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New Insights on the Systematics and Population Genetics of the White-Tailed Deer (*Odocoileus virginianus*) in the Neotropics with a New Genetic Dataset

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Abstract

The systematics of the white-tailed deer (*Odocoileus virginianus*), especially in the Neotropics, remains a subject of debate. Recently, a study showed the existence of at least eight genetically distinct groupings of *O. virginianus* using two molecular datasets (*mtCyt-b* gene and whole mitogenomes). Here, we analyzed 105 *O. virginianus* specimens using a dataset (*mtNADH5* and *mt12S rRNA)* that performed very well for samples with limited and poor-quality DNA, providing a phylogenetic signal like that of the aforementioned datasets. The main results were as follows: 1- Nine distinct groups of *O. virginianus* were detected, confirming the eight previously identified groups, plus the emergence of a new grouping in South America; 2- The eight previous groupings were detected but with some modifications. The original Group 2 was divided into two groups, Group 2a (southern Mexico, Belize, and certain areas of Guatemala) and Group 2b (certain areas of Guatemala and Honduras). The original Groups 4 and 5 (Colombia, Venezuela, and French Guiana) were merged into a single Group 4-5, which presented two different subgroups (one of lowland and Andean specimens and another exclusively of Andean specimens). The current Group 6, in addition to including all Ecuadorian specimens, included a strong subgroup comprised of specimens from Colombian eastern Llanos and Panama. In the current analysis, the specimens from the northern Peruvian Andes belonged to Group 7; 3- The origin of haplotype diversity in *O. virginianus* was estimated at the beginning of the Pleistocene (2.5 million years ago, mya), while the haplotype diversification within the nine detected groups ranged between 1.6 and 0.3 mya; 4- Some of these groups showed unequivocal signs of population expansions in the last 0.6 mya; 5- The spatial structure showed the existence of significant genetic patches but no clinal structure or isolation by distance was found. It is important to understand in depth the evolutionary history of a species as emblematic and of such high cultural and hunting value as the white-tailed deer.

Keywords: Mitochondrial *NADH5* and *12S rRNA* Genes; Neotropics; *Odocoileus virginianus*; Phylogenetic Analyses; Phylogeography; Population Genetics; Species and Subspecies; White-Tailed Deer

Introduction

Undoubtedly, one of the best-known deer species in the Americas is the white-tailed deer (*Odocoileus virginianus*), partly because it starred in the famous 1942 Walt Disney Productions film, Bambi, directed by David Hand (although the original novel of the same name was set in Austria and featured a roe deer, *Capreolus capreolus*). Therefore, the public primarily associates *O. virginianus* with its North American distribution (Canada, the USA, and Mexico). Nevertheless, the adaptability of this species allows it to occupy a wide variety of geographically and ecologically distinct habitats, including in the Neotropics [1-3]. In South America, *O. virginianus* lives in environments ranging from marshy savannas and islands at sea level to altitudes approaching 4,200 m in the Andes mountains [4]. Body size of South American deer is generally a little larger than those found in Central America and southern Mexico, but smaller than white-tailed deer found along the northern periphery of their North American range [5]. Additionally, *O. virginianus* is the species of deer in the Americas with the highest capacity to adapt to the environment [6] and can tolerate the effects of human activities on their populations and their habitat [7].

Based on the fossil record, ancestors of white-tailed deer crossed the Bering land bridge from Siberia to North America about 5-7 mya (late Miocene-early Pliocene). The ancestral form diverged and spread throughout the continent. The fossil record of Pliocene and Pleistocene deer in the Americas is scarce [8], although members of this genus were the most common cervids in the Blancan and Pleistocene faunas of North America. The oldest cervid fossils known from the New World are *Bretzia* and *Eocoileus* from around 5 mya [8-10]. *Bretzia pseudalces*, a medium-sized deer of the Plio-Pleistocene of North America, was assumed to be a descendant of Eurasian Pliocene deer with three-tined antlers, such as *Cervavitus* [8, 11]. The fossil record suggests a diversification within Odocoileini around 5.1 mya in North America [10, 12,13]. Molecularly speaking, radiations within the Odocoileini were dated in relationship with Pliocene-Pleistocene events (4.3–1.4 mya) [14]. Other estimations of the divergence of Odocoileini were dated around 5.0–3.5 mya [15]) as well as the split of Blastocerina and Odocoileina was estimated to 3.4 mya by Hassanin, et al. [13]) or around 4-5 mya [16,17]. Gilbert, et al. [10] estimated 15.5-11.5 mya for the initial diversification within Odocoileini, and around 12.8–7.3 mya for the split of Blastocerina and Odocoileina. These temporal estimations seem to conflict with the fossil record or the other molecular temporal split estimations. The split of *Odocoileus* was estimated to 3.98-1.3 mya, and the divergence time between *Odocoileus hemionus* and *O. virginianus* was estimated to 2.10-0.60 mya [15]. More recently, Ruiz-García, et al. [17] estimated the initial divergence between the two North American *Odocoileus* species and the beginning of diversification within *O. virginianus* at 2.18 mya (1.76-2.64 mya) using mt*Cyt-b* gene sequences and complete mitogenomes. The first unambiguous *Odocoileus* fossil is known from the early Pliocene (3.8–3.4 mya) of North America [11]. Nevertheless, some authors claimed that, from a paleontological perspective, the first fossil of *Odocoileus* apparently appeared in the Blancan period of North America around 4-3.5 mya [4,18]. It was thought that it descended from *Procoileus edensis* from the Eden Pliocene of California, which gave rise to *Odocoileus brachyodontus*, which, in turn, gave rise to *O. virginianus* around 2 mya also in the later Blancan period in North America [18]. All molecular split estimations fit well with the fossil record, with *O. brachyodontus* considered to be the ancestor of *O. virginianus* [3,17,19,20]. Nevertheless, Gustafson [11] considered *Bretzia* as closely related to *Odocoileus* and *Blastocerus* and suggested that *Odocoileus* replaced *Bretzia* around 3.5 mya. *O. virginianus* was not uncommon in the central and eastern USA states during the Irvingtonian (early to mid-Pleistocene) and was common and widespread in the Rancholabrean (late Pleistocene). Numerous remains at Holocene (Recent) occupation sites suggest *O. virginianus* was an important game animal to Paleo-Indians [18].

Neotropical cervids underwent a rapid adaptive radiation after migration into South America via the Panamanian Isthmus; absence of other ruminant artiodactyls allowed them to fill niches making them the most diverse group of ungulates in South America [3]. This rapid diversification makes untangling the complex evolutionary patterns challenging [15,17,21-27].

Traditionally, it was assumed that *O. virginianus* evolved in North America resulting in a later divergence of the red brocket deer because of isolation within South America [1,28]. Notwithstanding, recent phylogenetic reconstructions indicated to the contrary that *Odocoileus* originated from the red brocket deer clade [17,21-23,29].

In North America, there are two species of the genus *Odocoileus* as we discussed, *O. hemionus* and *O. virginianus*. Analyses using combined nuclear genes and SRY showed that both are sister species. Nevertheless, analyses carried out using mitochondrial genes have shown a different situation. Ruiz-García, et al. [17] observed two types of relationships between *O. hemionus* and *O. virginianus*. On the one hand, some *O. hemionus* were the sister group to *O. virginianus*, and, on the other hand, some *O. hemionus* carry *O. virginianus* mtDNA through genetic introgression or recent hybridization and are, therefore, embedded within *O. virginianus*. Therefore, discordant genetic divergence between mt and nuclear DNA sequences in both species of *Odocoileus* is due to old introgressive hybridization, lineage sorting of ancestral alleles, or recent hybridization

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interspecific [30].

Because it has such a wide geographical distribution (from Canada to northern Bolivia, passing through the rest of North America, all Central American countries, Colombia, Ecuador, Peru, Venezuela, the Guianas and northern Brazil), many subspecies or potential species have been described. There have been attempts to group *O. virginianus* subspecies into related clusters based on comparative morphology, which resulted in up to 40 subspecies [31]. Molecular studies of the last few decades suggest the presence, at least, of 26 subspecies [20,32-34], especially in USA and Mexico.

Currently, there are 38 recognized subspecies of white-tailed deer with many more described historically [4,20,35]. Thirty of them in North and Central America, and the remaining eight subspecies would be distributed throughout South America.

Recent genetic studies suggest little genetic basis for many subspecies [17,32,34,36]. In general, genetic studies often find fewer discrete genetic groups than expected based on the number of subspecies [17,34, 37,38]. Unraveling taxonomy is complicated because some populations of *O. virginianus* were restored through a series of translocation programs during the mid- to late-twentieth century. Nevertheless, the use of diverse source stocks has obscured genetic relationships among populations due to widespread admixture [36,39]. In the southeastern USA, breeding dates may vary by up to 2–3 months over short geographic distances, which appears in part to be a long-lasting phenotypic legacy of the mixing of diverse source stocks [40].

The primary reason for the designation of multiple subspecies is regional patterns of morphological variability. For example, in the southeastern USA, *O. v. clavium* is geographically and phenotypically distinct from continental populations and restricted to 17 islands in the lower Florida Keys that total about 98 km^2 [4]. Three subspecies of white-tailed deer have ranges spanning the USA–Mexico border: *O. v. couesi, O. v. texanus*, and *O. v. carminis*. Mandujano, et al. [41] and Ambriz-Morales, et al. [34] suggest that 13 of the 14 Mexican subspecies can be separated into two groups based on their morphologic features: the northern subspecies (*texanus, carminis, miquihuanensis, veraecrucis, mexicanus*, and *couesi*), which grow to larger sizes, and the southern subspecies (*sinaloae, thomasi, yucat anensis, truei, oaxacensis, acapulcensis*, and *nelsoni*) whose sizes are smaller. *O. virginianus* is also native to the Coiba Island (673 km^2) situated 24 km off the Pacific coast of Panama. This population on Coiba Island is considered a separate subspecies (*O. v. rothschildi*) and is similar in size to *O. v. clavium* [42]. Hershkovitz [19] was the first that suggested that this species originated in Central America, subsequently dispersing to North America, and to South America after the rise of the Panama Isthmus about 3 mya. The geographic expansion of *O. virginianus* toward greater latitudes was comparatively recent [19].

In the case of the South American putative subspecies derived from Cabrera [43], who recognized eight subspecies. Nevertheless, there is almost no information on the diagnostic characters of these subspecies, and disagreement as to their exact distribution. The eight most widely accepted subspecies of *O. virginianus* in South America are the following: (1) *O. virginianus cariacou* Boddaert 1784 [Type locality "'Gujania, Brasilia"; restricted to "Guyane, coastal French Guiana," by Hershkovitz [44]]. Its geographical distribution is Brazil, from the northeast to the mouth of the Amazon River, reaching all the northern Amazon basin to the low Rio Negro, included French Guiana following Cabrera [43] and Brokx [5]. Smith [20] restricted its distribution to the north of the Amazon River and the east of Guyana. (2) *O. virginianus gymnotis* Wiegmann 1833 [Type locality: "stammt aus Columbien"; restricted to the Orinoco region, Venezuela, by Osgood [45]]. Its geographical distribution is Surinam, Guyana, Venezuela (savanna regions), and Colombia, in the eastern Llanos [43]. According to Brokx [5], its geographical distribution is Surinam, Guyana, Brazil (north of the Negro River), Venezuela (including the states of Amazonas and Bolívar, and the Llanos to the north of the Orinoco and Apure rivers). According to Smith [20], its geographical distribution is Surinam, Guyana, Brazil (north of the Negro River) Venezuela (part of the western Llanos and the whole of the eastern Llanos, the state of Bolívar, the eastern half of the state of Amazonas). (3) *O. virginianus goudotii* Gay and Gervais 1846 [Type locality "vit dans les regions elevees de la Nouvelle-Grenade"]. Its geographical distribution is Venezuela (Mérida Andean highlands), and Colombia (Andean highlands in all cordilleras) following Cabrera [43]. The distribution according to Smith [20] is remarkably different: Brazil (northern basin of the state of Amazonas to west of the Negro River), Venezuela (western and central regions of the country, including the Caribbean coastal fringe, Andes, Llanos to the north and south of the Apure River, and the western half of the state of Amazonas), Colombia (the whole country except the Pacific region), Ecuador, (Amazonian region), and Peru (northern Amazonian region). Brokx [5] used *O. virginianus apurensis*, a nomen nudum, for the *Odocoileus* found south of the Apure River (Venezuela) and in the eastern Colombian Llanos, which, according to Smith [20], would be *goudotii*. (4) *O. virginianus margaritae* Osgood 1910 (Type locality "vicinity of Puerto Viejo, Margarita Island, Venezuela."). Its geographical distribution is Venezuela, Margarita Island [5,20,43]. (5) *O. virginianus curassavicus* Hummelinck 1940 (Type locality "Island of Curacao," Netherland Antilles). Its geographical distribution is Netherlands Antilles, island of Curaçao [5,20,43]. Husson

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[46] speculated that the description might be based on an introduced mainland specimen. Notwithstanding, this deer is small [5], which suggests that it is a truly insular form. (6) *O. virginianus tropicalis* Cabrera 1918 (Type locality "La María, en el valle del Dagua," Colombia). Its geographical distribution is Colombia and Ecuador, in Pacific region of both countries [5,20,43]. Tate [47] considered this subspecies to be closely related to the Panamanian white-tailed deer. (7) *O. virginianus ustus* Trouessart 1910 [Type locality "El Pelado, au nord de Quito (4,100m), sur la frontiere de Colombie; Chilla cocha, dans la Cordillere occidentale (3,800m), entre la ville de Machala et celie de Loja, province del Oro; Narihuiiia, dans la Cordillere occidentale, au Nord de la montagne precedente (4,000m), entre Machala et Cuenca, province de J'Azuay;"]. Its geographical distribution is Ecuador, in the Andean region, and possibly the southern end of the Andes in Colombia [5,20,43]. (8) *O. virginianus peruvianus* Gray 1874 (Type locality "Ceuchupate," probably Combapata, Cuzco, Peru). Its geographical distribution is Peru (the whole country excluding the Amazonian region) and Bolivia (the northwest of the country) [5,20]. Cabrera [43] questioned its presence in Bolivia. Different synonyms for these white-tailed deer taxa in the Neotropics can be seen in Table 1.

Subspecies	Synonym	Author	Locality
Odocoileus virginianus cariacou	1-*sylvaticus*	Kerr, 1792	No type locality given, but based on Barrere's (1741: 151) "Biche des Bois" from French Guiana
	2- *campestris*	Cuvier, 1817	No type locality designated; based on antlers from a Brazilian figured made by Daubenton (1756) and on a similar specimen ("qui se trouvait dans Ie cabinet de Tenon") without locality
	3- *spinosus*	Gay and Gervais, 1846	I'Amerique meridionale
	4-*suacuapara*	Miranda-Ribero, 1919	-
Odocoileus virginianus gymnotis	1-*savannarum*	Cabanis, 1848	British Guiana
	2- *Gymnotis wiegmanni*	Fitzinger, 1879	-
	3- *tumatumari*	Allen, 1915	-
Odocoileus virginianus goudotii	1-*columbicus*	Fitzinger, 1879	Mittel Amerika, Columbien, wo diese Form in der Republik Neu Granada vorkommt
	2- *lasiotis*	Osgood, 1914	Paramo de los Conejos, Sierra de Merida, [Táchira.] Venezuela
Odocoileus virginianus margaritae	None	-	-
Odocoileus virginianus curassavicus	None	-	-
Odocoileus virginianus tropicalis	None	-	-
Odocoileus virginianus ustus	1-*consul*	Lönnberg, 1922	-
	2-*abeli*	Spillmann, 1931	
	3-*gracilis*	Spillmann, 1931	-
	4-*antonii*	Spillmann, 1931	
	5-*aequatorialis*	Spillmann, 1931	-
Odocoileus virginianus peruvianus	1-*brachyceros*	Philippi, 1894	Cajamarca, Peru
	2- *philippi*	Trouessart, 1905	-

Table 1: Synonyms of the scientific nomenclature of the eight supposed subspecies of white-tailed deer (*Odocoileus virginianus*) in South America proposed by Cabrera (1961). Synonyms determined by Cabrera (1961) and Smith (1991).

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The notion that South American *Odocoileus* are only subspecies of *O. virginianus* originated in Lydekker's [48] "Catalogue of the Ungulate Mammals in the British Museum (Natural History)," and was formalized by Hershkovitz [44] and Cabrera [43]. Authors who believe there is more than one species of white-tailed deer in South America criticize Cabrera's [43] findings. This is because it contradicts what he had long maintained. Cabrera [49] had thought that there were notable differences in characters between North and South American *Odocoileus*. He had recognized three South American species of *Odocoileus* (*suacuapara* = *cariacou*; *columbicus*; *peruvianus*) and had even suggested the existence of a fourth species [50]. Cabrera [43] justified his subsequent change of opinion by noting that evidence had appeared which indicated that, contrary to what he had previously thought, some South American *Odocoileus* have a metatarsal gland, whereas some North American *O. virginianus* lack it. Méndez-Arocha [51] submitted evidence refuting the idea that some South American *Odocoileus*, namely those that Cabrera [43] called *O. v. cariacou*, possess a metatarsal gland. Based on this evidence, this author proposed that the deer from Colombia, Venezuela, and the north of the Guianas be considered a species separate from *O. virginianus*. For Venezuelan deer particularly, Méndez-Arocha [51] suggested the existence of three forms: *O. cariacou gymnotis*, *O. cariacou lasiotis* (Mérida Andes), and *O. cariacou margaritae*. The view of Méndez-Arocha [51] concerning the absence of a metatarsal gland in South American *Odocoileus* was supported by Hershkovitz [52], Brokx [53], and Baker [54], who noted that populations from Canada and USA are the only ones with a well-developed gland (more than 4 cm in diameter, with a prominent tuft of circumglandular hair; [55]), whereas in populations from southern Mexico to South America, the gland is absent or poorly defined. For South American deer specifically, Brokx [5] noted that a metatarsal gland appears to be always absent. Therefore, Molina and Molinari [56] and Molinari [2] considered that the current taxonomy of South American *Odocoileus* [43], which regards them as conspecific with North American *O. virginianus*, is based on the erroneous assumption of variability in the metatarsal gland in both North and South American *Odocoileus*. Both works [2,56] considered that the extensive variation in minor morphology has even led to the proposed existence of multiple species among populations traditionally referred to *O. virginianus*. For example, morphometric analyses and differences in the frequency of qualitative skeletal traits in North America and northern South America led Molina and Molinari [56] to propose that populations from these two continents are different species. Similarly, morphological variation occurs among even geographically proximate populations of Neotropical white-tailed deer, suggesting that they also might be different species [2]. Notwithstanding, the phylogenetic value assigned to these morphological characters does not necessarily provide a significant phylogenetic signal for defining species.

The only study of *O. virginianus* conducted in South America prior to that of Ruiz-García, et al. [17] was that of Moscarella, et al. [1] in Venezuela, who analyzed 730 base pairs, bp, of the mt control region for 26 specimens sampled from the three taxa of *O. virginianus* present in Venezuela (while Molina, et al. [56] treated them as full species, Moscarella, et al. [1] treated them as subspecies of *O. virginianus*). That study showed evidence of a significant population structure for *O. virginianus* in Venezuela. Phylogeographic analyses showed four lineages with the putative subspecies *O. v. gymnotis* appearing to be polyphyletic. The first lineage contained haplotypes from *O. v. goudotii* (Merida state), and haplotypes of *O. v. gymnotis* (Barinas, Lara, Falcón, Carabobo, and Monagas states). The second lineage clustered all haplotypes from Margarita Island (*O. v. margaritae*) and a haplotype from Sucre state (*O. v. gymnotis*), which were geographically proximal. The third lineage contained haplotypes from Guárico and Apure states. These haplotypes, belonged to *O.v. gymnotis*, which were located geographically in the Venezuelan Llanos. The fourth lineage was represented by haplotypes from Miranda, Zulia, Falcón, and Anzoátegui states. All these haplotypes belonged to *O. v. gymnotis* and were distributed along the Venezuelan coastline.

Recently, Ruiz-García, et al. [17] obtained new genetic results, particularly for two databases (mt*Cyt-b* gene, 90 specimens, and mitogenomes, 22 specimens) focused on Neotropical white-tailed deer. Using the mt*Cyt-b* gene and mitogenomes, the following eight genetically distinct groups of *O. virginianus* were detected: one in North America (USA and northern Mexico, Group 1), two in Central America (southern Mexico, Guatemala, Belize, and Honduras, Group 2, and Panama, Group 3), and five in South America [part of the central Colombian Andes, Group 4; part of the eastern Colombian Andes, Venezuela, and French Guiana, Group 5; Ecuador (both the Andean region and the coast), Group 6; the north-central coast of Peru, Group 7; and the central and southern parts of the Peruvian Andes, Group 8]. All analyses performed detected these eight groups, but the relationship between them varies depending on the phylogenetic procedures used. The genetic distances between these groups of *O. virginianus* were not extremely high. For the mt*Cyt-b* gene, the genetic distances ranged from 0.3% to 2.8%, while for mitogenomes they ranged from 1.2% to 3.5%. These values were not too high, and if no appreciable chromosomal differences are found among these groups of *O. virginianus* in future studies, it could be concluded that there is only one species of *O. virginianus*. The diversification of mitochondrial haplotypes at the origin of *O. virginianus* was estimated to have occurred during the early Pleistocene.

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Bayesian inferences and MJ networks yielded identical estimates around 2.2 mya.

White-tailed deer is considered a species of Least Concern according to the IUCN Red List and are not listed as Critically Endangered, Endangered, Vulnerable, or Near Threatened status [33]. The best estimate available suggested that populations of white-tailed deer throughout their range exceeded several million [57,58] to as many as 24 million [59].

Occasionally, the *O. virginianus* samples collected over the last 25 years consisted mainly of a few hairs or very small fragments of skin or bone, and the DNA obtained was scarce and of low quality, making it unsuitable for obtaining whole mitogenomes or nuclear gene sequences. Because of this, we were forced to amplify very small fragments of some mt genes to try to increase the number of *O. virginianus* specimens studied, especially in geographic areas that showed the possible existence of other clusters that could not be resolved by the study of Ruiz-García, et al. [17]. To this end, we analyzed 105 *O. virginianus* specimens from the USA, Mexico, Guatemala, Belize, Costa Rica, Panama, Colombia, Venezuela, French Guiana, Ecuador, Peru, and Bolivia. But which mt genes would be suitable? And could these small fragments of mt genes offer phylogenetic results like those obtained in Ruiz-García, et al. [17]?.

Based on the above, we selected two small fragments of mtDNA. One corresponds to the mt*NADH5* gene (medium-high evolution rate), and to the mt*12S rRNA* gene (low evolution rate). These two fragments could thus represent the average evolution rate of mtDNA. We must remember that, although mtDNA represents a single linked locus, se lection pressures and evolutionary rates are highly heterogeneous across it [60, 61].

As a consequence of all this, the main aims of the research presented here are: 1- To determine how many different groups of *O. virginianus* there are, especially in the Neotropics, and whether these groupings agree with those observed by Ruiz-García, et al. [17]; 2- To determine, if possible, the geographic origin of the ancestor of the current *O. virginianus*; 3- To estimate the possible genetic heterogeneity among the different geographical groupings of *O. virginianus* detected and to measure the magnitude of these genetic heterogeneities; 4- To determine if there is an exact correspondence between some of these genetic groupings found and the traditionally accepted subspecies; 5- To estimate the divergence times of the ancestor of *O. virginianus* and of the ancestors of the different genetic groupings found within this species and relate them to climatic or geological changes through their respective natural histories; 6- To estimate the levels of genetic diversity within each of the different genetic groupings found within *O. virginianus*; and 7- To determine possible demographic changes for the overall sample of *O. virginianus* and within each one of the genetic groupings found within this species through their respective natural histories.

Material and Methods

Samples Analyzed

Over the past 28 years, various tissues from white-tailed deer have been collected in different areas of the Neotropics. Most of the samples came from hunters' trophies (hair, small pieces of skin, antler fragments, or turbinate bone fragments) or from bushmeat consumed by locals in their respective geographic areas where the animals were hunted (muscle). Some of these samples, from which good-quality DNA was obtained, were analyzed in Ruiz-García, et al. [17]. Nevertheless, we also have a fraction of very small samples that provide DNA of very low quality and quantity (a few hairs, a few milligrams of antlers or turbinate bones), from which it was impossible to fully sequence the mt*Cyt-b* gene, let alone the complete mitogenomes. Therefore, we proceeded to sequence small fragments of mt*NADH5* and mt*12S rRNA* genes. Results were obtained for 105 specimens of *O. virginianus*, including some sequences obtained from GenBank (North American and north-central Mexican specimens). The analyzed specimens came from the following locations: six from the USA and north-central Mexico (five from GenBank), 13 from southern Mexico, three from Belize, four from Guatemala, two from Honduras, one from Costa Rica, three from Panama, 15 from Colombia, four from Venezuela, one from French Guiana, 21 from Ecuador, 31 from Peru, and one from Bolivia. Additionally, as outgroups, 10 specimens of *Mazama americana* (two from GenBank), two specimens of *Mazama temama* (one from GenBank), one specimen of *Mazama rufa* (from GenBank), one specimen of *Mazama nana* (from GenBank), one specimen of *Mazama jucunda* (from GenBank), three specimens of *Mazama pandora* (two from GenBank), three specimens of *Bisbalus citus* (one from GenBank), two specimens of *Mazama rufina*, four specimens of *Passalites nemorivagus* (one from GenBank), four specimens of *Subulo gouazoubira* (all four from GenBank) and one specimen of *Ozotoceros bezoarticus* (from GenBank) were analyzed, for a total of 32 sequences used as outgroups (Table 2).

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Location	NS	TT
White-tailed deer		
New specimens		
Odocoileus virginianus		
Mexico (n = 14)		
Quintana Roo State (Playa del Carmen)	8	Hairs
Yucatán State (Mérida)	3	Hairs
Campeche State	2	Hairs
Chiapas State (Palenque)	1	Hairs
Guatemala (n = 4)		
Petén Department (Petenitza)	2	hairs and bones
Izabal Department (Livingston, Chocón Machacas)	2	Hairs
Belize (n = 3)		
Río Bravo-Orange Walk-district	3	Hairs
Honduras (n = 2)		
Roatán Island	2	Hairs
Costa Rica (n = 1)		
Alajuela Province	1	Hairs
Panama (n = 3)		
Coclé Province (Cerro La Vieja, Penomeme)	2	Hairs
Chiriquí Province (Boquerón)	1	Hairs
Colombia (n = 15)		
Cundinamarca Department (PNN Chingaza)	2	Hairs
Caldas Department	3	Hairs
Risaralda Department	5	Hairs
Santander Department	1	Skin
Guainía Department	1	Bones
Meta Department (Villavicencio, Puerto López)	2	hairs and skin
Arauca Department	1	Bones
Venezuela (n = 4)		
Mérida State	2	Hairs
Falcon State	1	Hairs
Apure State	1	Hairs
French Guiana (n = 1)		
Sinnamary-Cayenne	1	Muscle
Ecuador (n = 20)		
Cotopaxi Province (Latacunga, PN Cotopaxi)	7	hairs and skins
Napo Province	3	Hairs
Azuay Province (Cuenca, Baños)	2	Hairs
Tunhuragua Province	1	Skin
Pichincha Province	1	Skin
Imbabura Province	1	Hairs
Manabi Province (Portoviejo, PN Machala)	2	hairs and skin

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Guayas Province	3	Hairs
Peru (n = 31)		
Cajamarca Department	2	Skins
Piura Department	3	Skins
Lambayeque Department	2	Skins
Lima Department	2	Skins
Ica Department	1	Hairs
Ayacucho Department	2	Skins
Apurimac Department	8	Hairs
Cuzco Department	4	Hairs
Puno Department	2	Skins
Huaraz Department	2	Hairs
Huancavelica Department (Collcabamba)	1	Skin
Junin Department (Mantaro)	1	Hairs
La Libertad Department	1	Skin
Bolivia (n = 1)		
La Paz Department	1	Skin
GenBank		
Odocoileus virginianus		
USA (n = 2)		
Texas and Washington States	2	KM612273 and KY928667
Mexico (n = 2)		
Veracruz and Tamaulipas States	2	KM612271 and KM612278
Odocoileus hemionus		
USA (n = 1)		
Arizona State	1	T1766

Table 2: Sources of the white-tailed deer collected and analyzed at the mitochondrial *ND5* and *12S rRNA* genes (105 specimens). NS = Sample sizes at the *ND5* and *12S rRNA* genes; TT = Type of tissue employed to obtain DNA.

Molecular Procedures

We extracted and isolated DNA and sequenced the mt*NADH5* and mt*12S rRNA* genes from animals hunted and consumed by rural communities (the major fraction of the samples). The tissues used to extract DNA were small pieces of skin, hair, small fragments of antlers, turbinate bones, and muscle using the NX-48S Tissue DNA kit (NextractorR NX-48S; Genolution, Seoul, Republic of Korea). The primers used to amplify the mt*NADH5* gene were the following: L12673: 5'- GGTGCAACTCCAAATAAAAGTA-3', and H12977: 5'-AGAATTCTATGATGG ATCATGT 3' (Waits et al., 1999). The primers used to amplify the mt*12S rRNA* gene were L1091: 5'- AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3'; H1478: 5'- TGACTGCAGAGGGTGACGGGCGGTGTGT-3' [62-64]. The total length of the analyzed sequences was 608 base pairs (bp). Both mtDNA strands were sequenced directly using BigDye Terminator v3.1 (Applied Biosystems, Inc., Foster City, CA, USA). Sequencing products were analyzed on anABI3730 DNA Analyzer system (AppliedBiosystems, Inc., Foster City, CA, USA).

Phylogenetic and Population Genetic Analyses

Phylogenetic Trees, BAPS analyses, and Divergence Times

jModeltest v2.0 [65], Kakusan4 [66], and MEGA X 10.0.5 [67] software were applied to determine the best evolutionary mutation model for the analyzed sequences of the mt*NADH5* and mt*12S rRNA* genes. The Bayesian information criterion (BIC) [68], the Akaike information criterion (AIC) [69], and the maximum likelihood criterion (lnL) [70] were used to determine the best evolutionary model for the relationships among the neotropical deer

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taxa studied. The BIC showed that the best nucleotide substitution model was TN93 + G (BIC = 10.963,64), while for AIC, the best nucleotide substitution model was GTR + G (8.375, 04) and for lnL, the best nucleotide was GTR + G + I (lnL = -3.905,62, respectively). The nucleotide substitution model used to obtain the phylogenetic trees was GTR + G + I.

We constructed three mitochondrial phylogenies for aligned sequences with maximum likelihood (ML) and Bayesian Inference (BI) procedures. For the ML tree, we used RAxML v8.2.12 software [71]. RAxML was run with the GTRGAMMA model of nucleotide evolution and default parameters, and 1,000 bootstrap replicates to calculate node support for the two ML trees obtained. 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% [23,72].

BI tree was completed with the BEAST v. 1.10.4 software [73]. 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 [73]. 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 [74] and TreeAnnotator v1.10.4 [75] was used to determine the best tree. A Yule speciation model and a relaxed molecular clock with an uncorrelated log-normal rate of distribution were used [76]. Posterior probability (*pp*) values provide an assessment of the degree of support of each node on the tree. Trees were visualized in FigTree v. 1.4.4 software [77].

The BI tree, obtained with the BEAST v. 1.10.4 software, was used to estimate the time to most recent common ancestor (TMRCA) for the different nodes found in this tree. We used a prior of 5 mya for the initial node of divergence between the Neotropical deer [16]. A second approach for inferring divergence times [78] is named 'borrowed molecular clocks' and uses direct nucleotide substitution rates inferred from other taxa. For this second approach, we used a Median Joining network (MJ network) with the help of Network 4.6.10 software from Fluxus Technology Ltd. (Colchester, UK) [79]. The ρ statistic [80] was estimated and transformed into years of divergence among the haplotypes. To determine the temporal splits, we estimated the mutation rate per sequence and per million years. For the mt*NADH5* and mt*12S rRNA* genes, the average mutation rate used was 0.69×10^{-6}, which is equivalent to one mutation every 239,154 years. Networks are more appropriate for intraspecific phylogenies than tree algorithms because they explicitly allow for the co-existence of ancestral and descendant haplotypes, whereas trees treat all sequences as terminal taxa [70].

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 [81], 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.

Genetic Heterogeneity Statistics Estimated for the White-Tailed Deer

We estimated the following statistical heterogeneity indices for the different groups of white-tailed deer analyzed: table of contingency, H_{ST}, K_{ST}, K_{ST}*, γ_{ST}, N_{ST}, and F_{ST} [82], and indirect gene-flow estimates were obtained assuming an infinite island model [83] for all the groupings taken together. Significance was estimated with permutation tests using 10,000 replicates. We also estimated genetic heterogeneity between white-tailed 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 [84,85]. We also used the absolute genetic distance (Da) to determine the percentage of genetic differences among the different white-tailed deer groupings.

Genetic Diversity Statistics and Possible Demographic Changes in the white-Tailed Deer Groupings using the mt*ND5* and mt*12S rRNA* Gene Dataset

The following genetic diversity statistics were applied to determine the genetic diversity for the different white-tailed deer groupings 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 [84,85].

To estimate possible demographic changes for all the groups considered of white-tailed deer, we used Fu and Li's D* and F* tests [86], Fu's F_S statistic [87], Tajima's D test [88], and R_2 statistic [89]. Ninety-five percent confidence intervals and probabilities were obtained with 10,000 coalescence permutations. Also, a mismatch distribution (pairwise sequence differences) was obtained [90]. We used raggedness (*rg*) to determine the similarity between the observed and theoretical curves. These demographic analyses were also

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carried out using the software DNAspv6.1 and Arlequin v3.5.1.2 software [84,85]. Based on the mismatch distribution analysis, it is possible to estimate how long ago a possible population expansion began. For this, the expression t = 2mt was used, where t is a parameter calculated by the DNAsp v6.1 software, m is the mutation rate per million years for the analyzed genes [0.69 %/million years (average of 1.25 %/million years for mt*NADH5* gene and 0.13 %/million years for mt*12S rRNA* gene, specific values for Cervidae, [91,92], respectively)] and t is the time in generations that have elapsed since the demographic change began [90]. Females can become sexually mature at about 6 months of age if they can reach a body mass of 30–40 kg. In Pennsylvania, 0–50% of fawns sampled across 22 management units were pregnant [93]. Most (>90%) females ≥1.5 years old become pregnant [93]. During a 13-year study on Anticosti Island, the conception rate in harvested females was 86% (n = 1089) [94]. Males 6 months of age can breed but are unlikely to be competitive for mates. Older males are more likely to breed, but 1- and 2-year-old males may collectively sire one-third or more of offspring [95]. Furthermore, multiple paternity in litters is common where Sorin [96] reported 22% of twins being sired by different males. For all these reasons, we consider that one generation would be around one year old.

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 [73,97]. 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). 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. Bayes factors were computed as twice the difference between the log of the marginal likelihoods (2[Log ML(BSP)–Log ML(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 [98]. The same occurs with small changes in the mutation rate (μ), which can greatly affect the effective number and, in turn, estimated divergence time [99].

Spatial Genetic Structure in the White-Tailed Deer

Different spatial analyses were applied to the genetic dataset analyzed in the white-tailed deer. The first spatial analysis was a Mantel's test. It was used to detect possible overall relationships between a genetic matrix among the individuals of white-tailed deer sampled in all the area studied (Kimura 2P genetic distance [100]) and the geographic distance matrix among the individuals. In this study, Mantel's test was normalized according to Smouse, et al. [101]. This procedure transforms this statistic into a correlation coefficient. The probability was estimated by means of 10,000 replicates.

A spatial autocorrelation analysis was carried out using the Ay statistic with 10 distance classes (DCs). Ten DCs were chosen because the number of comparison pairs within each DC were very similar and high enough to reach statistical significance. Ay can be interpreted as the average genetic distance between pairs of individuals that fall within a specified DC. Ay takes on a value of 0 when all individuals within a DC are genetically identical and a value of 1 when all individuals within a DC are completely dissimilar. The probability for each DC was obtained using 10,000 randomizations. The 10 DCs were as follows: 0–183 km, 183–350 km, 350–550 km, 550–714, 714–882 km, 882–1,029 km, 1,029-1,269 km, 1,269–1,589 km, 1,589-2,052 km, and 2,052-6,326 km. The real distances among the specimens studied are larger than those used in this analysis because we used geographic coordinates that do not consider the geomorphology of the area studied. These analyses were carried out with AIS v1.0 software [102]. Moreover, we used the genetic landscape interpolation analysis (GLIA), which was performed by means of the "inverse dis tance weighted" method [102], to view spatial genetic structure of the data in a three-dimensional space. Finally, another spatial analysis was carried out with the Monmonier's Algorithm Analysis (MAA) with the same software. This geographical regionalization procedure is used to detect the locations of putative barriers to gene flow by iteratively identifying sets of contiguous, large genetic distances along connectivity networks. A Delaunay triangulation was used to generate the connectivity network among sampling points. A graphical representation of putative "barriers" inferred by the algorithm is superimposed over the connectivity network to detect rapid identification of important geographical features reflected by the genetic dataset. In this case, we used this procedure to detect the 10 most important geographical barriers to the mt*NADH5* and mt*12S rRNA* genes analyzed for

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the white-tailed deer.

Both the results of the BAPS and MAA procedures were georeferenced in ArcGIS Pro 3.2.2 [103] matching the vectors and coordinates in the figures with a point layer including each sample geographic position, respectively. From this, vector layers were generated to visualize spatial genetic analyses overlaid on physical geography (https://pro.arcgis.com/es/pro-app/latest/get-started/download-arcgis-pro.htm)

Results

Phylogenetic Analysis of *O. virginianus*

The ML tree (Figure 1) showed the strong phylogenetic relationship of *Odocoileus*, especially with red brocket deer, as has been found in several previous studies. With the genes used, *M. pandora* did not behave as the sister group of *Odocoileus* (*O. pandora*) as reported by other authors [23,104]. Nor was a strong relationship found between *O. virginianus* and *M. pandora* with the mt*Cyt-b* gene in Ruiz-García, et al. [17]. In fact, in both analyses, *M. pandora* was the red brocket deer taxon most distantly related to *Odocoileus*, excluding *M. rufina*, which was more strongly associated with gray brocket deer (Blastocerina), as also observed with the mitogenomic data in Ruiz-García, et al. [17]. In this phylogenetic tree, the ancestor of a specimen from Guatemala (Livingston) was the first to diverge within the *O. virginianus* clade (Bootstrap: 72%), which could be related to the geographic origin of this species. Several analyses using the *mtCyt-b* gene and mitogenomes (shown and not shown in Ruiz-García, et al. [17]) also identified this specimen as the most divergent within *O. virginianus*. The next group to diverge consisted of three specimens from two different origins (96%), two from the Colombian Eastern Llanos (Arauca and Meta departments), and one from the Ecuadorian Pacific coast (Machala National Park, Manabí province). This group was absent from the analysis by Ruiz-García, et al. [17], and we call Group 9. The next ancestor to diverge was the one that gave rise to Group 2, comprised, in this case, of specimens from southern Mexico (Quintana Roo and Yucatán states), Belize, and Guatemala (Petén department) (93%). It was named Group 2a. Next, the ancestors of two groups diverged. One of these groups was made up of Group 1 (91%), which corresponds to specimens from the USA, northern Mexico, a specimen sampled in Palenque (Chiapas state, Mexico), and a specimen of *O. hemionus* introgressed by *O. virginianus*, a group already detected by Ruiz-García, et al. [17]. The second group consisted of two Honduran specimens from Roatán Island and another Guatemalan specimen (Chocón Machacas) (78%). It was named Group 2b because it was a split from Group 2, previously identified by Ruiz-García, et al. [17]. The next ancestor to diverge gave rise to a large grouping that included white-tailed deer specimens from a wide area of northern South America and southern Central America (89%). In the study by Ruiz-García, et al. [17], a significant portion of these specimens comprised Groups 4 (Colombian Central Cordillera) and 5 (Colombian Eastern Cordillera, the Colombian department of Guainía, the Venezuelan state of Mérida, and French Guiana). Nonetheless, in the present study, specimens from both groups appear intermingled, with the addition of new specimens from other geographic areas not analyzed in Ruiz-García, et al. [17]. This grouping was designated Group 4-5. It was composed of two moderately robust subgroups. On the one hand, a subgroup comprised specimens from the Venezuelan coast (Falcón State), Costa Rica (Alajuela province), Panama (Coclé province), Andean specimens from the Colombian Central Cordillera (Caldas and Risaralda departments), the Colombian Eastern Cordillera (Chingaza National Natural Park, Cundinamarca department), and the Orinoco-Amazonian area of the Colombian department of Guainía (74%). The other subgroup consisted exclusively of Andean specimens from the Colombian Central Cordillera (Caldas and Risaralda departments), the Colombian Eastern Cordillera (Cundinamarca and Santander departments), and the Cordillera de la Culata in Mérida State (Venezuela) (59%); these last two specimens formed a small, solid group (88%). The next ancestor to diverge belonged to Group 8 sensu Ruiz-García, et al. [17], which corresponded to the south-central Peruvian Andes (departments of Ancash, Junín, Ayacucho, Huancavelica, Apurímac, Cusco, and Puno), the northern Bolivian Andes (La Paz department), and the south-central coast of Peru (southern Lima and Ica departments) (94%). The next diverging ancestor formed Group 7 sensu Ruiz-García, et al. [17], composed of specimens from the north-central coast of Peru (northern Lima, Libertad, Lambayeque, and Piura departments) and the northern part of the Peruvian Andes (Cajamarca department) (96%). Unlike the findings in Ruiz-García, et al. [17], the two analyzed specimens from Cajamarca clustered robustly with each other (91%), forming the outermost subgroup of Group 7, while, in the cited study, one grouped with Group 7 and the other with Group 8. The last ancestor to diverge according to this procedure was that corresponding to Group 6 sensu Ruiz-García, et al. [17], which originally consisted only of specimens of Ecuadorian origin. With the mitochondrial genes studied in this case, some small subgroups appear in various areas of Ecuador (Pacific coast-Pichincha, 88%; Cotopaxi-Imbabura, 96%; Cotopaxi, 50%; Latacunga-Azuay, 95%), but the most striking subgroup (80%) was composed of two other small subgroups of non-Ecuadorian origin. The first was composed of two specimens from the Colombian and Venezuelan Llanos (Meta department, Colombia, and Apure state, Venezuela) (78%). The second group consisted of two Panamanian specimens from the province of Coclé (78%).

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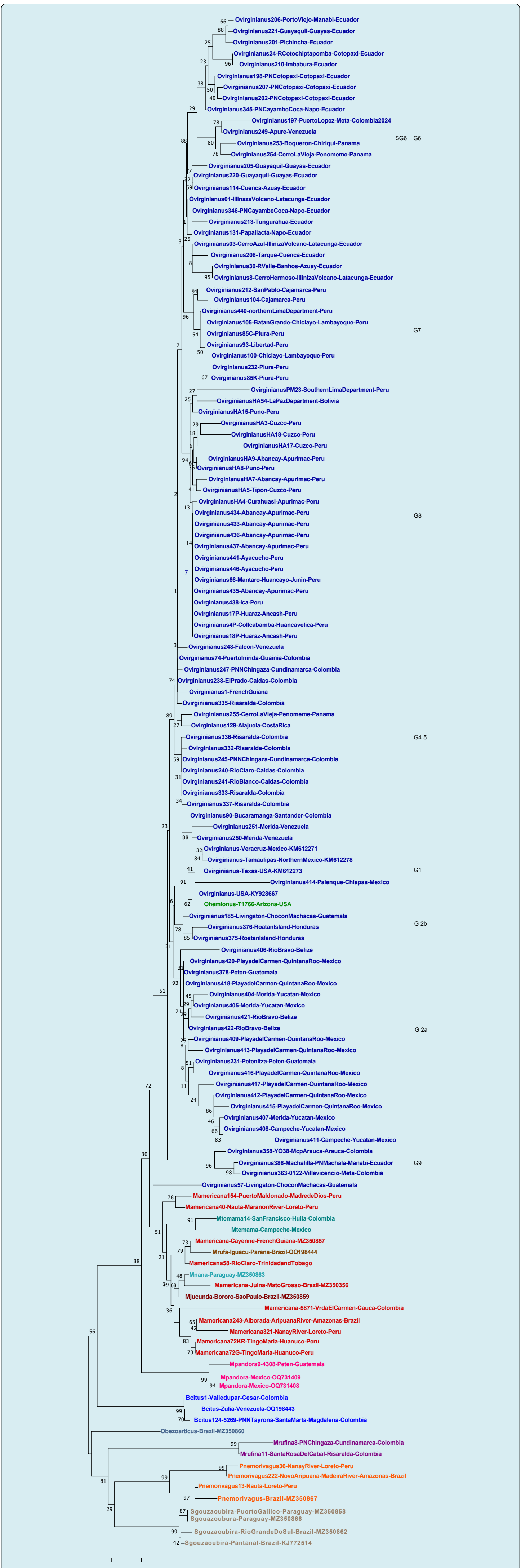


Figure 1: Maximum Likelihood (ML) tree for a total of 105 specimens of different populations of white-tailed deer (*Odocoileus virginianus*) sequenced at the mitochondrial *ND5* and *12S rRNA* genes. Nine genetically distinct groups were detected and are noted in the phylogenetic tree. Different brocket deer species were used outgroup. Specimens with numerical codes, their sequences were obtained in GenBank. In nodes, bootstrap percentages. Different colors represent different species.

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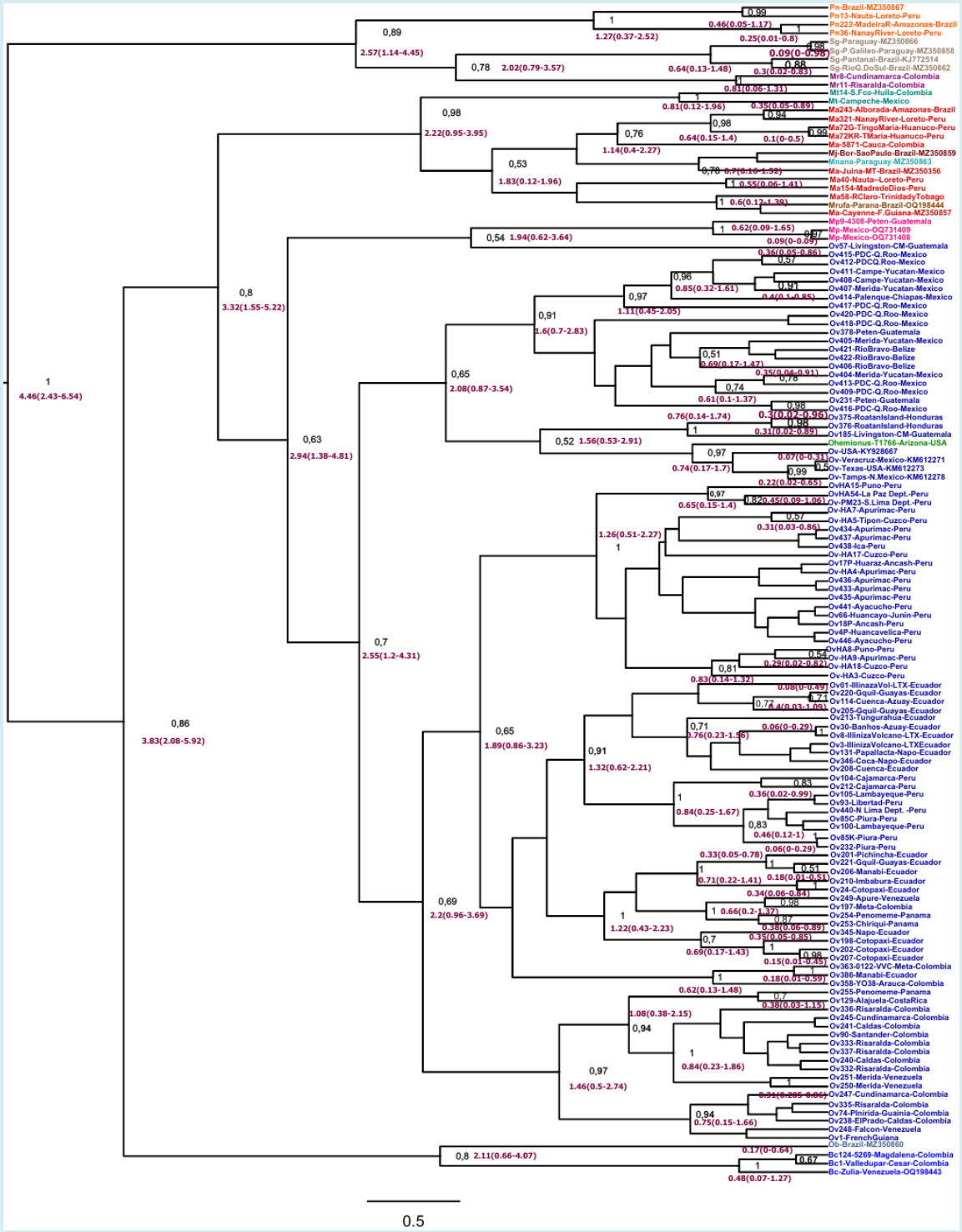


Figure 2: Bayesian Inference (BI) tree for a total of 105 specimens of specimens of different populations of white-tailed deer (*Odocoileus virginianus*) sequenced at the mitochondrial *ND5* and *12S rRNA* genes. Different brocket deer species were used outgroup. For specimens with numerical codes, their 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; Mrufa = Mazama rufa; Mj = Mazama jucunda; Mp = Mazama pandora; Ov = Odocoileus virginianus; Ohemionus = Odocoileus hemionus; Ob = Ozotoceros bezoarticus*; Bc = *Bisbalus citus*; Different colors represent different species.

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The BI tree (Figure 2) differentiated Blastocerina from Odocoileina (posterior probability, *pp* = 1), with one conspicuous exception. *B. citus* and *O. bezoarticus* were associated with Odocoileina, (*pp* = 0.86). The relationship between these two species was relatively strong (*pp* = 0.8), and the existence of *B. citus* was very robust (*pp* = 1). Within Blastocerina, the existence of *P. nemorivagus* was also robust (*pp* = 1), with two outstanding groupings distinguished within it (*pp* = 0.99 and 1, respectively), which probably represent two distinct taxa within *P. nemorivagus*. *S. gouazoubira* also showed very robust differentiation (*pp* = 1) with two well-differentiated subgroups within it (*pp* = 0.98 and 0.88, respectively), warranting a similar comment to the previous one. Interestingly, *M. rufina* was also associated with the Blastocerina (*pp* = 0.78), as found in other studies [17]. With the exceptions mentioned, two different groupings were formed within the Odocoileina (*pp* = 0.8). One of them contains all the red brocket deer of the *M. americana* complex and *M. temama* (*pp* = 0.98), the latter species being differentiated (*pp* = 1) from the other taxa found within the *M. americana* complex (different groups of *M. americana*, *M. jucunda*, *M. nana*, *M. rufa*). Subsequently, a large clade consisting of *M. pandora* and *O. virginianus* was found, although the statistical strength of this association was relatively weak (*pp* = 0.63). It is noteworthy that the *O. virginianus* specimen from Livingston, Guatemala, was associated with *M. pandora*, although the statistical robustness of this association was low (*pp* = 0.54). Nonetheless, the ancestor of this specimen would have been the first to diverge from all the studied *O. virginianus* specimens, coinciding, in this respect, with the ML tree analyzed. Within *O. virginianus*, two large groups were distinguished (*pp* = 0.7). One grouping (*pp* = 0.65) contained the Central American Group 2a (*pp* = 0.91), the Honduran and Guatemalan Group 2b (*pp* = 1), and the USA and northern Mexican Group 1 (*pp* = 0.97). On the other hand, there was a grouping (*pp* = 0.69) that contained the remaining groupings mentioned in the previous trees. Within this clade, the first grouping to diverge was Group 4-5 (*pp* = 0.97), which included two associated Central American specimens (Costa Rica and Panama) (*pp* = 0.7), plus specimens from different locations in Colombia, Venezuela, and French Guiana. A subgroup consisting exclusively of Andean specimens from Colombia (Cundinamarca, Risaralda, and Santander departments) and Venezuela (Mérida state) was particularly noteworthy (*pp* = 1). The two Venezuelan Andean specimens also showed a very significant association within the aforementioned Andean subgroup (*pp* = 1). The next grouping to diverge was Group 8 from the south-central Andes of Peru and northern Bolivia (*pp* = 1), followed in this divergence, although its position in this part of the BI tree is not very strong (*pp* = 0.38), by Group 9, which was a very strong association (*pp* = 1). Finally, the divergence of Groups 6 and 7 was observed, although in a different way compared to what was observed in the previous trees. Group 6 split into two groups. One of them (*pp* = 0.91) contained some of the Ecuadorian specimens and the Peruvian Group 7, this grouping being very statistically robust (*pp* = 1), with the two specimens from the northern Peruvian Andes (Cajamarca department) being the most divergent within it (*pp* = 0.83). The second grouping (*pp* = 1) also included other Ecuadorian specimens, plus the strong grouping (*pp* = 1) made up of two specimens from the Colombian Venezuelan Llanos (*pp* = 0.98) and Panamanian individuals (*pp* = 0.87) already mentioned in the previous trees. Therefore, certain differences between the ML and BI trees were observable.

The NJ network (Figure 3) yielded some interesting results worth highlighting. Like the trees discussed previously, *M. rufina* was associated with *S. gouazoubira* (Blastocerina), and *B. citus* was more closely associated with red brocket deer + *Odocoileus* than with gray brocket deer. Nevertheless, unlike what was observed in those trees, *P. nemorivagus* showed an interesting relationship with certain groups of *O. virginianus*. This NJ network revealed three possible alternatives (hypotheses) for determining the origin of *O. virginianus*. The first, which correlated with the more traditional view, suggested an ancestral relationship between the haplotypes of *M*. (*Odocoileus*) *pandora* and *O. virginianus*. If this were the case, the most ancestral haplotypes of the last species would have been those of Group 1, which would allow us to determine the origin in what is now the USA and north-central Mexico. From this grouping, Groups 2a, 2b, 3, and 4-5 would have formed, the latter being vital for the formation of the remaining groups of *O. virginianus* in South America. A second alternative was that the oldest haplotype was H98, which corresponded to the Livingston (Guatemala) specimen, as already detected in the previous trees. In this case, the origin of *O. virginianus* would be more closely related to that of other red brocket deer different from *M. pandora*. In this case, the next grouping that would have originated was Group 4-5, which was shown to be made up of two subgroups, and from which, on the one hand, Groups 1, 2a, 2b, and 3, typically North and Central American, and on the other hand, the remaining South American groups (Groups 8, 7, 6, and 9) would have originated. A third possibility was that the origin of *O. virginianus* was mediated by some type of genetic introgression with *P. nemorivagus* (see the issue addressed in Ruiz-García, et al. [17]). If this were the case, the first *O. virginianus* association to appear would have been represented by Group 9 (Colombian Llanos and the Ecuadorian Pacific coast), which would have given rise to the Ecuadorian Group 6 (which would include some haplotypes more localized in the Colombian-Venezuelan and Panamanian Llanos) and which, in turn, would have given rise to the Peruvian Groups 7 and 8 and Group 4-5, which would have been decisive in the generation of the Central and North American groups. As can be seen, this analysis offers highly conflicting hypotheses for determining the

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origin of *O. virginianus*. Furthermore, the same nine groups visualized in the phylogenetic trees were also detected with the NJ network. The star-shaped pattern of some of the *O. virginianus* groupings suggests the possibility of population expansion events. This was the case for Groups 6, 7, 8, 4-5, and 2a.

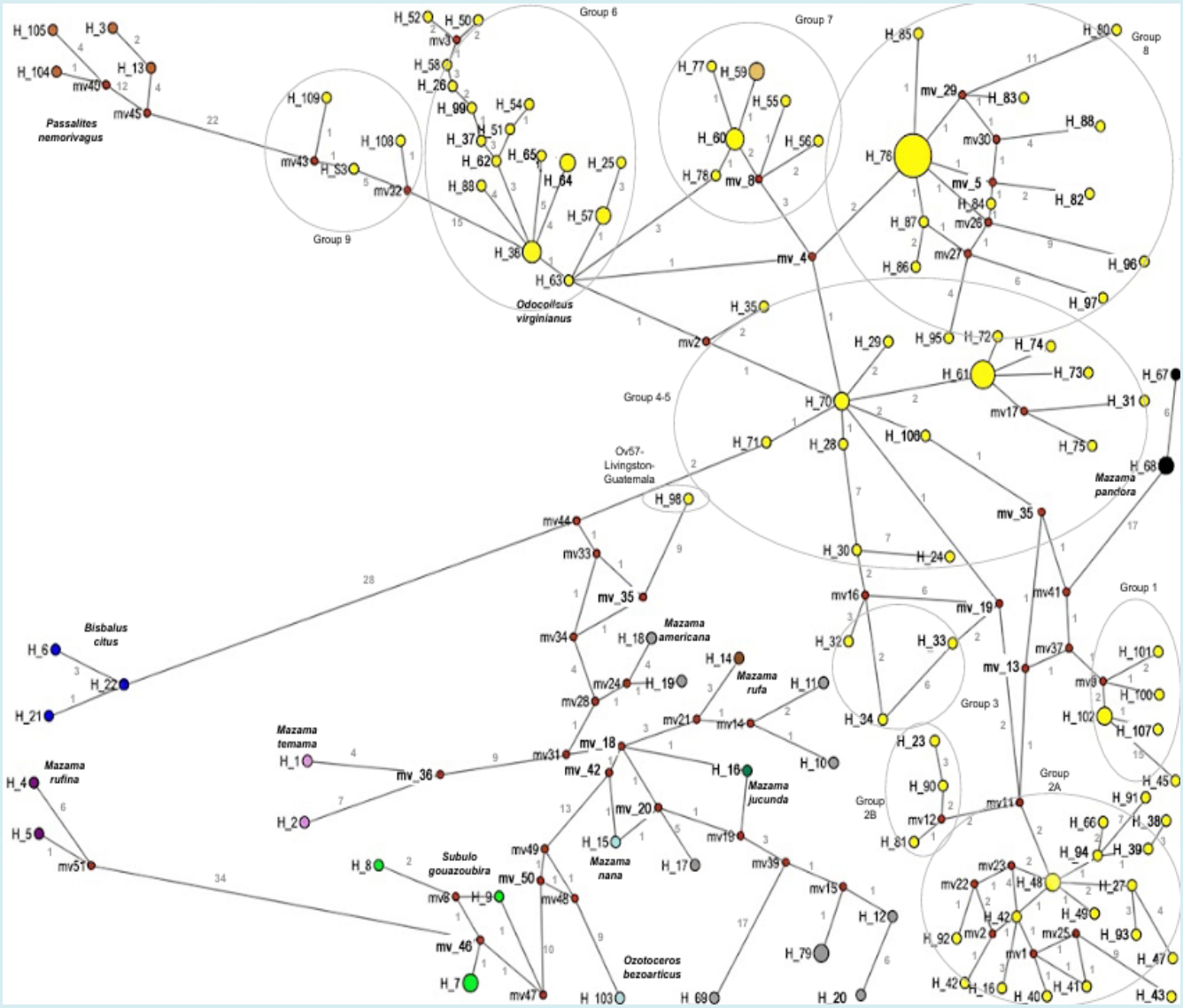


Figure 3: Median Joining Network (MJ network) containing the haplotypes of 105 specimens of different populations of white-tailed deer (*Odocoileus virginianus*) sequenced at the mitochondrial *ND5* and *12S rRNA* genes. Haplotypes of diverse brocket deer species were used to show their relationships with *Odocoileus*. Different species are represented with different colors. The yellow circles are those corresponding to *Odocoileus virginianus*. Red circles indicate undetected or extinct haplotypes. The size of the circles is proportional to the sample sizes of each haplotype. The numbers in the lines are the mutations among the haplotypes including the haplotypes not found or extinct (mv).

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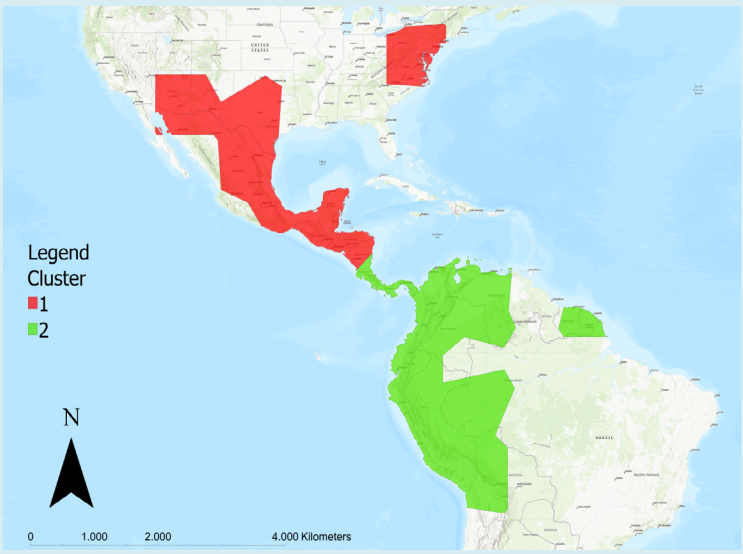


Figure 4: Different populations or groups of white-tailed deer (*Odocoileus virginianus*) were detected by means of BAPS from 105 specimens of white-tailed deer (*Odocoileus virginianus*) at the mitochondrial *ND5* and *12S rRNA* genes. Each different color indicates a different population or taxa. In red, the specimens sampled in USA and northern Mexico (Group 1) and in southern Mexico, Belize and part of Guatemala (Group 2a) and in other part de Guatemala and Honduras (Group 2a). In green, the specimens sampled in Costa Rica and Panama (included in Groups 4-5 and 6), Colombia, Venezuela, French Guiana (Groups 4-5, 6 and 9), Ecuador (Groups 6 and 9), and in Peru (Groups 7 and 8).

The BAPS analysis (Figure 4) showed the existence of two groups as the most optimal result [log (marginal likelihood) of optimal partition = -2,375.01; p = 1.00]. The two groups were easily identifiable geographically. One group comprised all specimens from North America and northern Central America, while the other included all specimens from southern Central America (Costa Rica and Panama) and all South America. Therefore, this analysis detected two large, geographically relevant groupings, but did not detect more localized groupings as was the case with the phylogenetic trees.

Divergence Times for Different Groups of *O. virginianus*

Two analytical methods were used to estimate divergence times for the origin of *O. virginianus* and for the haplotype diversification of the various groups found. The first method used BI. The separation between the ancestors of Blastocerina and Odocoileina was estimated at 4.46 mya (95% HPD = 2.43–6.54 mya). The divergence between the ancestors of red brocket deer (excluding *M. rufina*) and *Odocoileus* was estimated at 3.32 mya (1.55-5.22 mya). These estimates all placed the divergence during the Pliocene. The following estimates all placed it during the Pleistocene. The temporal separation between the ancestors of *M. temama* and those of the *M. americana* complex was estimated at around 2.22 mya (0.95-3.95 mya), with the estimated haplotype diversification within *M. temama* at approximately 0.81 mya (0.12-1.96 mya) and the onset of this diversification in the *M. americana* complex at 1.83 mya (0.72-3.2 mya). The divergence between the ancestors of *M. pandora* and *O. virginianus* was estimated at 2.94 mya (1.38-4.81 mya). In this analysis, the *O. virginianus* specimen from Livingston (Guatemala) was grouped with *M. pandora*, with an estimated time divergence between the two taxa of 1.94 mya (0.62–3.64 mya). The initial haplotype diversification in *O. virginianus* (excluding the specimen discussed) was established 2.55 mya (1.2–4.31 mya). The initial time divergence of Group 2a from southern Mexico, Belize, and part of Guatemala was estimated at 1.6 mya (0.7–2.83 mya). Group 2b diverged from the North American Group 1, in this analysis, at around 1.56 mya (0.53-2.91 mya), and the haplotype diversification within Groups 2b and 1 were 0.76 mya (0.14-1.74 mya) and 0.74 mya (0.17-1.7 mya), respectively. The remaining groups of *O. virginianus* began their haplotype differentiation at around 2.2 mya (0.96-3.69 mya). The beginning of diversification within Group 4-5 was established 1.46 mya (0.5-2.74 mya), and the two subgroups found within it began their haplotype diversifications at 1.08 mya (0.38-2.15 mya) and 0.75 mya (0.15-1.66 mya), respectively. The original separation of Group 9 from the remaining *O. virginianus* groupings was estimated at 1.72 mya (0.76-2.96 mya), with diversification within it beginning 0.62 mya (0.13-1.48 mya). The Peruvian Group 8 began its diversification process 1.26 mya (0.51–2.27 mya). An Ecuadorian subgroup of Group 6 began its haplotype divergence around 1.06 mya (0.4–2.0 mya), and the related Peruvian Group 7 began its diversification process 0.84 mya (0.25–1.67 mya). The other Ecuadorian subgroup of Group

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6 began its haplotype proliferation 1.22 mya (0.43–2.23 mya). Within this group, the subgroup formed by specimens from the Colombian Venezuelan Llanos and by Panamanian specimens would have begun its divergence around 0.66 mya (0.2-1.37 mya).

Using the MJ network, different estimates of temporal separation between the different taxa and groups of deer analyzed were also obtained. Some of the most interesting estimates obtained, which could be compared to those obtained with BI, were the following. The estimated temporal separation between *M. pandora* and Group 1 of *O. virginianus* was 2.631 ± 0.169 mya. The divergence within Group 4-5 was established 0.771 ± 0.214 mya, while the differentiation of the initial haplotypes of this group (found in Colombia) from the derived haplotypes found in Panama and Costa Rica was established at 0.6 ± 0.207 mya and 0.239 ± 0.113 mya, respectively. The second subgroup found within Group 4-5 began its differentiation 0.287 ± 0.095 mya ago. The Ecuadorian Group 6 began its haplotype proliferation around 1.244 ± 0.264 mya, while mitochondrial diversification began at similar times in Group 9 (1.036 ± 0.374 mya), Group 3 (1.275 ± 0.406 mya), Group 1 (0.957 ± 0.210 mya), and Group 2a (1.182 ± 0.209 mya). With this procedure, diversification within the Peruvian Groups 7 and 8 appears lower than with BI (Group 7: 0.292 ± 0.110 mya; Group 8: 0.561 ± 0.089 mya). Both methods detected the initial diversification of *O. virginianus* and the respective geographic groupings within the Pleistocene.

Genetic Heterogeneity Found among Different Groups of *O. virginianus*

Considering the nine groups found within *O. virginianus*, the existence of overall genetic heterogeneity among these groups (Table 3) and among all pairs of them (Table 4a) was analyzed. The internal subgroup of Group 6, which consisted of non-Ecuadorian specimens (Colombian Venezuelan Llanos and Panama), was differentiated in this analysis. Overall, the genetic heterogeneity found was highly significant for all the statistics analyzed, and the estimates of theoretical gene flow among these nine groups were very low, less than 1, which is considered a critical value in an infinite island model. For pairs of groups, the highest values corresponded to Group 7 vs. Group 9, Group 8 vs. Group 9, Group 2b vs. Group 9 (F_{ST} = 0.763, $p < 0.00001$), G6 in-group vs. Group 9, and Group 2b vs. Group 7. As can be seen, Group 9 is involved in many of the comparisons that showed maximum genetic differentiation. The lowest values detected (although all were significant) involved Group 1 vs. Group 2a, Group 2a vs. Group 4-5, Group 2a vs. Group 2b, Group 6 vs. Group 4-5, Group 2a vs. Group 6, and Group 6 vs. Group 8. It can also be seen that Group 2a and Group 6 are those that present the least genetic heterogeneity with respect to the other groups analyzed. If we analyze the Da values (Table 4b), the most differentiated pairs of groups were Inner Group of G6 vs. Group 9 (Da = 3.22%), Group 2b vs. Group 9 (Da = 2.48%), Group 7 vs. Group 9 (Da = 2.40%), Group 8 vs. Group 9 (Da = 2.40%), Group 2a vs. Group 9 (Da = 2.30%), Group 1 vs. Group 9 (Da = 2.30%), Group 6 vs. Group 9 (Da = 2.22%), and Group 4-5 vs. Group 9 (Da = 2.13%). Therefore, the greater genetic differentiation of Group 9 compared to the other *O. virginianus* groups detected in this analysis was confirmed. The lowest Da values were those corresponding to Group 4-5 vs. Group 8 (Da = 0.60%), Group 6 vs. Group 4-5 (Da = 0.65%), Group 2b vs. Group 4-5 (Da = 0.73%), Group 6 vs. Group 8 (Da = 0.74%), and Group 2a vs. Group 4-5 (Da = 0.78%). It is also observable how some groups consistently exhibit very small genetic distances, even though their genetic heterogeneities may eventually be significant. These are the cases of Groups 6, 4-5, and 2a.

Genetic heterogeneity and gene flow statistics	Values	Probabilities
X^2	828.625 *df* = 624	0.00001**
H_{ST}	0.082	0.00001**
K_{ST}	0.486	0.00001**
K_{ST*}	0.311	0.00001**
Z_S	1198.537	0.00001**
Z_{S*}	6.348	0.00001**
S_{nn}	0.973	0.00001**
γ_{ST}	0.523	0.001**
F_{ST}	0.606	0.001**
$Nm_{(\gamma ST)}$	0.46	
$Nm_{(FST)}$	0.33	

Table 3: Overall genetic heterogeneity among the nine groups of white-tailed deer (*Odocoileus virginianus*) found in this analysis with the mitochondrial *ND5* and *12S rRNA* genes. Nm = gene flow; df = degree of freedom; ** $p < 0.01$ (highly significant).

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Groups	1	2	3	4	5	6	7	8	9
1	-	0.88	0.93	0.9	1.6	1.77	1.86	1.53	2.31
2	0.358* (0.184)*	-	0.81	0.78	1.29	1.39	1.54	1.2	2.3
3	0.490** (0.373)*	0.389* (0.119)	-	0.73	1.35	1.43	1.59	1.27	2.47
4	0.490** (0.357)*	0.384* (0.257)*	0.536** (0.276)*	-	0.65	1.06	0.87	0.59	2.13
5	0.574** (0.369)*	0.463** (0.324)*	0.604** (0.258)*	0.430** (0.295)*	-	1.08	0.8	0.74	2.22
6	0.596** (0.498)**	0.480** (0.211)*	0.615** (0.551)**	0.549** (0.368)*	0.492** (0.255)*	-	1.87	1.53	3.22
7	0.692** (0.604)**	0.576** (0.339)*	0.754** (0.641)**	0.641** (0.452)**	0.518** (0.304)*	0.713** (0.637)**	-	0.94	2.36
8	0.619** (0.450)**	0.490** (0.352)*	0.667** (0.345)*	0.496** (0.351)*	0.464** (0.327)*	0.639** (0.414)**	0.656** (0.444)**	-	2.4
9	0.681** (0.557)**	0.624** (0.257)*	0.763** (0.727)**	0.742** (0.496)**	0.691** (0.354)*	0.762** (0.700)**	0.789** (0.704)**	0.764** (0.485)**	-

Table 4: Genetic heterogeneity statistics [F_{ST}, and γ_{ST} (into parenthesis)] for nine group pairs found of white-tailed deer (*Odocoileus virginianus*) at the mitochondrial *ND5* and *12S rRNA* genes below the diagonal; (b) Absolute distances, Da, (in %) among all the nine group pairs found of white-tailed deer above the diagonal. 1 = Group 1; 2 = Group 2a; 3 = Group 2b; 4 = Group 4-5; 5 = Group 6; 6 = internal subgrouping 6; 7 = Group 7; 8 = Group 8; 9 = Group 9. * $p < 0.01$; ** $p < 0.05$.

Genetic Diversity and Possible Demographic Changes in Different Groups of *O. virginianus*

Table 5 shows the values of various genetic diversity statistics obtained in the different groups of *O. virginianus* detected. Globally, for this species, both H_d and π were high (H_d = 0.984 ± 0.007; π = 1.89%), which is consistent with the existence of different groups that exhibit a certain degree of genetic differentiation among them. All nine groups detected had high H_d values, with the Peruvian Groups 7 and 8 showing slightly lower values (H_d = 0.889 ± 0.091 and H_d = 0.714 ± 0.108). The groups with the highest π values were Group 2a (1.89%), Group 1 (1.28%), and Groups 6 and the subgroup within Group 6 (both with 1.11% and 1.13%, respectively). The group with the least genetic diversity was the Peruvian Group 7 (0.38%).

Species	N	NH	Hd	π	θ per sequence
Overall sample of *O. virginianus*	105	80	0.984 ± 0.007	0.0186 ± 0.0012	26.978 ± 6.781
Group 1	6	5	0.933 ± 0.122	0.0128 ± 0.0054	9.635 ± 4.862
Group 2a	17	17	1.000 ± 0.020	0.0152 ± 0.0023	12.719 ± 4.796
Group 2b	3	3	1.000 ± 0.272	0.0113 ± 0.0050	4.000 ± 2.726
Group 4-5	18	13	0.928 ± 0.052	0.0060 ± 0.0011	6.396 ± 2.546
Group 6	20	16	0.974 ± 0.025	0.0111 ± 0.0016	6.483 ± 2.519
Subgrouping within Group 6	4	4	1.000 ± 0.177	0.0113 ± 0.0029	7.091 ± 4.153
Group 7	9	6	0.889 ± 0.091	0.0038 ± 0.0010	2.943 ± 1.543
Group 8	25	13	0.740 ± 0.097	0.0085± 0.0023	10.858 ± 3.809
Group 9	3	3	1.000 ± 0.272	0.0033 ± 0.0014	8.000 ± 6.000

Table 5: Genetic diversity for the overall sample of white-tailed deer (*Ododoileus virginianus*) and for the different groupings found in this study (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).

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Regarding possible demographic changes (Table 6 & Figure 5), the total sample analyzed showed significant signs of population expansion for all six statistical measures used (including mismatch distribution). Group 2 also showed signs of population expansion (five significant tests, including the mismatch distribution test), as did Group 4-5, which showed significant evidence of population expansion for all six tests performed. Groups 6 (two significant tests, including the mismatch distribution test), 7 (two significant tests), and 8 (four significant tests) also showed evidence of potentially significant population expansions throughout their natural history. For the groups that presented significant values of *rg* associated with the mismatch distribution test, the beginnings of these population expansions were as follows. In the case of the total sample (τ = 6.595), the beginning of the population expansion was estimated at 789,000 years ago, while in the cases of Group 2 (τ = 5.147), Group 6 (τ = 4.964), and Group 4-5 (τ = 2.347), the estimated beginnings of the population expansion were 615,500 years, 593,600 years, and 280,650 years, respectively.

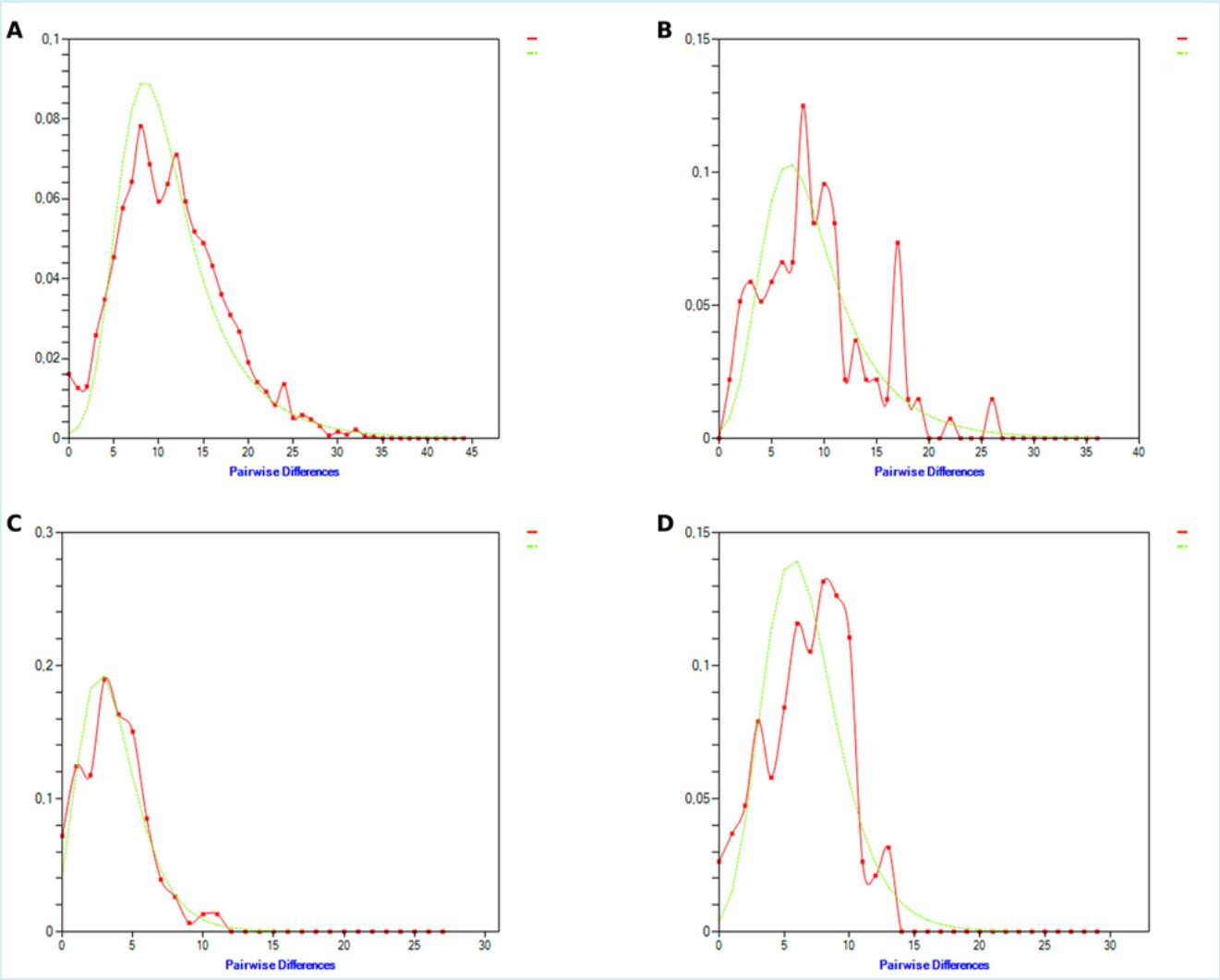


Figure 5: Analysis of significant mismatch distributions (pairwise sequence differences) at the mitochondrial *ND5* and *12S rRNA* genes: (A) *Odocoileus virginianus* (Overall sample); (B) *Odocoileus virginianus* (Group 2a: southern Mexico, Belize, and part of Guatemala); (C) *Odocoileus virginianus* (Group 4-5: Costa Rica, part of Panama, Colombia, Venezuela and French Guiana); (D) *Odocoileus virginianus* (Group 6: Ecuador). All of these significant cases were related to a significant female population expansion.

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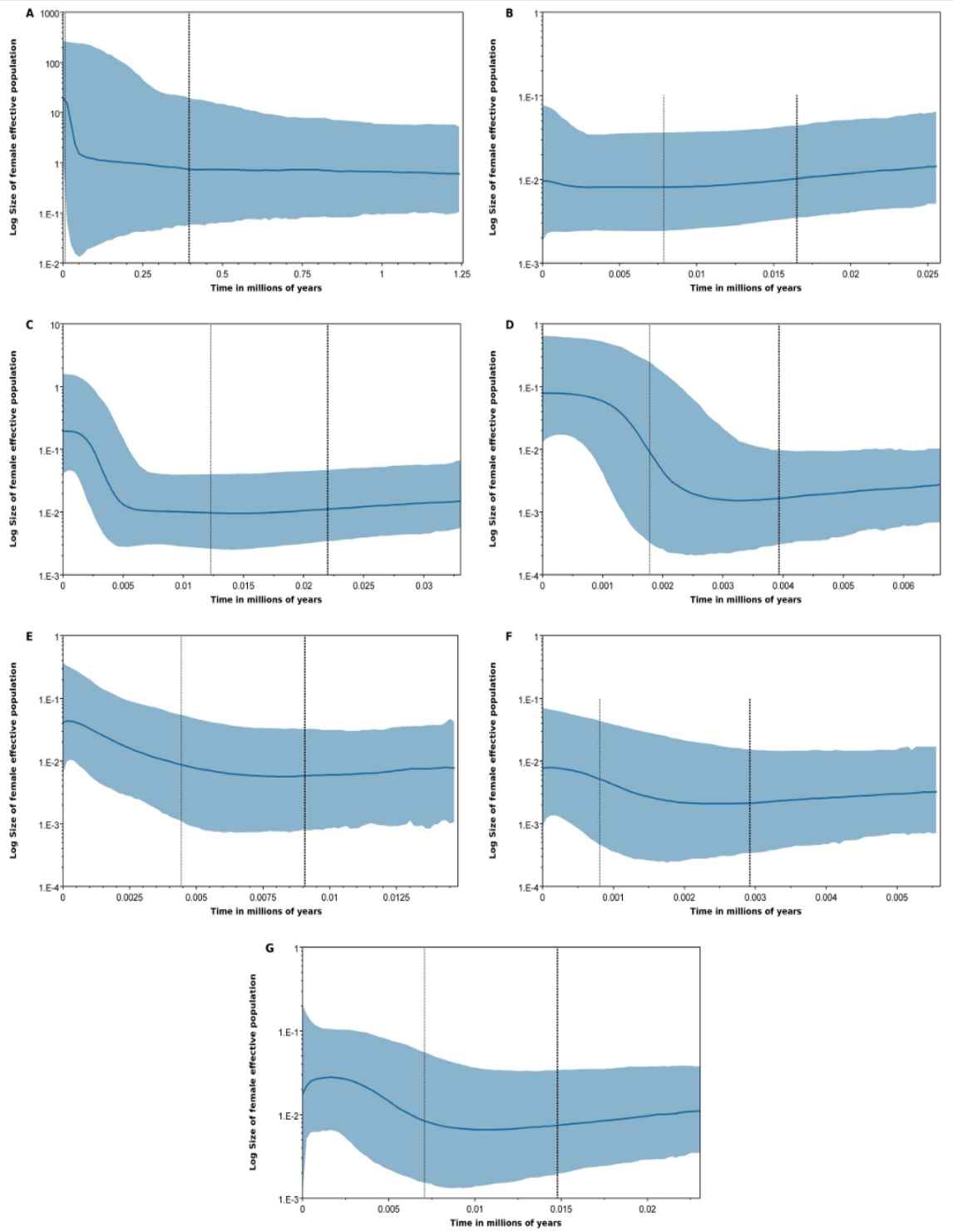


Figure 6: Bayesian skyline plot (BSP) analyses to determine the possible demographic changes across the natural history of different groups of white-tailed deer (*Odocoileus virginianus*) using the mitochondrial *ND5* and *12S rRNA* genes. (A) Overall sample of *O. virginianus*; (B) Group 1 (USA and northern Mexico); (C) Group 2 (joined 2a and 2b) (southern Mexico, Belize, Guatemala and Honduras); (D) Group 4-5 (Costa Rica, part of Panama, Colombia, Venezuela, French Guiana); (E) Group 6 (Ecuador); (F) Group 7 (central-northern coast and northern Andes of Peru); (G) Group 8 (central-southern Andes and southern coast of Peru and northern of Bolivia).

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	Tajima's D	Fu's Fs	Fu & Li's D*	Fu & Li's F*	R2	Raggedness (rg)
Overall sample of O. virginianus	-2.075 *p* = 0.0014*	-72.069 *p* = 0.0001*	-3.068 *p* = 0.0099*	-2.953 *p* = 0.0092*	0.953 *p* = 0.0049*	0.0016 *p* = 0.00001*
Group 1	-1.238 p = 0.105	0.391 p = 0.485	-1.242 p = 0.111	-1.246 p = 0.141	0.261 p = 0.859	0.12 p = 0.291
Group 2a	-1.35 p = 0.074	-9.897 p = 0.0010*	-1.779 p = 0.041*	- 1.73 p = 0.043*	0.093 p = 0.047*	0.019 p = 0.045*
Group 4-5	-1.681 p = 0.034*	-6.395 p = 0.0013*	-2.166 p = 0.033*	-2.148 p = 0.043*	0.06 p = 0.0001*	0.016 p = 0.049*
Group 6	-0.037 p = 0.545	-6.269 p = 0.0058*	-0.361 p = 0.321	-0.229 p = 0.378	0.132 p = 0.537	0.013 p = 0.011*
Group 7	-1.026 p = 0.194	-1.838 p = 0.047*	-0.731 p = 0.250	-0.817 p = 0.261	0.112 p = 0.0092*	0.114 p = 0.279
Group 8	-2.041 p = 0.0063*	-2.316 p = 0.162	-1.893 p = 0.042*	-2.085 p = 0.042*	0.063 p = 0.0049*	0.064 p = 0.551

Table 6: Demographic change statistics applied to the mitochondrial *ND5* and *12S rRNA* genes for the overall sample studied of white-tailed deer (*Odocoileus virginianus*) and for the different groups found. *p* = probability; * Significant.

The BSP procedure (Figure 6) was applied to determine possible demographic changes over the last tens of thousands of years in the *O. virginianus* populations where this analysis could be applied. For the overall *O. virginianus* population, the last million years were estimated. In this latter case, strong population growth was observed in the last 10,000 years (Bayesian Factor = 11.34) (Figure 6a). In the case of Group 1, a constant but very slight decrease was observed in the last 25,000 years, with a very slight increase in the last 1,500 years (Figure 6b). Group 2 also showed a slight but steady decline in the size of the female population over the last 35,000 years, but with significant population growth (Bayesian Factor = 12.89) that began 5,000 years ago and stabilized around 2,000 years ago (Figure 6c). Group 4-5 appeared to have undergone a similar demographic process. From 7,000 to 3,000 years ago, this population experienced a slight but steady decline. However, around 3,000 years ago, there was a significant increase in the population size of the female lineages (Bayesian Factor = 10.24), which stabilized around 1,000 years ago (Figure 6d). Group 6, analyzed over the last 15,000 years, showed a population increase from about 6,500 years ago until about 500 years ago, when population growth stopped. This population increase was not as explosive as in the cases of Groups 2 and 4-5 discussed previously, being much more gradual (Bayesian Factor = 6.1) (Figure 6e). Group 7 showed that over the last 5,500 years it had undergone a slight but constant population decline (like what was observed in most of the previous cases) and, also slightly but gradually, began population growth about 2,000 years ago, continuing to the present (Bayesian Factor = 5.8). The gradual (non-explosive) growth of Group 7 was very similar to that of Group 6 detailed above (Figure 6f). Finally, Group 8 showed a slight but progressive decline in its female population from 25,000 years ago until about 7,500 years ago, when a strong population growth began (Bayesian Factor = 9.69), followed by a decline in the last 500 years (Figure 6g). Therefore, it is observable in most analyses that all the groupings originated from a not very marked but constant population decline and that, during the Holocene, population growth occurred. This growth could have been very slight, as in Group 1, moderate, gradual, but constant, as in Groups 6 and 7, or very explosive, as in Groups 2, 4-5, and 8. In some of these groups, a population decline was observed in recent centuries (cases of Groups 6 and 8).

Spatial Structure in *O. virginianus*

Analyzing the sample of 105 *O. virginianus* specimens, the Mantel test (r = 0.0266; *p* = 0.22) did not detect a significant relationship between the genetic and geographic distances of these specimens. Therefore, the possible presence of events related to isolation by distance was not detected (Figure 7a). Similarly, the spatial autocorrelation analysis (Figure 7b) presented a correlogram that was not significant overall (V = 0.0258; *p* = 0.652), nor were any of its DCs. The correlogram profile showed the existence of different genetic patches from 0 to 2,000 km away and a slight decrease in genetic similarity from 2,000 km to 6,300 km away, but without significant spatial autocorrelation values for each DCs. GLIA analysis (Figure 7c) confirmed the existence of genetic patches, but without a clinal spatial structure or isolation by distance. Monmonier's algorithm (Figure 7d) showed 10 geographic areas with potential genetic barriers. The first barrier (dark blue) was detected in the Apurimac department in the south-central Peruvian Andes, within the territory occupied by G8. The second geographic barrier (green) extended over a vast

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area. It stretched from Washington state (USA) to French Guiana, encompassing Central America, parts of Colombia and Ecuador, and reaching as far north as Peru. Different genetic clusters of *O. virginianus* were found in this extensive geographic area. The third geographic barrier (blue green) extended from the southern Pacific coast of Ecuador and encompassed the north-central coast of Peru, corresponding to the location of Group 7. Barrier 4 (brown) covered the geographic area from the department of Cusco, through the department of Puno, to the department of La Paz in Bolivia. Specimens of Group 8 are found in this area. Barrier 5 (purple) extended from the department of Ancash, through the department of Ayacucho, to the southern coast of Peru (department of Ica). This area also included specimens of Group 8. Barrier 6 (brownish green) encompassed the geographic area from central Colombia to central Panama, coinciding with part of Group 4-5. Barrier 7 (black) corresponded to a small geographical area in the interior of the Cuzco department that included specimens from Group 8. Barrier 8 (dark gray) was in the Cajamarca department in the northern Peruvian Andes and coincided with the specimens from that area that were in Group 7. Barrier 9 (bright blue) was restricted to the heart of the Ecuadorian Andes, going from the province of Pichincha to the province of Tungurahua. This geographic area coincides with part of the distribution of Group 6. Finally, barrier 10 (bright green) essentially defined the eastern Colombian Andes Mountain range up to the Colombian eastern plains, including part of Group 4-5 and part of Group 9.

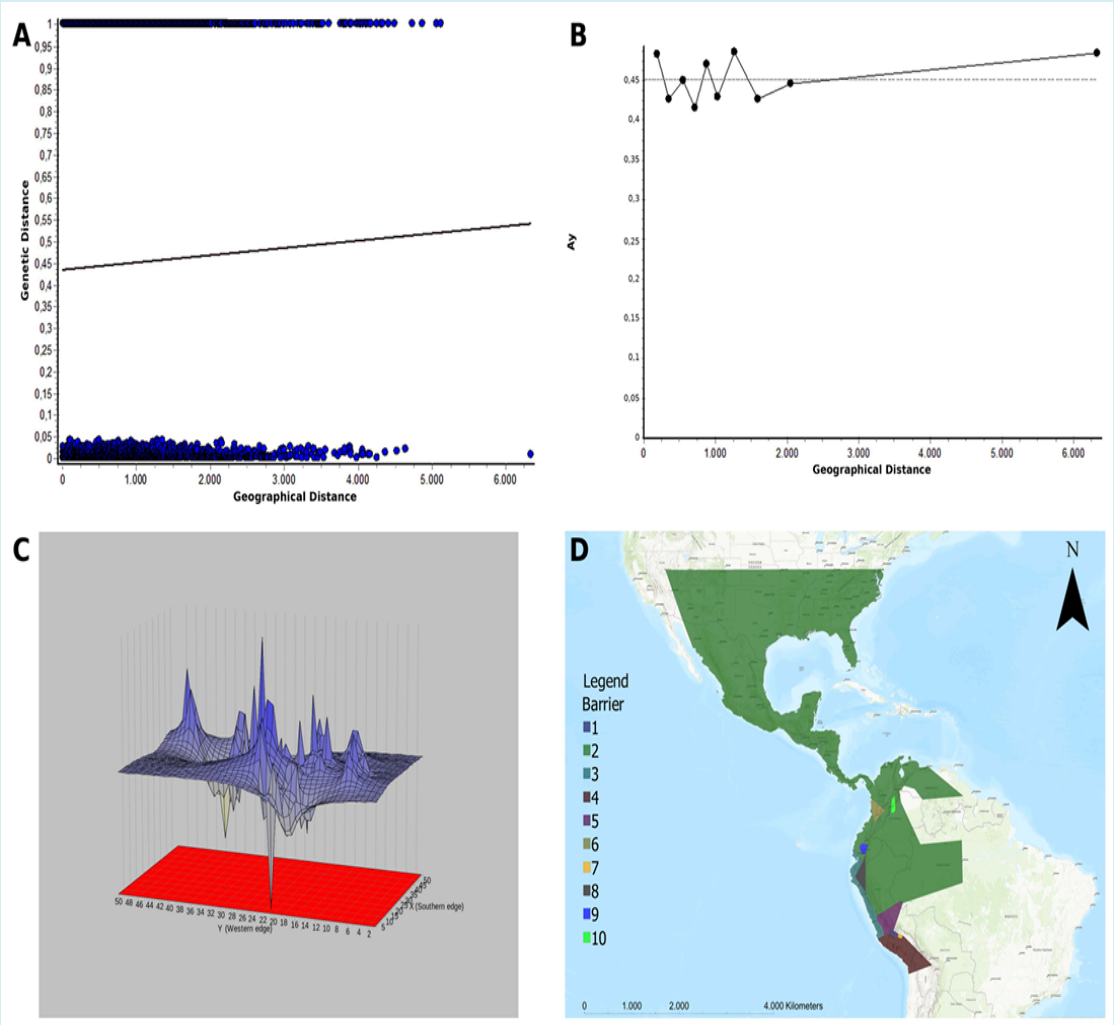


Figure 7: Analyses of the spatial structure of 105 specimens of white-tailed deer (*Odocoileus virginianus*) at the mitochondrial *ND5* and *12S rRNA* genes: (A) Mantel's test (B) Spatial autocorrelation analysis with ten distance classes (DCs); (C) Genetic landscape interpolation analysis (GLIA) across the area studied in North, Central and South America; (D) Monmonier's algorithm analysis (MAA) with ten detected geographical barriers; the map was created using the ArcGIS Pro 3.2.2 software (https://pro.arcgis.com/es/pro-app/latest/get-started/download-arcgis-pro.htm).

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It is worth noting that this procedure determined the existence of possible geographic subgroups within G8 (south-central Peruvian Andes, plus a sector of the south-central Peruvian coast; barriers 1, 4, 5, and 7), within G7 (northern-central Peruvian Pacific coast; barriers 3 and 8), and within G4-5 (Costa Rica, Panama, Colombia, Venezuela, and French Guiana; barriers 6 and 10). Nonetheless, barrier 2 did not differentiate genetic groupings within it despite its enormous geographic extent (from the USA to French Guiana).

Discussion

In this study, we expanded the sample size analyzed in Ruiz-García, et al. [17], especially for samples of Neotropical origin. Samples from Costa Rica and Bolivia were added, along with new samples from new geographic areas in Panama, Colombia, Venezuela, and Ecuador. Overall, the eight genetically distinct groups identified in Ruiz-García, et al. [17] were detected, but with some modifications. A new group (Group 9) appeared, and some geographic subgroups emerged that had not previously been identified within the larger groups.

As Ambriz-Morales, et al. [34] stated, the analysis of whole mitochondrial genomes increases the resolution of a high level of polytomy and decreases the number of branches with low support [105]. Notwithstanding, in many cases, the number of specimens from which complete mitogenome can be obtained is limited by the quality and quantity of DNA available from the samples and by financial investment. Therefore, it is extremely important to be able to use samples from many specimens that represent most of the geographic distribution of a species, with small fragments of sequences from various mitochondrial genes that possess a phylogenetic signal like that of complete mitogenomes, and with a much lower financial investment. This study shows how small fragments of various mitochondrial genes, obtained from very small biological samples that offer low-quality DNA in small quantities, provide results very similar to sequences obtained even from complete mitogenomes. Nevertheless, as discussed in Ruiz-García et al. [17], to confirm the taxonomic and systematic validity of these groups, it is necessary to analyze specimens from these mitochondrial groupings using nuclear gene sequences. Even more importantly, karyotypic studies should be carried out on specimens of each of these mitochondrial groupings to determine whether there are significant chromosomal differences between them and, therefore, whether they could constitute new species or, conversely, whether the absence of significant chromosomal differences would denote the absence of reproductive isolation barriers and, with the application of the Biological Species Concept (BSC) [106-108], all these molecularly differentiated *O. virginianus* groups would form a reproductive continuum and, therefore, a single species.

New Insights into the Phylogeny and Systematics of Odocoileina

Some authors have determined (using the *mtCyt-b* gene and mitogenomes) that *M. pandora* is the sister group to *Odocoileus* [23,104] and, therefore, that this brocket deer could be a new species of the genus *Odocoileus*, *O. pandora*. Nonetheless, Ruiz-García, et al. [17] did not obtain evidence of this association using the mt*Cyt-b* gene. In the present study, using the mt*NADH5* and mt*12S rRNA* genes and the ML tree, no evidence of an association between *M. pandora* and *O. virginianus* was obtained either. Nevertheless, the BI tree did detect an association between *M. pandora* and *O. virginianus* (especially with an *O. virginianus* specimen from Livingston, Guatemala) which, in several analyses, behaved as the specimen of this species most closely related to the origin of the initial mitochondrial diversification of *O. virginianus*. Notwithstanding, this association was statistically weak. The NJ network showed three possible origins of *O. virginianus* in relation to brocket deer. In one of them, *M. pandora* would be associated with *O. virginianus* via specimens of this species from Group 1 (North America). That is, if the original ancestor of the current *O. virginianus* occurred in North America, then *M. pandora* would be the sister group to *Odocoileus* and could be called *O. pandora*. The temporal separation between the ancestor of *Odocoileus* and *M. pandora* was estimated to have occurred at the end of the Pliocene, beginning of the Pleistocene (2.9-2.6 mya). Therefore, it is a priority to sequence nuclear genomes and perform karyotypic analyses to determine whether the use of *O. pandora* is sustainable.

In the present study, *M. rufina* behaved as Blastocerina in both phylogenetic trees and the NJ network. The same occurred when analyzing mitogenomic data that included *M. rufina* with Blastocerina [17]. Other analyses with individual mitochondrial genes placed it as the outermost taxon of the Odocoileina, justifying its status as a distinct genus from *Mazama* (*Andinocervus*) [109]. Conversely, *B. citus*, considered a Blastocerina, was associated with the Odocoileina as the outermost group in all three analyses (ML, BI trees, and MJ network). Therefore, it is important to conduct studies with nuclear genomes to accurately determine the correct taxonomic position of *M. rufina* and *B. citus* within Blastocerina or Odocoileina.

New Insights into the Origin of *O. virginianus*

As had already been shown [17], using the mt*Cyt-b* gene and whole mitogenomes, also with mt*NADH5* and mt*12S rRNA* genes, the presence of a specimen of *O. hemionus* within Group 1 of *O. virginianus* was observed. Particularly,

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old introgression and/or recent hybridization seem to be the likely explanations because natural hybridization and interbreeding between both species of *Odocoileus* have been documented [13,31]. Hybrids occur where the range of the two species overlaps, although hybrid ancestry is not always obvious. It has been reported that up to 24% of animals in local populations had detectable hybrid ancestry, and an average of about 5.6% on a regional basis [110]. The consequence of this is that the *hemionus* group detected by Gutiérrez, et al. [23] represents *O. hemionus* specimens that have introgressed with mitochondrial DNA specific to the North American *O. virginianus* and they quickly became embedded in this group. Ruiz-García, et al. [17] have dated the mitochondrial introgression of *O. virginianus* in *O. hemionus* to 0.59 mya (0.38–0.84 mya). This historical genetic introgression may also be accompanied by recent hybridization events. There are different studies that show hybridization in captivity [111-113], and it has also been found in natural populations of British Columbia [114], Alberta [115,116], the Southwestern USA [113], the Pacific Northwest [117], and Texas [118].

Ruiz-García, et al. [17] estimated the onset of mitochondrial diversification in *O. virginianus* at around 2.2 mya. In the current study, this timeframe was estimated to be around 2.5 mya. In both cases, this was at the beginning of the Pleistocene.

One of the aspects that could not be definitively clarified in Ruiz-García, et al. [17] was the precise geographic origin of the current *O. virginianus*. In that study, different analyses offered four alternative hypotheses: 1- A Central American origin (Guatemala or Panama); 2- A North American origin (USA and northern-central Mexico); 3- A South American origin in what is now the northern part of the Central Colombian Andes; and 4- A South American origin in the south-central part of the Peruvian Andes. Different analyses yielded different results, although the Central American origin was the most frequently obtained. In the present study, for example, Panama and the south-central part of the Peruvian Andes were ruled out as possible geographic centers of origin for this species.

In this study, three different hypotheses were generated: 1- The first again pointed to a presumed Central American origin. The ML tree identified the specimen from Livingston (Guatemala) as possessing the haplotype closest to that of the ancestor of the current *O. virginianus*. The BI tree also showed this specimen to be even closer to *M. pandora* than to the other *O. virginianus* specimens. The NJ network showed three different alternatives regarding the origin of *O. virginianus*. In one of them, this specimen could also have been involved in the initial differentiation of the *O. virginianus* haplotypes, being more closely related to the red brocket deer of the *M. americana* complex than to *M. pandora*. In the ML tree with mitogenomic data (not shown here), this specimen also appeared as the first to diverge within *O. virginianus*. In the ML tree, if we exclude the Livingston specimen (Guatemala) and the small and enigmatic Group 9, the first major group to diverge corresponds to G2a from southern Mexico, Belize and part of Guatemala. Therefore, different analyses point to the northern part of Central America as a key point in the origin of the current *O. virginianus*. 2- The second hypothesis pointed to North America as the geographic location of the initial diversification of the current *O. virginianus*. The MJ network showed that, if *M. pandora* is the sister taxon of *Odocoileus*, then the North American Group 1 was the first to have diverged within *O. virginianus*. Ruiz-García, et al. [17] also showed the same with an NJ network using mitogenomic data. 3- In this new analysis, a grouping not detected by Ruiz-García, et al. [17] appeared, named Group 9, comprised of two specimens from the Colombian Eastern Llanos and one specimen from the Ecuadorian Pacific coast. This new grouping played a relevant role in some analyses regarding the origin of the current *O. virginianus* or, at least, in the very early diversification of the haplotypes of this species. For example, in the ML tree, it was the next grouping to diverge after the Livingston (Guatemala) specimen. Similarly, one of the options presented by the MJ network indicated that Group 9 might have some kind of genetic relationship with *P. nemorivagus* and could be the origin of the other *O. virginianus* groups found in South, Central, and North America. Therefore, this hypothesis would place the origin of the current *O. virginianus* in South America or, at least, that from a very ancient Central American ancestor, the lineages that later colonized Central and North America originated in South America. A similar picture was obtained in one of the alternatives of the MJ network since the ancestor of the Livingston specimen (Guatemala) would have given rise to a couple of the haplotypes of Group 4-5, specifically H71 (Risaralda department, Colombia) and H70 (Caldas and Guainía departments, Colombia) that would have been fundamental for the remaining mitochondrial diversification of the other groups of *O. virginianus* in South, Central and North America.

Regarding the first two hypotheses, there appears to be more molecular evidence supporting a northern Central American origin for the current *O. virginianus*. In the present study, the nucleotide diversity (p) value was highest in G2a (π= 1.89%), while the North American Group 1 had a p = 1.28%. Similarly, in the study by Ruiz-García, et al. [17], mitogenome analysis showed that the northern Central American group had a substantially higher p value than the North American group (USA and north-central Mexico) (2.44% vs. 0.93%).

Many of the results obtained by Ambriz-Morales, et

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al. [34] reinforce this idea. These authors analyzed the complete mitogenomes of nine Mexican subspecies of *O. virginianus* (central-northern Mexico: *O. v. texanus*, *O. v. couesi*, *O. v. veraecrucis*, *O. v. sinaloae*, and *O. v. mexicanus*, and southeastern-southeastern Mexico: *O. v. acapulcensis*, *O. v. oaxacensis*, *O. v. toltecus*, and *O. v. yucatanensis*). They found that the four subspecies with the most mitogenomic variation were from southern Mexico (*O. v. yucatanensis*, 124 specific variations; *O. v. toltecus*, 68 specific variations; *O. v. oaxacensis*, 55 specific variations; and *O. v. acapulcensis*, 36 specific variations), while the four Mexican subspecies with the fewest specific mitogenomic variations were from northern Mexico (*O. v. sinaloae*, 17 specific variations; *O. v. veraecrucis*, 8 specific variations; *O. v. couesi*, 7 specific variations; and *O. v. texanus*, 5 specific variations). It is known that populations with higher levels of genetic diversity tend to be older populations. Similarly, Ambriz-Morales, et al. [34] showed that *O. virginianus* subspecies from northern Mexico were more like each other (*O. v. texanus*, *O. v. veraecrucis*, *O. v. couesi*, *O. v. mexicanus*, and *O. v. sinaloae*) than the subspecies from southern and southeastern Mexico (*O. v. oaxacensis*, *O. v. acapulcensis*, and *O. v. yucatanensis*). The smallest genetic distance was always between putative *O. virginianus* subspecies from north-central Mexico, with the cases of *O. v. texanus* vs. *O. v. veraecrucis* (Da = 0.1%), followed by *O. v. couesi* vs. *O. v. sinaloae* (Da = 0.2%), *O. v. texanus* from USA, *O. v. texanus* from Mexico, vs. *O. v. veraecrucis* (Da = 0.3-0.5%). Conversely, the most differentiated Mexican subspecies were from the south of the country, specifically *O. v. yucatanensis* vs. *O. v. toltecus* (Da = 2.4%), and *O. v. toltecus* vs. *O. v. oaxacensis* (Da = 2.3%). In an identical manner to what was found by Ambriz-Morales, et al. [34], Logan, et al. [119] reported the genetic heterogeneity of Mexican subspecies of *O. virginianus* by comparing 480 bp sequences from the *D-loop* marker of nine subspecies. They also found a significant genetic separation between the southeastern Mexican subspecies and the remaining Mexican subspecies of *O. virginianus*. De La Rosa-Reyna, et al. [38], using 12 nuclear microsatellite loci, also found a strong nuclear genetic differentiation in *O. v. yucatanensis* compared with other central-northern Mexican *O. virginianus* subspecies. Ambriz-Morales, et al. [34] showed that a ten base pair deletion in the mt*D-loop* region in three Mexican south-southeastern subspecies of *O. virginianus* and one specimen of *O. virginianus* from French Guiana was shared. These authors concluded that French Guianan *O. virginianus* and *O. v. yucatanensis* could be descendants from the same ancestor that lived during the radiation of white-tailed deer to South America during the Pleistocene. Nevertheless, our results are ambiguous in this regard. The results with mt*NADH5* and mt*12S rRNA* genes, for both ML and BI trees, did not show a special relationship between Group 2a and the specimen we studied from French Guiana (Group 4-5). In fact, Group 2a would be more phylogenetically related to North American Group 1 than to Group 4-5 according to these analyses. Conversely, the MJ network did detect a potentially significant relationship between Group 2a and the specimen from French Guiana. Ruiz-García, et al. [17] also showed a significant relationship between Group 2 and the French Guiana specimen for the ML tree and MJ network with the mt*Cyt-b* gene (but not for the BI tree with that mitochondrial marker) and for the BI tree and MJ network for whole mitogenomes. Nonetheless, our BAPS analysis with the current molecular dataset strongly differentiated the specimens from the USA-northern central Mexico and from southern Mexico and northern Central America from the specimens from southern Central America and South America, where the French Guianan specimen was enclosed. Therefore, the 10 bp deletion at the mt*Dloop* is not entirely conclusive regarding the relationship between the Group 2 specimens and the French Guianan one.

Logan, et al. [120] also found results showing that populations in northern Mexico generally exhibit levels of genetic diversity that are not particularly high. These authors, using the mt*Dloop* marker, analyzed 105 specimens of *O. virginianus* from northern Mexico, including the subspecies *O. v. texanus*, *O. v. veraecrusis*, *O. v. carminis*, and *O. v. miquihuanensis*. In southeastern Nuevo León state, the original subspecies of that geographic area is *O. v. miquihuanensis* [121]. Nevertheless, two mitochondrial haplotypes characteristic of *O. v. texanus* appeared in this population (this same haplotype, along with two other haplotypes typical of *O. v. texanus*, also appeared in *O. v. veraecrucis*). Haplotype diversity for this population was rather low (H_d = 0.56). Conversely, in Tamaulipas, haplotype diversity was very high (H_d = 0.90) due to the introduction of individuals from different subspecies and diverse populations into that area. Therefore, many populations within Group 1 may exhibit high genetic diversity due to the relatively recent reintroduction of animals from different geographic origins. This occurs because, as Mandujano, et al. [41] demonstrated, of the two groups of Mexican *O. virginianus* subspecies based on their morphological features, the northern subspecies (*O. v. texanus, O. v. carminis, O. v. miquihuanensis, O. v. veraecrucis, O. v. mexicanus, and O. v. couesi*), which grow to larger sizes and have more developed antlers, are more sought after by human hunters as hunting trophies, while the southern subspecies (*O. v. sinaloae, O. v. thomasi, O. v. yucatanensis, O. v. truei, O. v. oaxacensis, O. v. acapulcensis*, and *O. v. nelsoni*), whose sizes are smaller, are less coveted as hunting trophies. Therefore, the high genetic diversity of the latter does not result from the mixing of specimens from different geographical origins for hunting purposes.

Three theses [7,122,123] systematically analyzed 12 of the 14 subspecies of *O. virginianus* present in Mexico using the mt*Dloop* marker with relatively large sample sizes. Hernández [122] analyzed 67 specimens for three subspecies

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(*O. v. miquihuanensi*, *O. v. couesi* and *O. v. mexicanus*). Ortiz-Contla [123] analyzed 47 specimens belonging to four subspecies (*O. v. texanus*, *O. v. veraecrucis*, *O. v. thomasi*, *O. v. yucatanensis*). Serna-Lagunes [7] studied 105 specimens of eight different Mexican subspecies of *O. virginianus* (*O. v. acapulcensis*, *O. v. couesi*, *O. v. mexicanus*, *O. v. nelsoni*, *O. v. oaxacensis*, *O. v. sinaloae*, *O. v. thomasi* and *O. v. toltecus*). In all three theses, the nucleotide diversity values were unusually high because they were incorrectly calculated and, moreover, these values were misinterpreted. Nonetheless, even if the π values were misinterpreted, misexpressed, or miscalculated, the highest values always correspond to subspecies from southern Mexico (*O. v. yucatanensis*, *O. v. thomasi*, *O. v. sinaloae*, *O. v. nelsoni*), which provides further evidence that Group 2 is more closely related to the origin of *O. virginianus* than Group 1. In fact, Moscarella, et al. [1], for mtDNA, estimated H_d values much higher for Venezuelan *O. virginianus* than those reported for North American white-tailed deer [124,125]. Again, this agrees better with an origin of *O. virginianus* in the territory of present-day southern Mexico and northern Central America than with an origin in the current territory of the USA.

Differences with Respect to the Work of Ruiz-García, et al. [17] and the systematics of *O. virginianus*

In most cases, the results obtained by Ruiz-García, et al. [17] were corroborated by the results obtained in the present study. Nevertheless, by increasing the sample size and using a different molecular dataset, new results were observed that expand our knowledge of the evolutionary history of *O. virginianus*.

The North American Group 1 was constituted in the same manner as observed by Ruiz-García, et al. [17]. The sequences of two specimens from north-central Mexico (Tamaulipas and Veracruz) and Texas (USA) were very closely related. They probably corresponded to specimens of *O. v. texanus* (or to presumed subspecies that are not very genetically differentiated). The most noteworthy new result was the inclusion of a specimen from Chiapas (southern Mexico) in this group. In that geographic area, three possible subspecies of *O. virginianus* converge: *O. v. toltecus*, *O. v. thomasi*, and *O. v. nelsoni*. Serna-Lagunes [7] showed a striking divergence between *O. v. toltecus* and all the other subspecies he analyzed (F_{ST} values ranged significantly from 0.092 to 0.127). Conversely, the F_{ST} values among the other *O. virginianus* subspecies studied were considerably lower [F_{ST} values ranging from 0 (*O. v. nelsoni* vs. *O. v. sinaloae*) to 0.029 (*O. v. mexicanus* vs. *O. v. thomasi*)]. Therefore, *O. v. toltecus* differed strongly from both the north-central and southern Mexican subspecies following Serna-Lagunes [7]. Ambriz-Morales, et al. [34] obtained a very different result using mitogenomes, since based on geographic location, *O. v. toltecus* would be expected to group with the southern and southeastern Mexican subspecies (such as *O. v. thomasi* or *O. v. nelsoni*), but they found *O. v. toltecus* in the clade of the central and northern subspecies closely related to *O. hemionus* (our Group 1). Our results were identical to those of Ambriz-Morales, et al. [34] and differed from those of Serna-Lagunes [7]. This could be interpreted as meaning that this subspecies belongs to Group 1 and has penetrated the territory of Group 2 (or if the evolution was from south to north, then it could have belonged to a lineage originally from Group 2 that gave rise to Group 1), or that, due to hunting, humans have recently introduced specimens from north-central Mexico into the south of the country. Nevertheless, the branch corresponding to the specimen we analyzed from Chiapas is long, leading us to favor the first hypothesis.

The temporal genetic diversification within Group 1 was estimated at around 0.96–0.74 mya, coinciding with a very intense cold period (Pre-Pastonian, 1.30–0.80 mya). In a broad sense, this period coincides with the Middle Pleistocene Transition (MPT) (1.2–0.5 mya). In a stricter sense, the MPT (transition from the Lower Pleistocene, Calabrian, to the Middle Pleistocene, Chibanian) occurred 774,000–770,000 ya, formally linked to the Brunhes-Matuyama magnetic reversal, which was the last drastic change in Earth's magnetic polarity. Glacial cycles changed from a periodicity of 41,000 years to longer and more intense cycles of 100,000 years. Many of the haplotype diversification events in the various groups of *O. virginianus* coincide with this period.

Ruiz-García, et al. [17] detected Group 2, which consisted of specimens from southern Mexico (Campeche, Quintana Roo, and Yucatán states), Belize, Guatemala (not all specimens), and Honduras. In different analyses, Group 2 was associated with Group 4 (Colombian central Andean Cordillera). With whole mitogenomes, Group 4 appeared to be the origin of Group 2. In the current analysis, Group 2 is divided into two subgroups: Group 2a [comprising specimens from southern Mexico (Campeche, Quintana Roo, and Yucatán states), Guatemala (Petén department, but not all specimens from this country), and Belize] and Group 2b [comprising two specimens from Honduras (Roatán Island) and one Guatemalan specimen from the Chocón-Machacas, Izabal department]. In Ruiz-García, et al. [17], the only specimen from Roatán Island in Honduras was the outermost specimen of Group 2, but with the current database, the original grouping is subdivided. Group 2a is the grouping found with the highest nucleotide diversity. Group 2a would supposedly consist of specimens of *O. v. yucatanensis* Hays 1872, *O. v. truei* Merriam 1898, and *O. v. thomasi* Merriam 1898 [7,20]. Within this grouping, there was no significant spatial structuring, so the genetic differences between these putative subspecies were very small or corresponded to a

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single subspecies. Our result fully agrees with that found by Ortiz-Contla [123], who estimated a negligible F_{ST} value (= 0.0006) and an Nm value (gene flow) of infinity between the two supposed subspecies (*O. v. yucatanensis* and *O. v. thomasi*). Therefore, pending nuclear and karyotypic analyses, we consider the existence of a single subspecies for this group (*O. v. yucatanensis*). Haplotype diversification in Group 2a was one of the oldest recorded for *O. virginianus* (1.6-1.2 mya).

Group 2b contained three specimens that could potentially correspond to the subspecies *O. v. nelsoni* Merriam 1898 or *O. v. truei* Merriam 1898. We have already seen that the presumed *O. v. truei* from southeastern Mexico is indistinguishable from *O. v. yucatanensis*; this would lead us to postulate that the subspecies associated with Group 2b is *O. v. nelsoni*. An alternative hypothesis is that the Honduran specimens do not actually belong to *O. v. nelsoni* (originally defined in Guatemala) but rather to the true holotype of *O. v. truei*, since the actual description and original type locality correspond to Honduras, previously established in 1888 by Frederick W. True who was indeed the first to describe this deer under the name *Cariacus clavatus*. Nevertheless, the specific name *clavatus* had already been used by Charles Hamilton Smith (*Cervus clavatus*), leading to a homonym conflict. Noting that the original name had already been used, Merriam [126] proposed a new name (*Odocoileus truei*) to honor True. Therefore, the only way to resolve which of the two names might correspond to Group 2b would be to be able to sequence the holotypes (or new neotypes) of *O. v. nelsoni* and *O. v. truei*. What is clear from our results is that *O. v. truei* does not exist in the extreme southeast of Mexico. Another interesting aspect of Group 2b is that in certain analyses it was more closely associated with Group 1 than with Group 2a (ML and BI trees, but not with the MJ network or genetic distance analyses). This could lead to two hypotheses related to what was previously discussed regarding *O. v. toltecus*. The first is that Group 2b represents a wedge of penetration of Group 1 into northern Central America or, alternatively, that Group 1 originated from an ancestor of this Central American Group 2b if a south-to-north colonization occurred, as suggested by Ruiz-García, et al. [17]. Using ML and BI trees, the strong relationship between Group 2 and Group 4, as visualized in Ruiz-García, et al. [17], was not observed. Nonetheless, the MJ network still detected a strong possible relationship between Group 2a and Group 2b with the new G4-5 clustering. Haplotype diversification in Group 2b was circumscribed around 0.76 mya, which coincides with the MPT discussed above.

Therefore, there is clear genetic heterogeneity in Mexico for *O. virginianus* (Group 1 and Group 2a), which agrees with different authors [17,34,38,41]. Ortíz-Contla [123], who included in his study two subspecies of *O. virginianus* from north-central Mexico and two subspecies from south-southeast Mexico, using an AMOVA, detected 20% of genetic variation attributable to differences between the analyzed subspecies and 80% genetic variance within the subspecies, which agrees with the above. Completely different from the results discussed, which agree with the existence of strong genetic heterogeneity in *O. virginianus* in Mexican territory, were the results of Serna-Lagunes [7]. Despite the unusually high genetic differentiation of *O. v. toltecus* in comparison to the other subspecies discussed above, he found, using AMOVA, an extremely low genetic variance attributable to the subspecies (two subspecies from northern Mexico and six from southern Mexico were analyzed), of only 2%, while most of the genetic variance resided among individuals within the subspecies (98%). Consistent with this, the phylogenetic trees showed the haplotypes of these eight subspecies completely intermingled and the absence of spatial structure for *O. virginianus* in the interior of Mexico (Mantel test, r = -0.326, p = 0.101). The discrepancy of this work with respect to all the others is difficult to explain unless there was some error in the sequence analysis.

This strong genetic heterogeneity among different groups of *O. virginianus* in Mexico may be a product of the existence of two biogeographic regions, the Nearctic and Neotropical, which converge in central Mexico as mentioned by Ambriz-Morales, et al. [34]. These regions give rise to a great diversity of environments and endemism. The transition zone between both regions comprises potential geographical barriers such as the Sierra Madre Occidental, the Sierra Transvolcanica Mexicana (responsible for various important vicariance events, [127,128]), and the Sierra Madre del Sur [127]. In particular, the Sierra Transvolcanica Mexicana may have played a significant role in the decoupling of Group 1 and Group 2 of *O. virginianus*. Similarly, climatological changes during the Pleistocene could have contributed to this conspicuous differentiation by creating distinct genetic patches [124] due to the presence of Pleistocene refugia [129-131]. Likewise, *O. hemionus* has also been found to exhibit a complex pattern of recolonization for subspecies of mule deer and black-tailed deer after the Last Glacial Maximum [32]. The differentiation of *O. v. yucatanensis* may have been influenced by the isolation of this population from the mainland during sea level rises between Pleistocene glaciations, like what has been suggested for *O. virginianus* in Florida [124]. In fact, at least two refuges (Arc and Soconusco refuges) have been in southern Mexico during the Pleistocene [132].

Ruiz-García, et al. [17] defined Group 3, consisting of two specimens from Panama, which, in some analyses, even played an important role in the possible origin of *O. virginianus*. Apparently, this Group 3 seemed to correspond very well to the subspecies *O. v. chiriquensis* Allen 1910. One

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of the analyses presented here (NJ network) again detected this cluster on this occasion (comprising three Panamanian specimens: two from the province of Coclé and one from the province of Chiriquí). Nevertheless, both ML and BI trees broke this cluster, such that one specimen from Coclé and the one from Chiriquí were strongly associated with each other, along with two specimens from the Colombian Venezuelan Llanos and within Group 6, which was primarily Ecuadorian. The other specimen from Coclé was strongly linked to a specimen from the province of Alajuela (Costa Rica), both within Group 4-5, which was primarily a group from northern South America. In fact, the BAPS analysis included the three Panamanian specimens and the Costa Rican one in the South American cluster. But this means that in southern Central America (especially in Panama) there are, at least, two different mitochondrial lineages more closely related to South American lineages than to those present in northern Central America. The holotype of *O. v. chiriquensis* should be used to determine which of the two lineages it belonged to. Notwithstanding, one of the specimens we analyzed comes precisely from Chiriquí, the place where the holotype of this subspecies was defined and we can use it as a neotype. Therefore, we could conclude that this specimen, along with one of the two specimens from Coclé, could represent *O. v. chiriquensis*. But there is a problem: this clade is within a larger clade composed mainly of Ecuadorian specimens that would correspond well to *O. v. ustus* Trouessart 1913 and is associated with two specimens from the Colombian Venezuelan Llanos that, according to traditional taxonomy, could also correspond (as will be discussed below, *O. v. apurensis* Brokx 1972) to another distinct subspecies. Since the oldest of the three names involved is *O. v. chiriquensis*, then the Ecuadorian Group 6, which would include Group 3 sensu Ruiz-García, et al. [17], plus a couple of specimens from the Colombian Venezuelan Llanos, should be named *O. v. chiriquensis*, while *O. v. ustus* and *O. v. apurensis* would simply be synonyms and should be removed. If Group 6 were elevated to species status, then the existence of *O. chiriquensis chiriquensis* (Panama), *O. chiriquensis ustus* (Ecuador), and *O. chiriquensis apurensis* (part of the Colombian Venezuelan Llanos) could be established, but no reproductive or chromosomal study has revealed any reproductive isolation mechanisms among these three populations of *O. virginianus* (BSC definition). Here we observe the poor fit that often occurs between molecular evolutionary population genetic analyses and classical taxonomy.

Ruiz-García, et al. [17] found two large groups in the northernmost part of South America: Group 4, restricted to the central Colombian Andes, and Group 5, widely distributed along the eastern Colombian Andes, the transition zone between the eastern Llanos and the Colombian Amazon, Venezuela (Mérida state), and French Guiana. Notwithstanding, the present study intermixed specimens from these two groups. Therefore, we refer to this grouping as Group 4-5 in the current analysis. Within this grouping, two subgroups were identified. One of these includes specimens from the central Colombian Andes (Risaralda and Caldas departments), the eastern Colombian Andes (Cundinamarca department), the transition zone between the eastern Colombian Llanos and the Colombian Amazon (Guainía department), the Venezuelan coastal zone (Falcón state), French Guiana, and southern Central America (Costa Rica and Panama). In the other subgroup, all specimens were of Andean origin, including the central Colombian Andes (Risaralda and Caldas departments), the eastern Colombian Andes (Cundinamarca and Santander departments), and the Andes in the state of Mérida (Venezuela). The two specimens from the Mérida Andes formed a strong cluster. This is probably the geographic area of the *O. virginianus* distribution that still requires a higher sampling density to determine exactly how many genetically distinct *O. virginianus* clusters exist within it. The start of diversification for Group 4-5 was estimated at 1.46 mya, while internal subgroups began their diversification at around 1.08 mya and 0.75 mya, respectively, coinciding with what was discussed for other groupings.

Molina and Molinari [56] and Molinari [2] when studying from a morphological and morphometric point of view various populations of *O. virginianus* in Venezuela distributed in very different biomes and environments concluded with the existence of three fully differentiated species.

The first of these was named *O. lasiotis*. Its distribution is restricted to the Sierra Nevada and the Sierra de La Culata, in the northern half of Mérida state, inhabiting páramo environments above 3,200 meters on sea level (mosl). The estimated distribution of Andean páramos (including shrubby, herbaceous, and desert páramos) in the central section of the Mérida Andes yields an area of less than 1,600 km^2. The best-studied population consists of between 240 and 250 individuals and is in the vicinity of Mucubají Lagoon [133,134].

Following Molina, et al. [56], the Mérida Andean *Odocoileus* differs from the rest of the mainland Venezuelan *O. virginianus* specimens most notably by different discrete cranio-mandibular characteristics: the presence of a posterior mental foramen (0 vs. >39%) and a gabled nasion (89 vs. <38%). Other cephalic characteristics which differentiate the Mérida Andean *O. virginianus* from other populations Venezuelan *O. virginianus* included: 1- a broader skull; 2- a distinctive pattern of interorbital sutures; 3- pedicles serving as the base for antlers more anteriorly directed than in other Venezuelan specimens of *O. virginianus*; and 4- robust and quiet antlers, with tips pointing forward. Molina, et al. [56] also claimed that the Mérida *O. virginianus* looks very different from its Venezuelan congeners: a conspicuous blackish mask

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covers most of the frontal region, the coloration is dominated by intense gray (as opposed to tawny or ochraceous), the head is massive, the limbs are short and strong, and the body is robust, with the back relatively straight in profile (as opposed to delicate head and limbs, and the back arched posteriorly).

Molina, et al. [56] commented that without offering taxonomic proof, Brokx [5] placed the Mérida Andean *O. virginianus* as closely related to lowland congeners from around the Maracaibo Lake and the Venezuelan Caribbean coastal. Brokx [5] also commented that there is some kinship between the Mérida *O. virginianus* from those of the islands of Curaçao and Margarita, based on some shared color tones in the dorsal pelage.

Molina, et al. [56] discussed possible names for this supposed full species of *Odocoileus* that they considered to exist in the Andes of Mérida. They commented that the names to consider were: 1- *goudotii*, highlands of Colombia; 2- *peruvianus*, Peruvian Andes; 3- *columbicus*, Colombia; 4- *ustus*, Andean Ecuador at 4,100 mosl; 5- *lasiotis*, at ca. 3,000 mosl in Mérida (Venezuela). Molinari, et al. [56] also commented that applying any of these designations implies that the Andean form from Mérida is conspecific with any of them, and since they consider this form to be a distinct and endemic species of that locality, the only valid option is *O. lasiotis*, even though they believe that all these gray forms of Andean *Odocoileus* must be more closely related to each other than to any lowland *Odocoileus* form. They also explained that the Andean form from Mérida cannot be the same as the one that inhabits the páramos of the eastern Colombian Andes. The justification was that the páramos of both locations were never in contact due to the arid Táchira Depression, which consists of valleys 60 km wide and 960 m deep [135,136]. This depression would have separated *lasiotis* (Venezuela) from *goudotii* (Colombia) at species level makes because there is a 150-km xeric gap, although Ruiz-García, et al. [17] showed that this alleged barrier did not exist for other mammal species, including *M. rufina-M. bricenii*.

Nevertheless, the molecular genetic results from Ruiz-García, et al. [17] and the current study do not agree with all of this. As discussed [17], the work of Molina, et al. [56] and Molinari [2] is commendable and meticulous in distinguishing morphological and morphometric differences in the skulls and mandibles (sizes and shapes) of numerous *O. virginianus* specimens from different Venezuelan regions. Nevertheless, although these morphological and morphometric traits were significantly different among various *O. virginianus* populations in Venezuela, this does not mean they are different species. Medium-sized or large mammal species with very wide geographic distributions generally exhibit morphological and morphometric differences due to the rapid action of differential natural selection occurring in different environments and possible action of genetic drift by founder events. In fact, Moscarella, et al. [1] estimated the genetic diversity of the white-tailed deer from the Andes of Mérida (four haplotypes) and detected a high H_d value (= 1) but a rather low nucleotide diversity value (π = 0.5%). The low nucleotide diversity value may be related to the fact that the ancestral populations of the Andes of Mérida might have been small and that the effects of genetic drift in small populations led to a loss in genetic diversity [137]. Nonetheless, the high levels of haplotype diversity suggested population expansion after a population bottleneck because it is expected that haplotype diversity recovers faster than nucleotide diversity [138, 139]. Therefore, this population appears to have undergone a founder effect with loss of genetic diversity. Taking all of that into consideration, the assumption that these different phenotypic characteristics necessarily imply different species has been a mistake made in many cases. For example, Merriam [140] considered the existence of 86 supposed species of brown bears in North America alone, analyzing craniometric data and different aspects of the skins studied. His methods of analysis were correct, but by using a typological approach, his interpretation was clearly erroneous. But our own species (*Homo sapiens*) is the best proof of this. Since their departure from Africa 120,000-60,000 years ago [141,142], their arrival in South Asia and Oceania 65,000-50,000 years ago [143], their arrival in Europe and North Asia 50,000-40,000 years ago [144], their arrival in America around 15,000 years ago [145], and the conquest of the Pacific Ocean islands between 4,000 and 1,000 years ago [146], the morphological and morphometric changes in human populations are fully evident due to the rapid action of natural selection adapting to different climates, plus the effects of genetic drift in some small and historically isolated populations. Nevertheless, we apply Mayr's BSC [106-108] to ourselves, and since there is no evidence of reproductive isolation barriers between the human groups with the most conspicuous morphological and morphometric differences, we consider ourselves a single species. Until the existence of significant chromosomal differences and/or the existence of post-, but above all, pre-zygotic reproductive barriers between different populations of *O. virginianus* can be demonstrated, we cannot assert that the *lasiotis* form (or any other) is a distinct species of *O. virginianus*.

With the knowledge we currently possess and without specific chromosomal and reproductive studies that allow us to analyze possible mechanisms of isolation between the various forms of *O. virginianus* found in Venezuela, the name of the population of the Andes of Mérida should be *O. v. goudotti* since genetically it is differentiable from *O. v. ustus* and from *O. v. peruvianus*; moreover, *goudotti* Gay and

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Gervais 1846 is earlier than *columbicus* Fitzinger 1879 and *lasiotis* Osgood 1914. The nucleotide diversity of the 10 Andean specimens that form the second subgroup within Group 4-5, comprised of specimens from the central and eastern Colombian Andes plus the two specimens from the Mérida Andes, was very low ($\pi = 0.38\%$), indicating that the sequences of these 10 specimens were remarkably like each other. While some statistical analyses slightly differentiated the two Mérida Andes specimens from the remaining eight Colombian Andes specimens ($F_{ST} = 0.31$, Nm = 0.56; Z* = 2.51, $p = 0.023$), others did not ($G_{ST} = 0.12$, Nm = 1.83; $x^2 = 10$, df = 5, $p = 0.08$). Without further evidence, it appears that *O. v. goudotti* was indistinguishable from *O. lasiotus*, and based on historical priority, *O. v. goudotti* seems to be the correct choice.

The second full species considered by Molina, et al. [56] and Molinari [57] was the white-tailed deer from Margarita Island (Venezuela), *O. margaritae*. Molina, et al. [56] concluded, using discrete craniomandibular characteristics, that the Margaritan *Odocoileus* differs from the rest of the Venezuelan taxa of *O. virginianus* mainly by the presence of a mylohyoid arch (38% vs. > 83%), naso-premaxillary contact (13% vs. < 8%), and a supraorbital bridge (12% vs. > 62%), its diminutive body size and the external appearance. Notwithstanding, Molinari [2] himself commented that, due to Margarita's contact with the mainland during the last glacial maximum, it is reasonable to expect that the living populations of Margarita Island (*margaritae*) and Araya (mainland *O. virginianus*) will show traces of mutual introgression that occurred 17,000–13,000 years ago. This possibly indicates the absence of pre-zygotic reproductive isolation barriers, which, using BSC, argues for the non-existence of distinct species. Moscarella, et al. [1] estimated the genetic diversity for *O. virginianus margaritae* from six specimens with six haplotypes, $H_d = 1$ and $\pi = 1.6\ \%$, which showed that, although this island population is small, it exhibited high genetic diversity. Moscarella [147] claimed that the separation of the Margarita *O. virginianus* from its upland congeners occurred at least 118,000 years ago. Nevertheless, later, Moscarella, et al. [1] commented that the last glacial maximum began in Venezuela between 19,000 and 16,000 years ago and ended around 13,000 years ago [148]. During this period, the populations of *margaritae* (insular) and *lasiotis* (= *goudotii*) (Andean) may have related to the lowland *O. virginianus* (= *gymnotis* or *cariacou*). If this hypothesis was correct, the strong cranial and size differences between *margaritae* and the *O. virginianus* forms of mainland Venezuela would have arisen only in the last 13,000 years. According to Molinari [2], these strong morphological differences could not have arisen in such a short time (13,000 years) since evolution would have had to be unusually rapid. Notwithstanding, there is evidence of very significant morphological changes in a very short time in multiple cases of vertebrates influenced by both natural selection and genetic drift in extremely small founding populations. For example, there are many examples of island dwarfism in mammals that are not representative of different species. Some examples are the following: The extinct Japanese wolf (*Canis lupus*) and the extinct Balinese tiger (*Panthera tigris balica*) were significantly smaller than their continental counterparts. Mitochondrial DNA analysis showed that the Japanese wolf was indistinguishable from North American wolf lineages [149,150]. Similarly, mtDNA evidence indicated that the Balis's tiger was indifferentiable from the Sumatra tiger (*Panthera tigris sondaica*; [151]). There are other examples too, such as the goats (*Capra hircus*) introduced into the Juan Fernández archipelago (Chile) during the 16th and 18th centuries by conquerors and pirates. These goats are half the size of the goats from which they were derived (Muñoz-Pedreros et al. 2003). Recently, Ruiz-García, et al. [153] showed that the agouti from the Roatán Island (*Dasyprocta ruatanica*; Honduras), also considered a different species by its size, is molecularly indifferentiable from the Central American agouti, *Dasyprocta punctata*. Indeed, both forms of agoutis can breed without problem (Ruiz-García, unpublished observations). Similarly, Jaramillo and Ruiz-García [154] and Ruiz-García, et al. [155] showed how the form of *Nasua narica* from Cozumel Island (18 km off the Mexican mainland coast), considerably smaller than the coati form from the Yucatán Peninsula, is not molecularly distinguishable from the purported distinct species, *Nasua nelsoni*, according to various Mexican researchers. It has been shown that artificial, natural, and sexual selection can drastically change the morphology of different organisms without speciation in just a small number of generations (the foxes of Belyaev, [156]; the Mediterranean lizard, *Podarcis sicula*, [157]; or the guppy, *Poecilia reticulata*, in Venezuela and Trinidad and Tobago; [158-161]). Additionally, the average height of people within indigenous populations of different Southeast Asian islands (Andaman, Luzon, Panay, and Mindanao islands) is considerably less than that of humans from the Asian continent [162,163]. Nevertheless, no one considers each of these insular dwarf human populations as different species. Thus, insular dwarfism in mammals is not a sufficient requirement to differentiate a species, as is the case of the white-tailed deer from Margarita Island. Having said this, however, a thorough molecular study of the white-tailed deer of Margarita Island with a significant sample size is urgently needed, as is a study of the other forms of *O. virginianus* in Venezuela using a wide variety of molecular markers and karyotypic analyses. Likewise, because its separation from mainland congeners in a similar way to that of the Margaritan white-tailed deer, it is important to analyze the case of the Curaçao white-tailed deer to investigate if it could be a full species, *O. curassavicus* as suggested Molinari [2].

The third full species considered by Molina and Molinari

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[56] and Molinari [2] was the white-tailed deer from the Venezuelan coast, Venezuelan western and eastern Llanos, Bolivar and Apure states (basically all the lowlands of Venezuela), which they named *O. cariacou*. Moscarella, et al. [1] named this taxon *O. v. gymnotis*. Molina, et al. [56] and Molinari [2] detected some heterogeneity within this supposed species, which led them to consider that there might be distinct subspecies of *O. cariacou* in Venezuela. Regarding the discrete craniomandibular characteristics that studied the Caribbean coast *Odocoileus* differed from specimens from the states of Bolívar, Llanos, and Apure in the presence of a posterior mental foramen (87 vs. < 63%), parietal foramen (75 vs. < 63 %), supraglenoid foramen (75 vs. < 61%), and emissary sphenoidal foramen (87 vs. > 97%). Additionally, Molina, et al. [56] found that, contradicting Brokx [5], their data indicated that the Caribbean coast *Odocoileus* has a closer relationship to deer from Bolívar, the Llanos, and Apure than to the white-tailed deer from the Mérida Andes. Therefore, Molina and Molinari [56] concluded that the Caribbean coast deer has genetic intergradation with congeners from the Llanos. Nonetheless, this deer has distinctive characters which suggest that it is different, at least subspecifically, so *O. cariacou* would contain several taxa within it as previously mentioned. Moscarella, et al. [1] analyzed 16 specimens of this taxon and obtained high estimates of genetic diversity (13 different haplotypes) with $H_d = 0.967$ and $\pi = 2.9\%$. Therefore, both estimates of genetic diversity were very high, which may be consistent with the morphological heterogeneity found by Molina, et al. [56].

In our study, we analyzed a specimen of *O. virginianus* from the Venezuelan coast (Falcón state). This specimen was included in the first subgroup found in Group 4-5, along with the specimen from French Guiana, two specimens from southern Central America (Costa Rica and Panama), and several Andean *Odocoileus* specimens from both the central and eastern Colombian Andes Cordilleras. The molecular differences between them were minimal, indicating that this grouping is widespread throughout northern South America and southern Central America, in both lowlands and highlands, and is closely related to the other subgroup of exclusively Andean origin already mentioned within Group 4-5. The supposed specific differentiation proposed by Molina, et al. [56] and Molinari [57] between highland and lowland *Odocoileus* in South America was not supported by these molecular data. Because this group includes specimens from such ecologically diverse locations, there is a proliferation of possible names for the specimens in this subgroup. Since the specimen from French Guiana was included in this subgroup, it seems that the group should be named *O. v. cariacou* Boddaert 1784, as Hershkovitz [44] determined that the type locality was the coast of French Guiana. It is interesting to note how Tate [47] considered the white-tailed deer of the Pacific region of Colombia and Ecuador to be closely related to the Panamanian specimens. We have already mentioned that in Panama we find two different genetic lineages. If the former corresponds to *O. v. chiriquensis*, this will show that *O. v. cariacou* is widespread not only throughout most of northern South America but also, at least, throughout southern Central America (Panama, Costa Rica). In this sense, Molina, et al. [56] and Molinari [2] would be correct in stating that *O. v. cariacou* would be the taxon found mainly in the lowlands of northern South America and extending into Central America, but it was incorrect to say that it would be exclusive to lowlands (it is also found in the Colombian Andes where it coexists with *O. v. goudoutti*), and its distribution does not reach southern Mexico and northern Central America, but only southern Central America. Other possible collateral hypotheses also arise. The first of these is that ancestral polymorphisms exist that are shared by specimens from very diverse geographic areas in northern South America and southern Central America. This hypothesis suggests that the isolation of these populations was relatively recent, and the processes of genetic differentiation could still be occurring (category III of phylogeographic patterns; [164]). The apparent relationship among haplotypes from unrelated geographic localities could be evidence of this hypothetical widespread ancestral population [165]. If genetic differentiation is so recent, it is unlikely that these groupings are fully differentiated species, as Molina, et al. [56] and Molinari [2] maintain, unless drastic chromosomal changes have occurred, as seen in brocket deer. Therefore, karyotypic studies of the different *O. virginianus* groupings found are crucial. A second possibility is the existence of historical introgression and/or recent hybridization among specimens of these groupings, as we have previously reported between *O. hemionus* and *O. virginianus* in North America. For example, we might consider that all the *O. virginianus* specimens found in the two Colombian Andean Cordilleras mentioned (central and eastern) are Andean specimens belonging to *O. v. goudotti*, but some of these specimens carry mtDNA from lowland *O. v. cariacou* because their ancestors were genetically introgressed by animals from that group. To distinguish this hypothesis from the first, nuclear genes would need to be studied. But if this second hypothesis were true, it would also be revealed that there have been no reproductive isolation mechanisms between these different taxa, thus supporting the existence of a single species of *O. virginianus* at least in southern Central America and northern South America.

Ruiz-García, et al. [17] identified a group of exclusively Ecuadorian specimens (Group 6). Most of these specimens originated in the Andes, while four were from the Ecuadorian Pacific coast. Three of these latter specimens formed a small, slightly distinct subgroup within Group 6, but the fourth

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remained grouped with an Andean specimen from the province of Azuay. In the present study, we also observed some small groupings of specimens from the Ecuadorian Pacific coast. For example, in the ML tree, a grouping of three specimens was found, two of them from the Ecuadorian coast (Guayas and Manabí provinces) and one from the province of Pichincha, extending towards the Ecuadorian coast. A second small grouping was found containing two more specimens from the Ecuadorian Pacific coast (Guayas province) but with one specimen of Andean origin (Azuay province). Tomiati, et al. [166] postulated that *O. v. ustus* gave rise to a lowland species of *Odocoileus* (*salinae*) in Ecuador. Molinari [2] criticized this claim. Notwithstanding, the existence of two distinct groupings, one in the Ecuadorian Andes and the other on the Ecuadorian Pacific coast, was not observed, contradicting Molinari's [2] concept that lowland and Andean white-tailed deer should constitute different species. In fact, the white-tailed deer of the Ecuadorian Andes and coast are closely related. In the analysis by Ruiz-García, et al. [17], Group 6 exhibited the lowest haplotype diversity. Nevertheless, in the present study, Group 6 showed a high haplotype diversity value, as all the other groups detected. The haplotype proliferation of Group 6 was estimated to be between 1.24-1.06 mya as considered in other groups of *O. virginianus*.

The most striking aspect of the new analysis was the emergence of a statistically robust subgroup within Group 6, which was itself composed of two other subgroups: one exclusive to the Colombian Venezuelan Llanos (Meta department and Apure state, respectively) and another, already discussed, containing two Panamanian specimens (*chiriquensis*). The Ecuadorian specimens correlated well with *O. v. ustus*, but the supposed subspecies *O. v. tropicalis* was not differentiated on the coast, apparently. The case of the two specimens from the Colombian Venezuelan Llanos deserves detailed commentary. The Venezuelan specimen was sampled from a population on the southern banks of the Apure River. Brokx [5,53] postulated that the Apure *O. virginianus* is a subspecies, which he referred to as *O. v. apurensis*. Nevertheless, Molina, et al. [56] concluded that this form was never formally described as *apurensis* and therefore lacks taxonomic validity. The results on discrete cranial–mandibular characters [56] did not support this, because the Apure white-tailed deer appeared to be tightly clustered with specimens from Bolívar and the Llanos. Nonetheless, continuous mandibular characters did indicate two groupings in Venezuelan Llanos, which might eventually be related to the presence of some differentiated taxon in the Apure River area. Therefore, Molina, et al. [56] concluded that the Apure white-tailed deer, with the characteristics they included in their analysis and the absence of any geographic (other than the Apure River) or ecological barrier separating it from specimens inhabiting the western Llanos, did not support Brokx's [5,53] contention that it should be considered a separate subspecies. Nonetheless, Molinari [2] later determined a certain degree of cranial differentiation in the Apure white-tailed deer (which is also distinguished by its light-colored coat and small size; [5]) compared to other specimens from the Llanos. The molecular result obtained here seems to confirm that in this Venezuelan region, extending into the Colombian Llanos, there exists a genetically distinct lineage that could be named *O. v. apurensis*. Some authors have commented that the white-tailed deer of Apure are related to specimens from the Colombian Llanos, and those from Bolívar to the Guianas and northern Brazil [5,20,43]. Indeed, the relationship of the first two seems confirmed by the molecular result found, while it would be possible that specimens from the state of Bolivar (not analyzed here) were more related to *O. v. cariacou*. Therefore, the new results presented here show that in Group 6 there would be three potential subspecies, highly related to each other, *O. v. ustus*, *O. v. apurensis*, and *O. v. chiriquensis*.

Ruiz-García, et al. [17] detected a Group 7 that extended along the north-central coast of Peru, where it was unclear whether it also expanded naturally into the northern Peruvian Andes, and which was strongly related to the Ecuadorian Group 6. In the present study, this group also appeared occupying the same geographic area, and on this occasion, the two specimens from the northern Peruvian Andes (Cajamarca department) were the most distinct specimens within this group. In this analysis, it was also showed a strong relationship with Group 6 and, in the BI tree, they were even grouped internally with some of the Ecuadorian specimens of Group 6. Group 7, in the present analysis, was the group that presented the lowest value of nucleotide diversity, which could be related to a relatively recent origin. Related to this, the onset of haplotype diversification of this Group 7 was estimated to be between 0.83 and 0.3 mya, slightly lower than that of other groups discussed.

Ruiz-García, et al. [17] detected a second genetically distinct group in Peruvian territory, extending across the south-central Peruvian Andes and part of the south-central Peruvian coast, Group 8. In the current analysis, this group also appeared strongly differentiated, including a specimen from northern Bolivia (La Paz department). This indicates that Cabrera's [43] comment regarding the absence of this species in Bolivian territory was inaccurate. Furthermore, the present results indicate that Molinari's [2] assertion that only *O. v. peruvianus* inhabits Peru and Bolivia, and that on the Pacific slope it is not restricted to high mountains, since in the central Andes, from the puna to the coast, there is continuous open vegetation suitable for *Odocoileus*, is incorrect. Really, two genetically distinct groups exist in Peruvian territory: one primarily coastal and the other primarily Andean, unlike what was found in Ecuador. Group

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7 could be related to *O. v. brachyceros* Philippi 1894 whose type locality was Cajamarca (Peru), and it is a senior name in reference to *O. v. philippi* Trouessart 1905, while Group 8 corresponds to *O. v. peruvianus* Gray 1874, whose type locality was Ceuchupate which corresponds to Combapata, a place adjacent to the Urubamba/Vilcanota valley, Canchis province, Cuzco department. It is worth noting that the MMA detected possible micro-geographic range subdivisions within Group 8. Of 10 geographic barriers that could exert some type of genetic fragmentation, four of them were located within the geographic distribution range of Group 8 (barriers 1, 4, 5, 7), while, curiously, this technique only detected one genetic cluster that extended from the east-west of the USA to French Guiana, encompassing all of Central America, Colombia, Venezuela and French Guiana itself delimited by barrier 2. Despite this possible fragmentation within Group 8, this grouping showed the lowest haplotype diversity value of all the detected groupings, unlike what was observed in Ruiz-García, et al. [17]. The haplotype diversification of this group was estimated to be between 1.26 and 0.6 mya, values like those of other *O. virginianus* groups.

In the present study, a Group 9 emerged that was not recorded in Ruiz-García, et al. [17]. This group consists of three specimens (two from the Colombian Llanos and one from the Ecuadorian Pacific coast) that occupied highly variable positions depending on the analyses performed. Using the ML tree, Group 9 was the second to have an older ancestor within *O. virginianus*. This could lead to speculation that there was an initial colonization from northern Central America to South America and that Group 9 is an ancient South American grouping, of which some ancestral haplotypes remain scattered across a wide area of northern South America, and that from Group 9, different colonization events occurred to the north and south (coinciding with one of the MJ network hypotheses). In the BI tree, Group 9 would have arisen from a common ancestor with Groups 6 and 7. Therefore, the exact relationship of this group to all the others remains an unknown that must be resolved with a higher sampling density in the Ecuadorian and Colombian Pacific and in the Colombian Venezuelan Llanos. What is clear is that in the Colombian Eastern Llanos, at least three distinct molecular lineages are present: one related to Group 9 (*O. v. tropicalis*?, *O. v. gymnotis*?), Group 4-5 (*O. v. cariacou*), Group 6 (*O. v. apurensis*), which makes the taxonomic situation of *O. virginianus* very complex in this region of South America. The haplotype diversification of Group 9 was estimated to be between 1 and 0.62 mya, values like those of other *O. virginianus* groupings.

This study detected several significant population expansions in various groups of *O. virginianus* during the Pleistocene. The groups that showed significant evidence of these demographic processes were the analyzed global sample of *O. virginianus*, Group 2 (expansion 0.615 mya), Group 4-5 (expansion 0.281 mya), Group 6 (expansion 0.594 mya), Group 7, and Group 8. In the three groups where the onset of these population expansions could be estimated, it coincided with the following Pleistocene events. Between 0.6 and 0.3 mya, the Earth experienced the definitive consolidation of the modern Ice Age pattern, characterized by three massive glacial periods of extreme intensity and long duration (100,000-year cycles), separated by brief warm interglacial periods. Beginning with the Mindel glaciation (Kansas; 0.58–0.39 mya), one of the most intense glaciations of the Pleistocene, followed by an interglacial period (MIS 11) during which the planet experienced extreme heat for 50,000 years [167]. This may have led to a significant dispersal of different haplotype lineages of *O. virginianus* that had fragmented in Pleistocene refugia in previous epochs. Subsequently, the glaciation associated with MIS 10 occurred, followed by the onset of another intense glaciation, the Riss glaciation (Illinois; 0.2–0.14 mya) [168]. This succession of very cold periods interspersed with some very warm interglacial resulted in significant genetic diversity within the haplotype lineages of *O. virginianus*.

Are there Really Different Species of *O. virginianus*?

Molina, et al. [56] asserted that the North American white-tailed deer and the Neotropical white-tailed deer (from northern Central America to South America) are two distinct species. They named the former *O. cariacou* and the latter *O. virginianus*. According to them, 13 discrete cranial-mandibular characters demonstrated that the Venezuelan *Odocoileus* constitutes a very divergent group with respect to North American *O. virginianus*. The characteristics that they found to differ significantly between the North American *O. virginianus* and the Venezuelan *Odocoileus* differed markedly in the presence of a mylohyoid arch (< 28 and > 38%, respectively), a hypoconulid spur (< 3 vs. 100%), nasopremaxillary contact (< 16 vs. > 13%), pterygopalatine dehiscence (< 88 vs. 100%), and posterior palatine foramina (< 41 vs. 100%) and the fact that South American *Odocoileus* do differ from North American *O. virginianus* in lack of metatarsal gland [51-54]. Similarly, our BAPS analysis identified two groupings that could be considered equivalent to *O. virginianus* (in this case, including North American specimens and those from southern Mexico and northern Central America) and *O. cariacou* (from southern Central America and South America). Nevertheless, in the present study, the range of genetic distances found among the different *O. virginianus* groupings ranged from 0.60% to 3.22%. These values were relatively low, although they may be significant. In fact, Kartavtsev [169] analyzed sequences of mt*COI* from 20,731 vertebrate and invertebrate animal species and obtained 0.89% ± 0.16% for populations within

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species, 3.78% ± 1.18% for subspecies or semispecies, and 11.06% ± 0.53% for full species within a genus. For species of different genera, this value should be around 16% or higher [169]. Bradley, et al. [170] claimed, for mt*Cytb*, that values <2% would equal intra-specific variation, values between 2% and 11% would merit additional study, and values >11% would be indicative of specific recognition. Molinari [2] criticized Moscarella, et al. [1] because the latter considered the white-tailed deer taxa of Venezuela to be subspecies, and not full species as Molinari [2] claimed, since the genetic distances were around 3%. This comment is irrelevant if the existence of reproductive isolation barriers is unknown. For organisms with chromosomal fragility (such as brocket deer), 3% divergence with markers of a neutral nature can be a high value that could underlyingly indicate the existence of different species due to reproductive incompatibility. Conversely, 3% genetic divergence in species that do not possess chromosomal speciation processes can be practically an intrapopulation value. All the differences presented in this section cannot resolve whether the different groups of *O. virginianus* are different species. Notwithstanding, the chromosomal differences between North American specimens and those from the Colombian Eastern Cordillera are minimal [171], and behavioral, physiological, or embryological incompatibilities have never been reported, as in the case of brocket deer [172]. Therefore, obtaining karyotypes of all the mitochondrial groups found is a priority to establish a first approximation of whether any type of reproductive incompatibility might exist, and to analyze possible pre-zygotic behavioral incompatibilities among specimens from the different groups of *O. virginianus*. Without obtaining these results, any speculation about the possible existence of different species within *O. virginianus* lacks merit. Therefore, the initial null hypothesis is that it constitutes a single species.

It is extremely important to obtain karyotypes and nuclear sequences from *O. virginianus* specimens from the nine identified clusters. The evidence from mtDNA suggests that these clusters do not correspond to different species. Notwithstanding, this discussion cannot be completely closed until karyotypes and nuclear sequences have been generated. It is also crucial to increase sampling density, especially from Honduras to Panama and from Colombia to French Guiana and northern Brazil, using all types of molecular markers, because it is possible that more clusters or sub-clusters could be detected within some of the nine main groups already identified in these geographic areas.

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Disclaimer/Conflict of Interest

We, the authors of this manuscript, declare that we have no conflict of interest.

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