EXPLORATORY USE OF MITOCHONDRIAL MARKERS TO INVESTIGATE A SUSPICIOUS CASE OF IMPORTING GIRAFFES TO BRAZIL

USO EXPLORATÓRIO DE MARCADORES MITOCONDRIAIS PARA INVESTIGAR UM CASO SUSPEITO DE IMPORTAÇÃO DE GIRAFAS PARA O BRASIL

USO EXPLORATORIO DE MARCADORES MITOCONDRIALES PARA INVESTIGAR UN CASO SOSPECHOSO DE IMPORTACIÓN DE JIRAFAS A BRASIL

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Abstract

Giraffes are ruminant mammals native to Africa. With their natural populations declining by around 40% over the last 30 years, giraffes have a "vulnerable" status on the International Union for Conservation of Nature Red List. For a long time, giraffes were considered to belong to a single species, *Giraffa camelopardalis*, although recent studies suggest the existence of four species and seven subspecies, with distinct distribution. In November 2021, 18 giraffes supposedly coming from wild populations of South Africa arrived in Brazil to be used in a conservation project. Shortly after their arrival, problems with their management in captivity led the Brazilian Federal Police to open an investigation into mistreatment and irregularities in the import process. Biological material from the giraffes was sent to the Federal Police DNA Laboratory for species identification and possible inferences about geographical origin through sequencing of fragments of the subunit I of cytochrome c oxidase (COI) and cytochrome b (cyt b) mitochondrial genes. Although not conclusive and indicate the need for further studies, the results showed that the giraffes were more similar to individuals from populations that occur naturally in the south of the African continent.

Keywords: Forensic, genetic identification, COI, cyt b, DNA.

Resumo

Girafas são mamíferos ruminantes nativos da África. Com as suas populações naturais diminuindo cerca de 40% nos últimos 30 anos, as girafas têm o status de "vulnerável" na Lista Vermelha da União Internacional para a Conservação da Natureza. Durante muito tempo, as girafas foram consideradas pertencentes a uma única espécie, *Giraffa camelopardalis*, embora estudos recentes sugiram a existência de quatro espécies e sete subespécies com distribuição distinta. Em novembro de 2021, 18 girafas supostamente provenientes de populações selvagens da África do Sul chegaram ao Brasil para serem utilizadas em um projeto de conservação. Logo após sua chegada, problemas com o manejo em cativeiro levaram a Polícia Federal a abrir uma investigação sobre maus-tratos e irregularidades no processo de importação. O material biológico das girafas foi enviado ao Laboratório de DNA da Polícia Federal para identificação das espécies e possíveis inferências sobre a sua origem geográfica por meio do sequenciamento de fragmentos da subunidade I dos genes mitocondriais citocromo c oxidase (COI) e citocromo b (cyt b). Embora não possam ser considerados conclusivos e apontem para a necessidade de mais estudos, os resultados mostraram que as girafas eram mais semelhantes a indivíduos de populações que ocorrem naturalmente no sul do continente africano.

Palavras-chave: Forense, identificação genética, COI, cyt b, DNA.

Resumen

Las jirafas son mamíferos rumiantes originarios de África. Con sus poblaciones naturales disminuyendo alrededor del 40% en los últimos 30 años, las jirafas tienen un estatus "vulnerable" en la Lista Roja de la Unión Internacional para la Conservación de la Naturaleza. Durante mucho tiempo se consideró que las jirafas pertenecían a una única especie, *Giraffa camelopardalis*, aunque estudios recientes sugieren la existencia de cuatro especies y siete subespecies con distinta distribución. En noviembre de 2021, 18 jirafas supuestamente provenientes de poblaciones silvestres de Sudáfrica llegaron a Brasil para ser utilizadas en un proyecto de conservación. Poco después de su llegada, problemas con el manejo en cautiverio llevaron a la Policía Federal a abrir una investigación por malos tratos e irregularidades en el proceso de importación. El material biológico de las jirafas fue enviado al Laboratorio de ADN de la Policía Federal para identificar las especies y hacer posibles inferencias sobre su origen geográfico a través de la secuenciación de fragmentos de la subunidad I de los genes mitocondriales citocromo c oxidasa (COI) y citocromo b (cyt b). Aunque no pueden considerarse concluyentes y apuntan a la necesidad de realizar más estudios, los resultados mostraron que las jirafas eran más similares a los individuos de poblaciones naturales del sur del continente africano.

Palabras clave: Forense, identificación genética, COI, cyt b, ADN.

1.Introduction

Giraffes are ruminant mammals of the Giraffidae family known for their long neck and distinctive spotty coat pattern. Native to Africa, their natural populations have suffered a 36-40% decline over the past 30 years, most likely due to habitat loss or degradation, climate change, hunting, and other factors (MULLER et al., 2018; O'CONNOR et al., 2019). Giraffes currently have a "vulnerable" status on the Red List of the International Union for Conservation of Nature (IUCN) and are listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), making a control of their trade between countries mandatory (IUCN, 2022; CITES, 2022).

For a long time, giraffes were considered to belong to a single species, *Giraffa camelopardalis*, and its various subspecies. Although this classification is still used by many, including the IUCN, genetic studies in recent years have shown that there is great variation between populations, indicating that the genus would not be monotypic (BOCK et al., 2014; FENESSY et al., 2016; WINTER; FENESSY; JANKE, 2018; PETZOLD; HASSANIN, 2020). In a recent work based on whole genomes analysis, the existence of four species and seven subspecies with distinct geographical distributions along the African continent was suggested (COIMBRA et al., 2021)

In November 2021, 18 giraffes identified as *G. camelopardalis*, supposedly coming from South African wild populations, arrived in Brazil. The import of the animals, which cost more than one million US dollars paid to an intermediary, was carried out by a Brazilian company that manages and operates wildlife parks under the justification of using them in a conservation project. Shortly after the arrival of the giraffes, problems with their handling in captivity in the state of Rio de Janeiro began to surface, including the news of the death of three individuals. After that, the Brazilian Federal Police opened a procedure to investigate the case. In addition to proving mistreatment, characterized by inadequate enclosures and insufficient food, the investigation sought to check the veracity and flaws in the information provided by the importers during the process of bringing the animals to Brazil, especially the country of origin.

Given the situation, biological material from the imported giraffes was sent to the DNA Laboratory of the Brazilian Federal Police for species testing and, if possible, to provide information about their geographic origin. The technique normally used for species testing consists of sequencing molecular markers that vary between species and comparing them with homologous sequences of known identity kept in databases (PEREIRA; CARNEIRO; AMORIN, 2008). For animal identification in the forensic area, the most common markers are the subunit I of cytochrome c oxidase (COI) and cytochrome b (cyt b) mitochondrial genes, which have been used for a long time in taxonomic and phylogenetic studies (ALACS et al., 2009; JOHNSON; WILSON-WILDE; LINACRE, 2014). Although there are differences and limitations depending on the group considered, both markers can correctly reconstruct known phylogenies and evolutionary relationships of many mammalians (TOBE; KITCHENER; LINACRE, 2010). Its use in geographic origin investigation, however, is not very common, especially due to the lack of robust populational reference databases.

Even considering that the value of COI and cyt b to investigate geographic origin can be very restricted, they can still expose a genetic pattern that are clearly incompatible with those find natural populations of giraffes from the declared place of origin of the imported animals. Therefore, the main objective of this study was to carry out some exploratory analyses to obtain information that can be useful for the investigation, also pointing out some aspects that require further examination. The case received great attention from the Brazilian media and can be considered unique in the case history of the Brazilian Federal Police, since it involved large exotic charismatic animals protected by international convention.

2. Methodology

In May 2022, the Laboratory received biological material from the 18 giraffes imported into Brazil. The items were identified by ear tag numbers or by the location of the exhumation site (in the case of dead animals). From the 15 giraffes that were still alive an oral/nasal swab was collected, whereas from the dead animals either a blood swab or 1 cm2 muscle tissue fragment obtained during their necropsies were collected. Swabs from live animals were collected through luring them to a more accessible point of the enclosure fence with some fresh leaves, avoiding any kind of containment and minimizing the stress caused by the procedure. All samples were obtained by a veterinarian. It is important to mention that Brazilian laws do not require approval by an ethics committee for animal studies carried out by official forensic laboratories, which are legally obliged to test animals in the context of criminal investigations.

DNA from samples (5993Q1-5993Q18) was extracted with the PrepFiler Express™ extraction kit using an AutoMate Express™ system (Applied Biosystems) following the manufacturer's instructions. Genetic identification followed standard protocols and primers used by the Laboratory. Segments of COI and cyt b genes were amplified with primers FishF1/FishR1 (WARD et al., 2005) and Mcb398/Mcb869 (VERMA; SINGH, 2003), respectively. 25 µl PCR reactions were performed in tubes containing 2U of AmpliTaq Gold® (Applied Biosystems), 1.5 mM of MgCl2 (Applied Biosystems), 0.2 mM of dNTPs (Promega), 0.4 mM of each primer (IDT), and 3 µl of DNA (not quantified). For the 420 base pairs (bp) cyt b fragment the cycling parameters were: an initial step of 94ºC for 11 min, 34 cycles of 94ºC for 20 s, 51ºC for 30 s, and 72ºC for 40 s; and a final step at 72º C for 10 min. For the 650 bp COI fragment, the initial and final steps were the same, but the intermediary steps con-

sisted of 35 cycles of 94ºC for 30 s, 54ºC for 30 s, and 72ºC for 1 min. Positive and negative controls were used to check for failed PCRs and contamination. Amplification products were purified with FastAP™ (Thermo Scientific) and Illustra™ Exonuclease I (GE Healthcare), sequenced on both ways using the Big Dye™ Terminator v3.1 (Applied Biosystems) sequencing kit, and purified by EDTA/ethanol precipitation, always following the manufacturers' protocols. Capillary electrophoresis was performed using an ABI 3500 genetic analyzer (Applied Biosystems). Sequences were assembled and had their quality assessed using SeqScape 3 (Applied Biosystems) and MEGA 11 (TAMURA; STECHER; KUMAR, 2021) software. To obtain similarity values (%) and check the identity of the samples, Cyt b and COI sequences were queried in GenBank (BENSON et al., 2013), and COI sequences also queried in the Species Level Barcode Records database of BOLD (RATNASINGHAM; HEBERT, 2007). The identification methodology was tested in advance with samples from three giraffes belonging to Brazilian zoos. These individuals, a female from the Brasília Zoo and two males from the Beto Carrero Zoo, in Santa Catarina, had already been analyzed previously and associated to *Giraffa giraffa* (FALKOWSKI, 2020).

Subsequently, to access a possible indication of populational origin, phylogenetic analyzes were carried out with sequences from the questioned samples and homologous cyt b and COI sequences obtained from GenBank. We used sequences of *Giraffa* individuals identified to the level of species or subspecies and *Okapia johnstoni* (okapi) as outgroup. We also included in the analyzes sequences obtained in this study from the three giraffes of Brazilian zoos. Maximum Likelihood (ML) trees were constructed using the Hasegawa-Kishino-Yano (HKY+G) model (HASEGAWA; KISHINO; YANO, 1985), and 500 bootstrap replications with MEGA 11 software.

DNA aliquots of all individuals sequenced and sexed in the present study are stored in the Laboratory at -20ºC.

3. Results

All 18 questioned samples originated sequences with the expected size, although some of them slightly shorter than others due to suboptimal sequencing readings of small segments immediately adjacent to the primers annealing sites. When comparing the same positions, however, all cyt b and COI sequences showed to be identical, and for the subsequent analyzes the sequences identified as "5993Q3" from each fragment was considered representative of the others. Samples from the Brazilian zoos also originated sequences with the expected size. GenBank accession numbers are OQ745815-OQ745818 and OR256269-OR256285 for COI sequences, and OQ744028-OQ744031 and OR259413-OR259429 for cyt b sequences.

Querying of sequence "5993Q3" of both fragments in the two databases resulted in high similarity with giraffe sequences. Querying the COI sequence in BOLD resulted in 99.38% of maximum similarity with *Giraffa camelopardalis* (3 first hits of the list of results provided by the database), emphasizing that in this database there are only 11 sequences of *Giraffa*, all identified as *G. camelopardalis* (four of them with attribution of the subspecies). On the other hand, querying the COI sequence in GenBank resulted in 100% similarity the subspecies *G. giraffa giraffa* (five first hits of the list provided by the database), although sequences of *G. tippelskirchi* also showed high similarity (>99%). Querying the cyt b sequence in GenBank resulted in 100% similarity with sequences of the subspecies *G. giraffa giraffa* (14 first hits of the list provided by the database). Two sequences from *G. g. angolensis* and two of *G. tippelskirchi* also showed the same percentage of similarity (100%).

Phylogenetic analyzes using the two markers resulted in similar clustering patterns. In both situations the questioned sequences, represented by the sequence "5993Q3" in the constructed trees, formed monophyletic clades with sequences identified as *G. g. giraffa* and the reference sequence from the Brasília Zoo (previously associated to *G. giraffa*). We decided to construct the trees with only one questioned sequence ("5593Q3"), since the use of identical sequences add no information for the phylogenetic trees and increases the computation time. The results are supported by the bootstrap values, especially for the cyt b marker (Figures 01 and 02).

4. Discussion

The cyt b and COI sequences of the 18 individuals showed to be identical, except for small differences in the total length. Although the markers used here are not suitable for kinship analyses, since they normally have low interspecific variability (TOBE; KITCHENER; LINACRE, 2010), the possibility that all individuals belong to the same maternal lineage cannot be discarded, since not a single base difference was found. If this is the case, the reproduction of these individuals in captivity would have to consider the negative consequences of inbreeding, including an increase in homozygosity, which allows for the expression of harmful recessive alleles that can affect the survival and fertility rates of the offspring of related individuals (CHARLESWORTH; WILLIS, 2009). Further studies using STRs and other markers would be recommended to clarify this issue.

As expected, querying the cyt b and COI sequences in the two databases used in this study allowed the association of all individuals to *Giraffa sp.* The results obtained here corroborate previous studies that successfully used these markers and databases to identify mammals for regulatory and forensic purposes (EATON et al., 2009; DALTON; KOTZE, 2011; SANCHES et al., 2012, D'AMATO et al., 2013, CHEN et al., 2015, DALTON; KOTZE, 2020). It is important to mention, however, that the representation of the genus *Giraffa* in GenBank is much higher than in BOLD's Species Level database, which makes it more informative in cases where animals from this group are investigated.

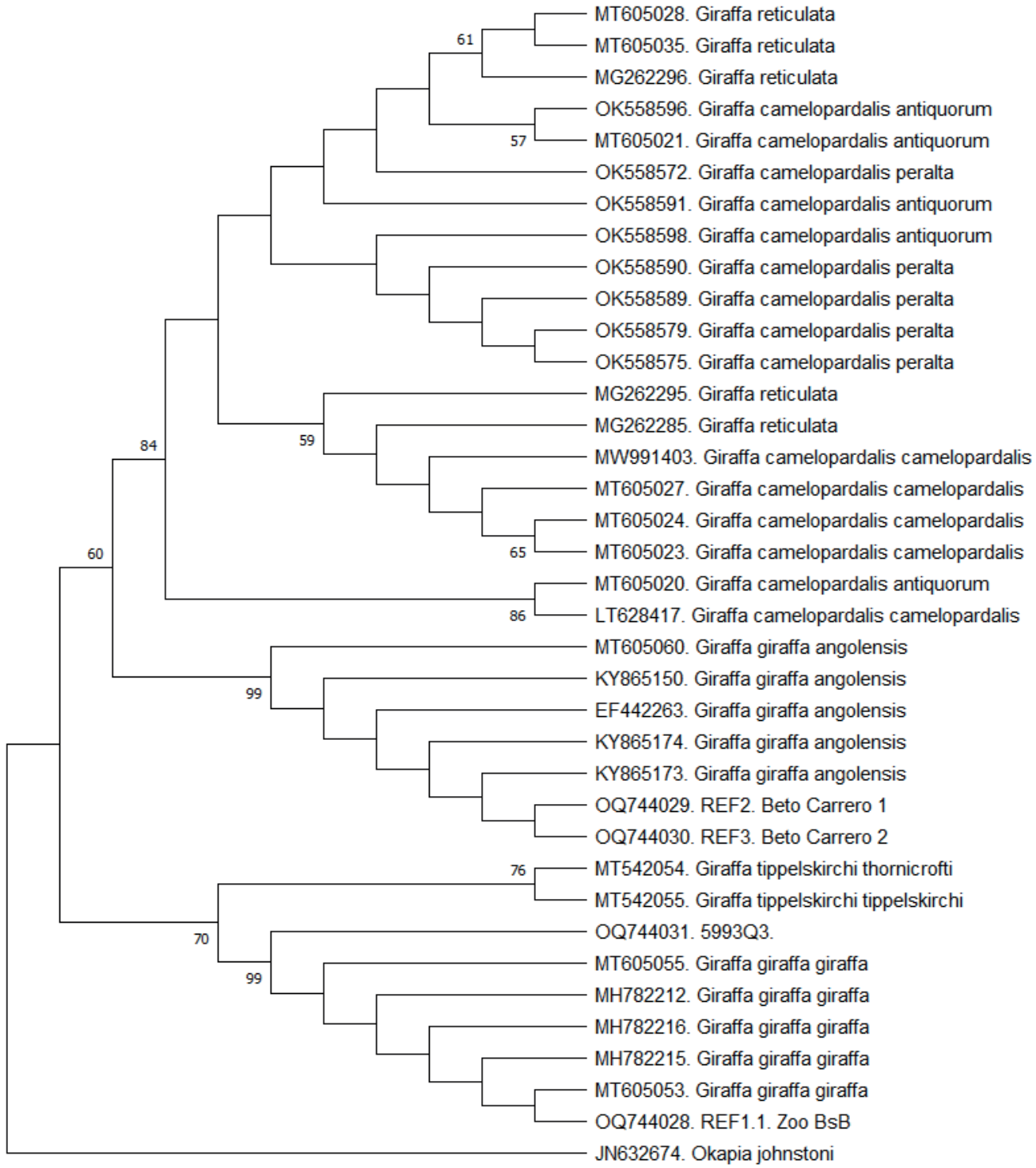

Figura 01: *Cyt b ML tree (HKY+G), bootstrap 500 (values below 50 are omitted). In the figure, "5993Q3" is the representative sequence of the 18 sequenced animals, and "REF" refers to the sequences of the animals from the Brazilian zoos sampled for this study (GenBank accession numbers are provided).*

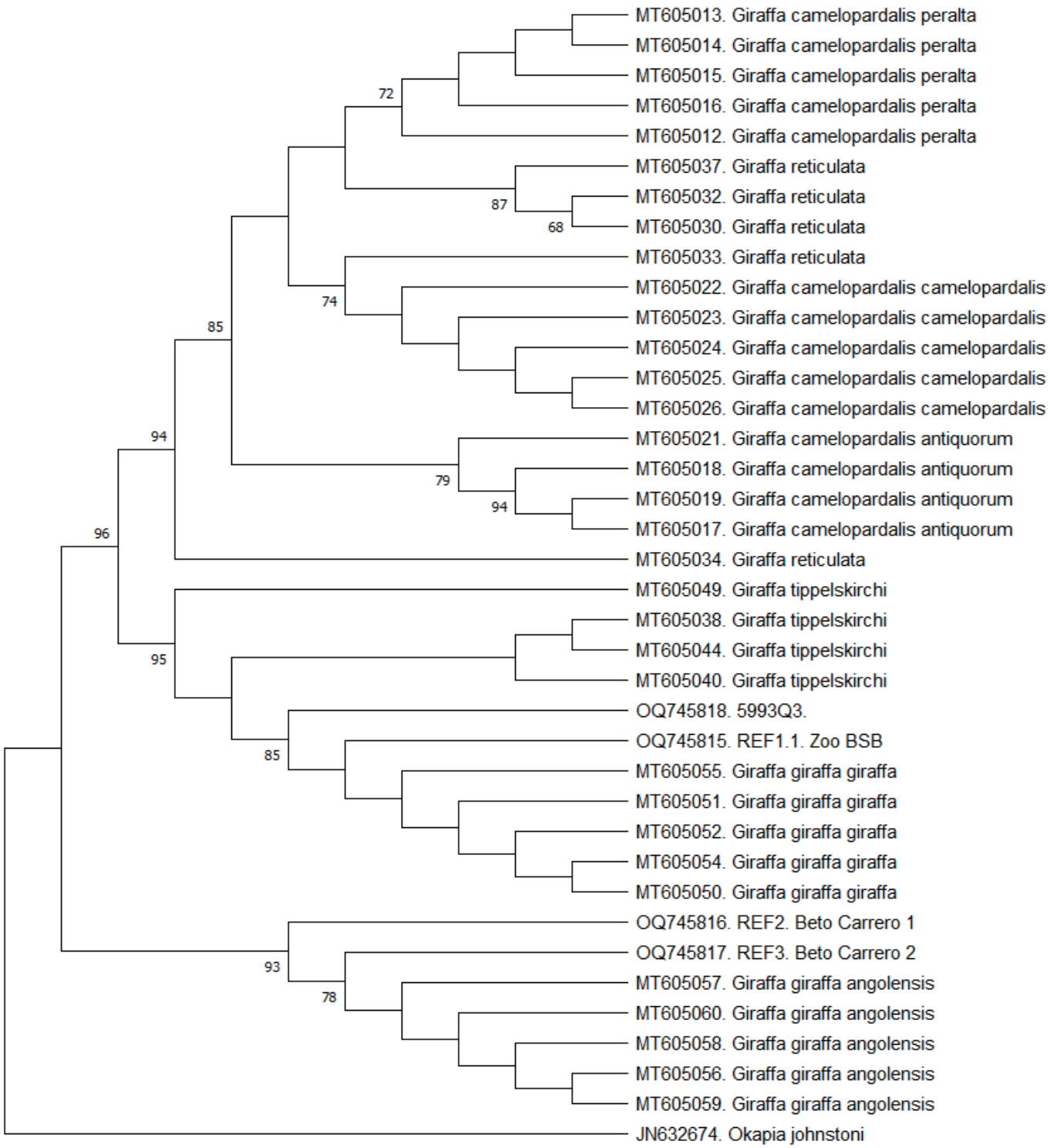

Figura 02: *COI ML tree (HKY+G), bootstrap 500 (values below 50 are omitted). In the figure, "5993Q3" is the representative sequence of the 18 sequenced animals, and "REF" refers to the sequences of the animals from the Brazilian zoos sampled for this study (GenBank accession numbers are provided).*

Nevertheless, even results obtained with the use of highly representative databases, such as GenBank, must be considered with care, depending on the group. In the present case, besides *G. g. giraffa*, sequences of *G. tippelskirchi* also presented high similarity with the queried COI sequence, which could make identifications based solely on this marker problematic. Considering the queried cyt b sequence, in addition to *G. g. giraffa*, sequences from two other subspecies/species (*G. g. angolensis* and *G. tippelskirchi*) also showed the same percentage of similarity (100%). However, bearing in mind that there are dozens of other sequences from *G. g. angolensis* and *G. tippelskirchi* in GenBank that showed lower similarity (<99%), and that phylogenetic analyzes conducted here resulted in these subspecies/species grouping into clades other than the one formed by 5993Q3 and *G. g. giraffa*, it is possible that these sequences are from more genetically diverse or misidentified individuals.

Coimbra et al. (2021) stated that *Giraffa camelopardalis* would comprise three subspecies, *G. c. antiquorum*, *G. c. camelopardalis* and *G. c. peralta*, and would be found in North Africa, in the limits of the sub-Saharan region. *Giraffa reticulata*, with no subspecies, and *G. tippelskirchi*, with two subspecies, *G. t. tippelskirchi* and *G. t. thornicrofti*, would occur further to the center of the continent. Finally, *Giraffa giraffa*, with two subspecies, *G. g. angolensis* and *G. g. giraffa*, would be found in the south, with populations in South Africa, Namibia, Angola, Botswana, Zimbabwe, Zambia, and Mozambique. Although the taxonomy of the group is still an object of discussion by some authors, the results of the phylogenetic analyzes carried out showed that giraffes imported to Brazil are more similar to individuals of the subspecies *G. g. giraffa*, which are mainly found in south of the African continent. Therefore, it is possible that the giraffes brought to Brazil were, in fact, originated from South African wild populations, as indicated by importers. It is noteworthy that the analyzes performed here are not conclusive, since they were based on only two mitochondrial markers and with a very limited number of reference sequences. The use of nuclear markers, or even entire genomes, associated with a greater number of reference sequences from different giraffe populations is essential to safely determine the place of origin of the animals.

According to a study by Falkowski (2020) with mitochondrial markers, the 14 individuals of *Giraffa sp*. existing in Brazilian zoos in 2020 would belong to two distinct species, *G. giraffa*, and *G. camelopardalis*, showing greater or lesser genetic proximity to their different evolutionary branches. Given the results, the author makes recommendations for more efficient breeding of these individuals, avoiding deleterious results for the species, such as hybridization. In this sense, the absence of more precise taxonomic information regarding the imported giraffes, based on genetic tests, for example, represents a serious problem. In addition to preventing a better understanding of the proposed conservation action, it would pose risks in terms of reproductive success if the imported animals were used in crossings with the existing giraffes in Brazil.

5. Conclusions

Although not conclusive in many aspects, the analysis of DNA sequences made possible, among other things, to indicate that the animals are more similar to individuals from populations that occur naturally in the south of the African continent. These data provided some elements to help the investigation conducted by the Brazilian Federal Police related to the import process but suggest further analyzes to confirm the results presented here and to clarify other aspects of the case. In general, the results confirm the importance of using molecular tools in the forensic area, especially in the investigation of wildlife crimes. In megadiverse countries, where this type of occurrence is quite common and represents a serious risk to the survival of the native species, the widespread use of these techniques would be very important and should be encouraged.

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