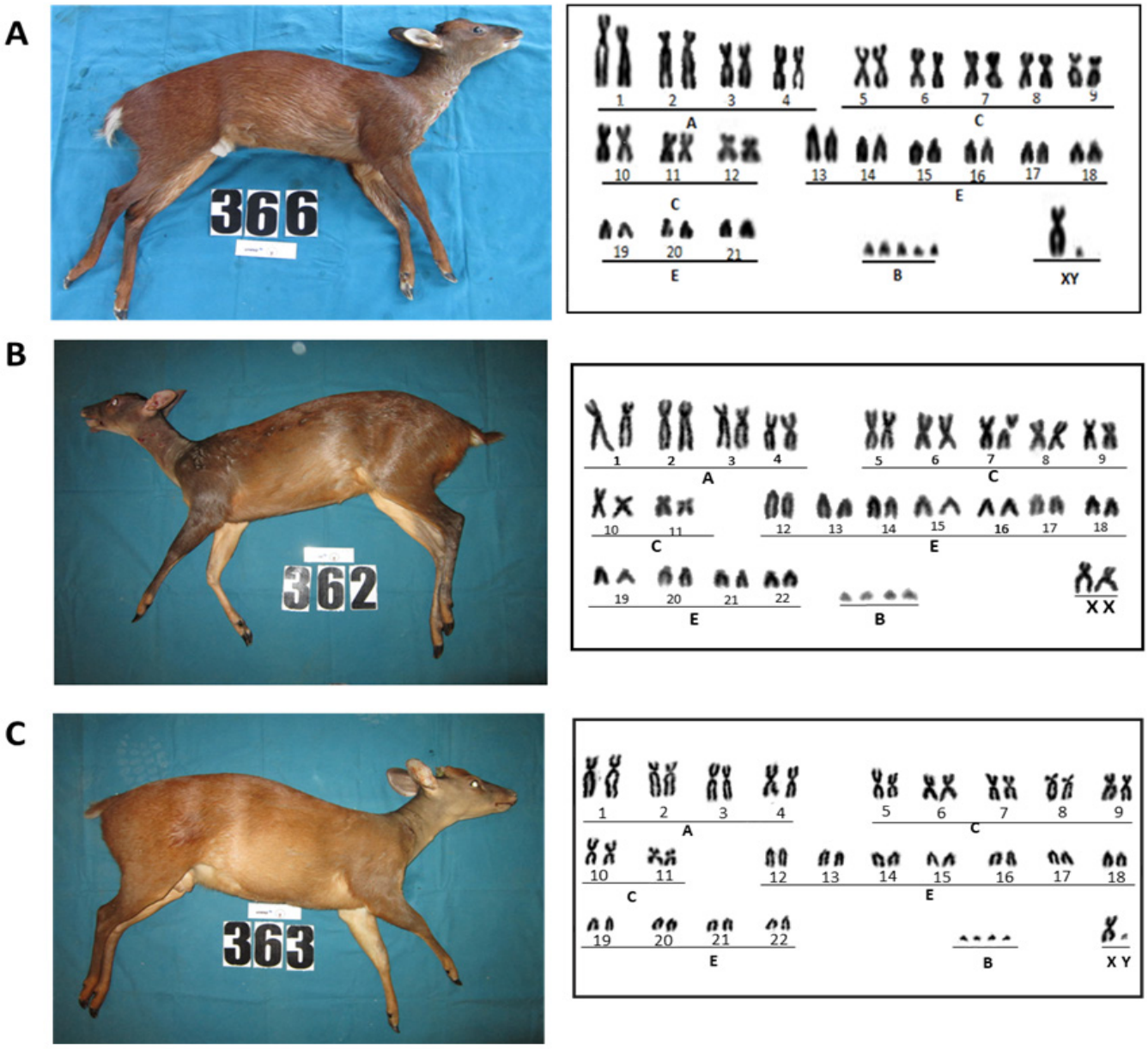

Figure 1. *Mazama temama* specimens analyzed cytogenetically. A) Male neotype from Veracruz, México (2n = 44 + 4-5Bs, FN=70 ID: T366). B) female from Campeche (CAM; 2n = 47 + 1-4Bs, FN = 70), ID: T362). C) male from Campeche (CAM; 2n = 46 + 1-4Bs, FN = 70 ID: T363). Groups of chromosomal relative lengths: A - large biarmed autosome; C – small biarmed autosomes; D – large acrocentric autosome; E – small acrocentric autosome). Adapted from Sandoval *et al.* 2022.

female displayed it in heterozygous (Figure 2A). All other chromosomes, including the composition of sex chromosomes, were homologous in all Campeche individuals.

Upon comparing the *M. temama* neotype (VER) with individuals from Campeche (CAM), various rearrangements were identified in the formation of their karyotypes. We observed that 10 pairs of chromosomes are involved in different rearrangements between VER and CAM karyotypes, involving both tandem and centric fusions (Figure 2): the proximal region of VER1 was homologous to CAM8 (Figure 2B), and the distal region to the shorter arm (q) of CAM6 (Figure 2C); the q arm of VER5 showed homology to the distal region of CAM2 q arm, while the shorter arm (p) of VER5 was homologous to the q arm of CAM10 (Figure 2D); the p arm of VER7 was homologous to CAM16 while the q arm was homologous to the proximal region of CAM2 q arm (Figure 2E); the p arm of VER10 was homologous to the p arm of CAM6 while the q arm was homologous to the p arm of CAM10 (Figure 2c); finally, the p arm of VER11 was homologous to CAM15 and the q arm to the CAM2 p arm (Figure 2F). The remaining chromosomes in VER were homologous to a sole chromosome in CAM, encompassing the two-armed pairs VER2, 3, 4, 6, 8, 9, 12 and the acrocentric pairs VER13, 14, 15, 16, 17, 18, 19, 20, 21. The composition of sex chromosomes was conserved and homologous between VER and CAM. A summary of these differences is highlighted in schematic Figure 3 by the colored chromosomes pairs. It is important to note that besides B chromo-

somes have been observed in all *M. temama* individuals, we did not represent it in schematic Figure 3 due to the intra and inter individual variation characteristic of this supernumerary chromosomes.

The Bayesian Inference tree of mitochondrial gene Cyt-b showed *Mazama temama* individuals forming a monophyletic clade separated from the other species of the genus with 98% of branch value (Sandoval *et al.* 2022; Escobedo-Morales *et al.* 2016). Within *M. temama* clade, the tree topology recovered two subclades (100 % branch value), one formed by the Mexican individuals from Veracruz and the other, by Campeche individuals of the study.

Table 2. List of mitochondrial gene Cytochrome b (Cytb) used in the phylogenetic analysis.

Species	Access number	Origin
Mazama temama	KP954719	Veracruz, México
Mazama temama	MZ350864	Veracruz, México
Mazama temama	OP712670	Veracruz, México
Mazama temama	MZ362858	Campeche, México
Mazama temama	MW047255	Campeche, México
Mazama americana	MZ350856	Juina, Mato Grosso, Brazil
Mazama americana	MZ350857	Cayenne, French Guiana
Mazama americana	JN632656	French Guiana
Mazama americana	MZ488872	Roraima, Brazil
Mazama rufa	MZ488852	Parana, Brazil
Mazama nana	MZ350863	Paraguay
Mazama nana	DQ789210	Parana, Brazil
Mazama jucunda	MZ350859	Brazil
Mazama rufina	JN632661	Colombia
Odocoileus virginianus	KM612278	México
Odocoileus virginianus	JN632671	French Guiana
Odocoileus hemionus	JN632670	USA
Odocoileus pandora	BK062825	México
Odocoileus Pandora	OQ731410	Campeche, México
Subulo gouazoubira	MZ350858	Paraguay
Subulo gouazoubira	KJ772514	Brazil
Passalites nemorivagus	MT008225	French Guiana
Passalites nemorivagus	MZ350861	Brazil
Ozotoceros bezoarticus	MZ350860	Brazil
Ozotoceros bezoarticus	JN632681	Uruguay
Blastocerus dichotomus	OQ196442	Brazil
Pudu puda	JN632692	Chile
Hippocamelus antisensis	JN632646	Argentina
Rangifer tarandus	KM506758	China

Discussion

Mazama temama species was first named by Kerr (1792) from Hernández (1651) descriptions of Mexican temamaçame (temazate) with type locality designated in El Mirador, Veracruz in east-central México (Hershkovitz 1951; Miller and Kellogg 1955; Cabrera 1960). However, the distribution ranges of the species have been reported from northeastern México to western Colombia, with records in Nicaragua, Guatemala, and Nariño state in Colombia (Allen 1915; Gutiérrez *et al.* 2017; Ramírez-Chávez *et al.* 2021). Across its wide distribution, a previous study showed karyotypical variants in México based on a neotype from Veracruz compared with specimens from Campeche, at southeast of México, with more than two pairs of central fused acrocentric chromosomes (Sandoval *et al.* 2022). Here, the BAC probes derived from cattle genome allowed to record the chromosomal differences between these two populations (Veracruz and Campeche) and confirmed the efficiency of using this technique in Neotropical deer species (Galindo *et al.* 2021a; Bernegossi *et al.* 2022). The integrated analysis of hybridization of both karyotypical variants showed the real differences among the specimens with 10 chromosomes involved in rearrangements between them. The karyotypical differences were one tandem fusion, four centric fusions formed by different chromosomes and a heterozygotic centric fusion. Although the occurrence of chromosomal rearrangements has been highly described among Neotropical deer species, mainly centric fusion in heterozygosis, its accumulation has been discussed as decreasing the reproductive fitness by leading to errors in meiosis and recombination suppression (Dobigny *et al.* 2017; Galindo *et al.* 2021a; Peres *et al.* 2021). Other studies have considered the difference of one tandem fusion as deleterious between karyotypes related to the fertility reduction of balanced gamete (Salviano *et al.* 2017, Galindo *et al.* 2021). Based on that, the evidence of at least 15 rearrangements, involving centric and tandem fusions, between *M. americana* and *M. rufa* supported the recognition of the later as a valid species within *Mazama* (Peres *et al.* 2021). Similar to this, the enormous karyotypic differences between Mexican red brockets from Veracruz and Campeche may be efficient reproductive barriers compromising the meiotic pairing between individuals of these populations and leading to the discussion of the biological species concept.

When compared with the karyotype described for captive individuals in San Diego Zoo with probable origin from Tamaulipas in México (Jorge and Bernishke 1977), it is important to consider that the authors misidentified four B chromosomes as acrocentric pairs 23 and 24. A later edition by Duarte and González (2010) allocated one pair of acrocentric autosomes as B chromosomes, however, the variation described between female and male in the previous study together with the small size of the pair 23 indicate that the karyotype should be newly edited to designate these two small acrocentric as B chromosomes. With this reorganization, the diploid and fundamental number of the red brocket deer from Tamaulipas will be corrected to 46 and 72, respectively. This karyotype differs with one more acrocentric pair and one less bi-armed chromosomes pairs from the Campeche individuals of our study. It is important to note that authors have discussed a 2n = 70 as the hypothetic ancestral karyotype of Cervids (Neitzel 1987;

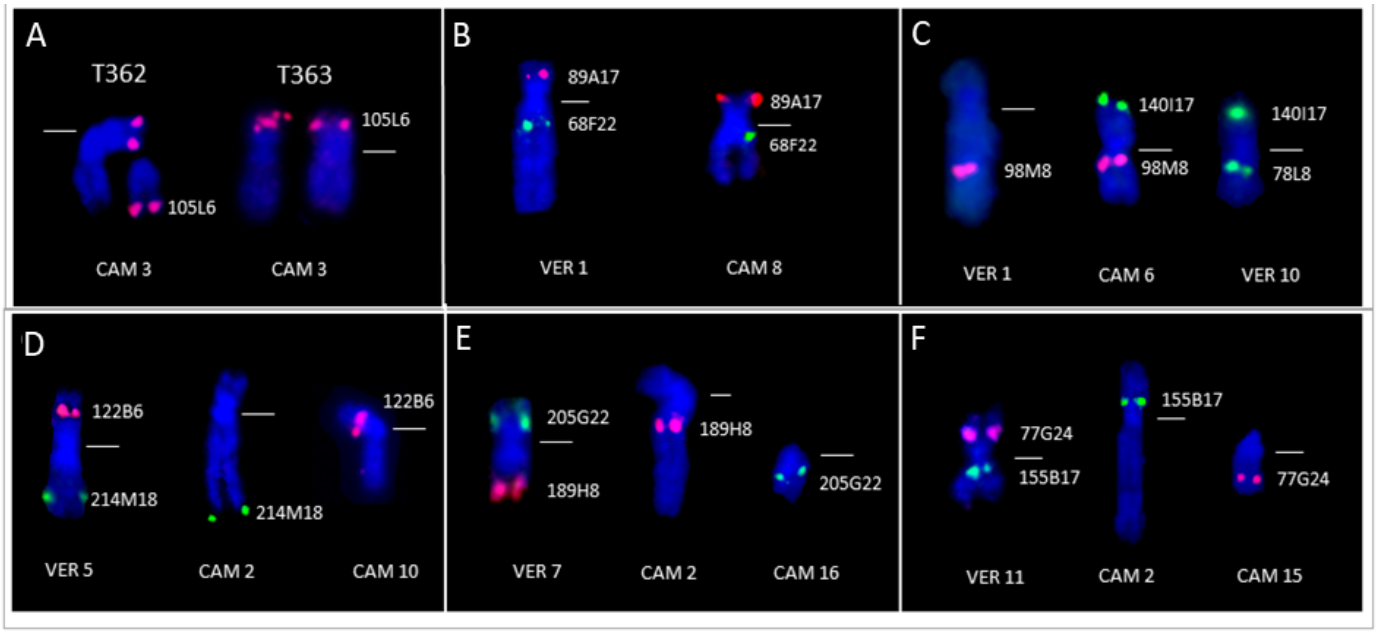

Figure 2. FISH results revealing distinctive chromosomal rearrangements among *Mazama temama* individuals from Veracruz (VER) and Campeche (CAM), as highlighted by the BAC clones specified on the right. A) Chromosomal polymorphism observed in individuals from CAM; T362 displays a fusion in heterozygosity, while T363 exhibits in homozygosity, as demonstrated by BAC105L6 (in pink). Chromosomal correspondence between. B) VER 1 proximal and CAM 8 revealed by BAC 89A17 (in green) and 68F22 (in pink). C) VER 1 q arm and CAM 6 revealed by BAC 98M8 (in pink), and between VER 10 and CAM 6 p arm revealed by BAC 140I17 (in green). D) q arm VER 5 and q arm CAM 2 highlighted by BAC 214M18 (in green), and between p arm VER 5 and CAM 10 p arm revealed by BAC 122B6 (in pink). E) q arm VER 7 and q arm CAM 2 indicated by BAC 189H8 (in pink), and between p arm VER 7 and CAM 16 revealed by BAC 205G22 (in green). F) q arm VER 11 and p arm CAM 2 revealed by BAC 155B17 (in green), and between p arm VER 11 and CAM 15 demonstrated by BAC 77G24 (in pink). The positions of centromeres are demarcated by dashed white lines.

Fontana and Rubini 1990; Dementyeva *et al.* 2010). Based on this, karyotypic evolution in Cervids is established by chromosomal fusions, such as Robertsonian translocations and tandem fusions, leading to a reduction in the number of chromosomes and changes in the fundamental number of chromosomal arms (Neitzel 1987; Fontana and Rubini 1990). Thus, it may suggest that a different rearrangement, probably a centromeric shift, an inversion of one acrocentric pair, or a tandem fusion between one acrocentric and a bi-armed chromosome, differentiates these karyotypes. FISH study should be performed in metaphasic cells of the Tamaulipas karyotypes described by Jorge and Bernishke (1977) to certainly identify the chromosomal divergences with the Campeche and Veracruz karyotypes. In any case, our results confirm that as other red brockets, *M. temama* evolved from successive tandem and centric fusions, in complex chromosomal rearrangements involving more than one chromosome (Jorge and Bernishke 1977; Sarria-Perea 2012; Sandoval *et al.* 2022). It is important to consider that accumulation of chromosomal rearrangements causes meiotic segregation errors with chromosome pairing limitations (Villagómez and Pinton 2008; Dobigny *et al.* 2017).

Specifically, the difference of more than two pairs of chromosomes has been described as a reproductive barrier in *M. americana* cytotypes, with tandem fusion as the mean causes of reduction in hybrid fertility considering the chance of successful reproduction in a backcross breeding with animals from the parent populations (Cursino *et al.* 2014; Salviano *et al.* 2017; Carranza *et al.* 2018). A recent study evaluated the meiotic segregation of hybrids with one heterozygotic tandem fusion in the *M. americana* complex, including a hybrid between the Carajás and Paraná cytotypes obtaining a rate of ~30 % gametic unbalance (Galindo *et al.* 2021b). Thus, the difference of one tandem fusion between populations is considered an efficient post-zygotic reproductive barrier and it was the mean key to suggest the validation of *M. rufa* species as separated from *M. americana sensu stricto* and Carajás cytoype (Galindo *et al.* 2021b; Peres *et al.* 2021).

The great morphological similarity between the neotype from Veracruz (east-central México) and Campeche individuals (southeast México) analyzed here, contrasted with the chromosomal differences among them. Our findings suggest that central American red brocket should be considered a cryptic complex with morphologically indistinguishable characterization and considerable cytogenetic diversity. The karyotypical rearrangements between *M. temama* populations analyzed here were greater than those that allowed the recognition of *M. temama* and *M. bororo* as valid species (Jorge and Bernishke 1977; Duarte and Jorge 2003).

These karyotypical differences among populations could be associated with geographic isolation array that may have occurred by habitat fragmentation that promoted genetic differences between populations (Abril *et al.* 2010). Sarria-Perea (2004) considered that chromosome and evolution rearrangements of *Mazama* species occurred at a faster rate than the molecular changes. Thus, the first phylogeographic study of *M. temama* also showed differences in genetic diversity of two Mexican populations of the species, with individuals from Oaxaca with a lower number of polymorphic sites compared to the population of Vera-

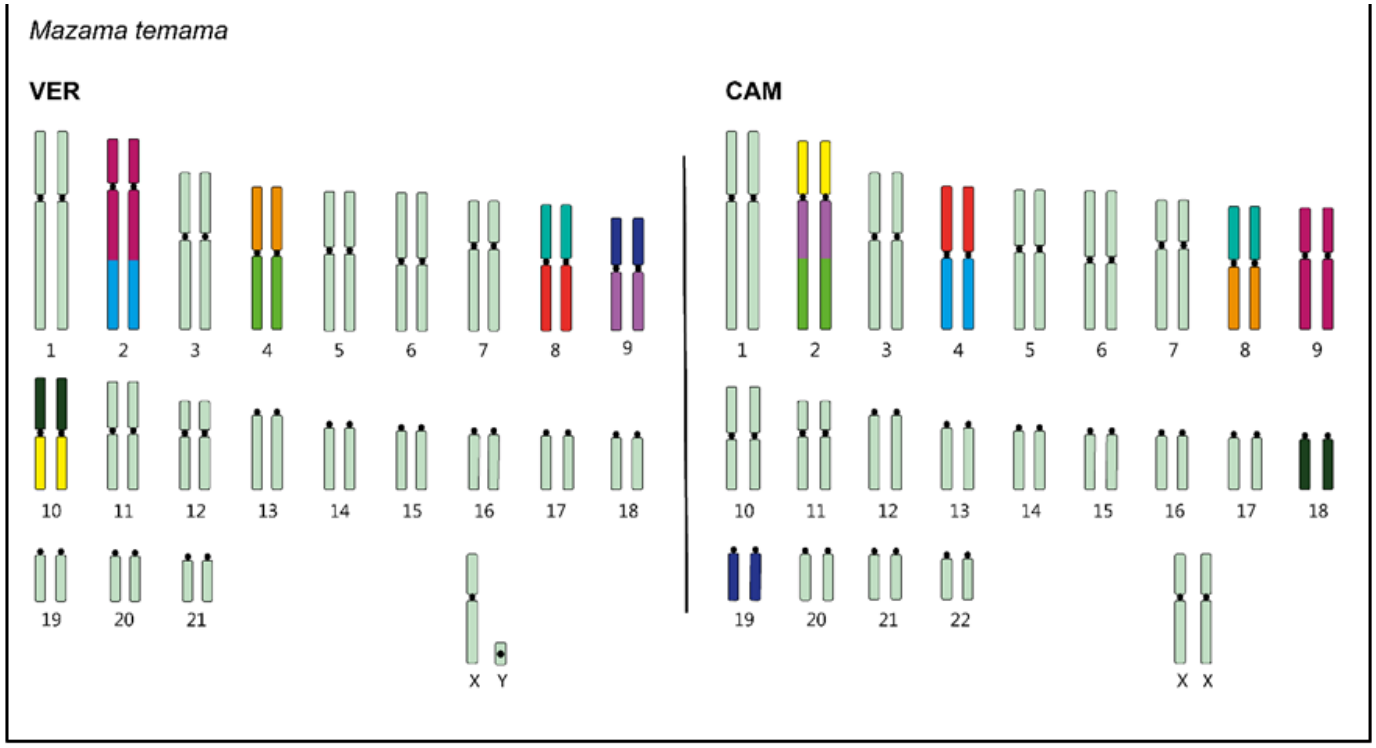

Figure 3. Comparative cytogenetics analysis of *Mazama temama* neotype from Veracruz (VER; 2n = 44 + 0 - 4 Bs, FN = 70) and *M. temama* specimens from Campeche (CAM; 2n = 46-47 + 1 – 4 Bs, FN = 70). The colored chromosomes indicate the karyotypes differences, each color representing one homologous chromosome in VER and CAM.

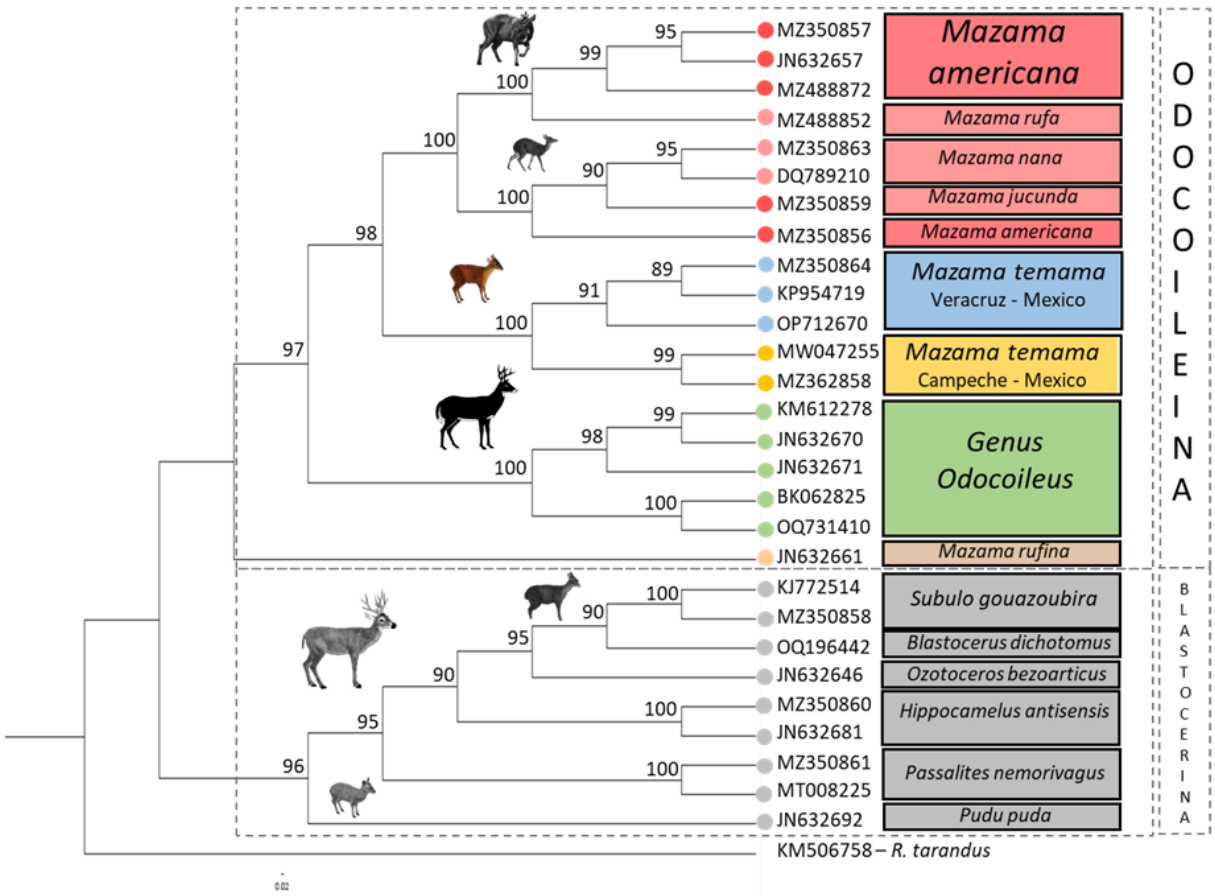

Figure 4. Bayesian inference tree of Cytochrome b (Cytb) gene from several Neotropical deer including *Mazama temama* individuals from Veracruz and Campeche localities in México. Odocoleina subtribe represented by: *M. americana*, *M. jucunda*, *M. nana*, *M. temama*, *M. rufina* and the genus *Odocoileus*. Blastocerina subtribe represented by *Subulo gouazoubira*, *Blastocerus dichotomus*, *Hippocamelus antisensis*, *Ozotoceros bezoarticus*, *Passalites nemorivagus,* and *Pudu puda*. Outgroup: *Rangifer tarandus*. The value above the clade represents the posterior probability of the analysis.

cruz (Serna-Lagunes *et al.* 2021). The authors hypothesized that populations underwent a genetic bottleneck and rapid population expansion, evidencing that geographic and reproductive isolation is due to the low gene flow between the two studied *M. temama* populations (Serna-Lagunes *et al.* 2021). Our phylogenetic results, in addition to confirm the monophyletic clade of Mexican red brocket within the genus *Mazama* (Escobedo-Morales *et al.* 2016, 2023; Sandoval *et al.* 2022), the Cyt-b tree presented here revealed the existence of potentially genetic structure between Veracruz and Campeche populations with high branch support. A similar result was observed in the phylogenetic analysis of partial ND5 and COI genes that positioned Campeche individuals in a separate subclade relative to one individual from Veracruz, however, the low support values preclude the recognition of existence of genetic structure (Sandoval *et al.* 2022). The distinction among *M. temama* populations was also discussed when Mexican and northern Central American populations were analyzed, in which a recent study suggested to keep *M. t. temama* (Kerr 1792) and *M. t. reperticia* (Goldman 1913) for the two morphological and ecologically differentiated groups based on craniometrical and environmental niche differences (Escobedo-Morales *et al.* 2023).

The existence of genetic variants between *M. temama* populations could be confirmed by molecular cytogenetic markers as shown in this study, demonstrating they are an efficient tool to study the evolution of brocket deer (Abril *et al.* 2010; Galindo *et al.* 2021a, b; Bernegossi *et al.* 2022). Although the number of samples is limited, our results suggest that central American red brocket populations have differentiated karyotypes. The possibility of crossing these *M. temama* individuals with these karyotypic differences could produce hybrids carrying tandem fusion and centromeric fusion, both in heterozygosis resulting in increased rates of unbalanced gametes (Galindo *et al.* 2021a). Thus, based on cytogenetic and molecular data, we assert that these *M. temama* populations should be individually considered from a management and conservation perspective, but is imperative to classify and taxonomically delimit these species. Therefore, it is important to increase cytogenetic sampling within the entire area of occurrence in order to understand this variation at the population level, and the limits of distribution of each karyotype and species.

The BAC probes were efficient to assist in chromosomal differentiation of *M. temama* individuals. The morphological similarity of the specimens contrasted with great chromosomal rearrangements between karyotypes from both localities, suggesting that *M. temama* could be a cryptic species complex with karyotypical differences ultimately resulting in a reproductive barrier. Conservation strategy efforts should be considered separately for each population of red brocket in México and Central America.

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