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Molecular sexing of *Panthera onca* and *Puma concolor* (Mammalia, Felidae) for forensic purposes

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Resumo

A onça-pintada (*Panthera onca*) e a onça-parda (*Puma concolor*) são, respectivamente, o primeiro e o segundo maiores felinos das Américas. Embora protegidas por lei, ambas as espécies enfrentam ameaças significativas no Brasil, incluindo a perda de habitat e a caça ilegal. Considerando essa situação, novas ferramentas que auxiliem as autoridades responsáveis pelo monitoramento e investigação de crimes envolvendo esses dois grandes felinos são de grande importância, incluindo protocolos de sexagem molecular. Na área forense, esses protocolos podem ser usados para esclarecer aspectos das investigações de crimes contra a vida selvagem e podem ter influência nas penas aplicadas aos responsáveis. Estudos anteriores já demonstraram que protocolos baseados no gene SRY são eficientes para a determinação do sexo em muitas espécies. Os principais objetivos deste estudo foram testar para *P. onca* e *P. concolor* um protocolo simples de sexagem molecular previamente descrito para outras espécies de mamíferos e aplicá-lo em amostras forenses, tais como ossos e dentes, recebidas para análise pelo Laboratório de DNA da Polícia Federal. Os testes foram bem-sucedidos e permitiram a sexagem de todas as amostras, confirmando a eficiência da região SRY para esse propósito e a aplicabilidade da metodologia proposta para onças-pintadas e pumas.

Palavras-Chave: Sexagem molecular, Felidae, Conservação, Forense, Brasil

Abstract

The jaguar (*Panthera onca*) and the puma (*Puma concolor*) are, respectively, the first and second largest felids in the Americas. Although protected by law, both species face significant threats in Brazil, including habitat loss and illegal hunting. In this context, tools that help authorities responsible for monitoring and investigating crimes involving these two big cats are of great importance, including molecular sexing protocols. In forensic investigations, these protocols can clarify aspects of wildlife crime cases and may influence the penalties applied to offenders. Previous studies have demonstrated that SRY-based protocols are efficient for sex determination in many species. The main objectives of this study were to test for *P. onca* and *P. concolor* a simple molecular sexing protocol previously described for other mammal species and to apply it in bones and teeth samples received for analysis by the Brazilian Federal Police DNA Laboratory. The tests were successful and determined the sex of all samples, confirming the utility of the SRY region for this purpose and demonstrating the applicability of the proposed method to jaguars and pumas.

Keywords: Molecular sexing, Felidae, Conservation, Forensics, Brazil

1. INTRODUCTION

The jaguar (*Panthera onca*) and the puma (*Puma concolor*) are, respectively, the largest and second largest felids in the Americas [1]. While the former species occurs from the southern United States, where it is almost extinct, to northern Argentina, the latter is distributed from southwestern Canada to the southern tip of South America. Both species occur in most of Brazil [2,3]. Due to a suspected 20-25% decline in area occupancy in the last 21 years, the jaguar is classified as "Near threatened" by IUCN [3]. It is also listed on CITES Appendix I, which includes all species threatened with extinction that are or may be affected by trade [4]. Although it is also experiencing a population decline, the broad distribution of the puma causes the species to be listed as Least Concern by the IUCN [2] and on CITES Appendix II, with some populations on Appendix I [4].

In Brazil, the jaguar is classified as "Vulnerable", while the puma has been recently removed from the list of endangered species [5]. Both species are protected, and their killing or unauthorized capture is considered a crime according to the country's laws. Despite the protection status, jaguars and pumas continue to face significant threats in Brazil, including illegal hunting, whether for sport or in retaliation for livestock predation, and habitat loss. Habitat loss by anthropogenic modifications in the landscape includes deforestation, wildfires, conversion of native grasslands and wetlands for intensive agriculture and livestock farming, in addition to the flooding of vast areas to create reservoirs for hydroelectric power plants [6]. In recent years, in Brazil, many hunting expeditions involving these species have been posted on social media, some of them presenting scenes of extreme cruelty [7,8]. Another threat to the species that must be considered is the trafficking of feline parts to international markets. A recent study identified an increase of seizures of jaguar body parts, mostly teeth, from Central and South American countries to supply the Chinese market, where they are used as jewelry or decorative items [9].

Considering this scenario of threats to wild populations, new tools to support authorities in monitoring and investigating crimes involving these two big cats are of great importance, including molecular sexing protocols. These protocols have been used in several situations, such as ecological studies, conservation genetics, and forensics, where they can be essential in determining the occurrence of a crime when biological evidence is limited [10]. For example, a study in Tanzania with leopard skins taken by trophy hunters found that almost 30% of them were from females, even though only males were legally allowed to be hunted [11].

Although hunting any native species is illegal in Brazil and current legislation does not consider the sex of the animal as an aggravating factor, sex identification can help persuade judges to apply more severe penalties for the killing of females, especially in species where they have a pivotal ecological or demographic importance. Females of many felines are the sole providers of parental care and play a crucial role for the survival of their offspring until they reach independence, which may take many months or years in some cases [12,13]. In addition, molecular sexing protocols can be applied in other situations, such as to access patterns of sex-based animal poaching and trafficking or helping to determine the minimum number of animals in a killing site. In the absence of individualizing techniques, molecular sexing of animal remains can determine the presence of at least two individuals at a crime scene if different sexes are identified. This kind of information may have legal implications in Brazil, since the number of animals involved influences the criminal case and the penalties applied to the offenders.

For mammals, molecular sexing protocols use sex-specific markers, such as the Y chromosome sex-determining gene (SRY), which is responsible for the development of male gonads and consequent phenotypic masculinization [14]. Previous studies have demonstrated that SRY-based protocols are efficient for sex determination in many species, including jaguars and pumas [15]. Therefore, the main objectives of this study were to test for *P. onca* and *P. concolor* a simple molecular sexing protocol previously described for other mammal species and to access its suitability for application to forensic samples, such as bones and teeth, received for analysis by the Brazilian Federal Police DNA Laboratory. The tested protocol represents an option for studies with both species and has the potential to contribute to their conservation.

2. METHODS

Molecular sexing was conducted using a protocol described for South and Southeast Asian mammals [16]. This protocol uses the primers Y53-3C/Y53-3D [17] to amplify a 225 bp fragment of the SRY gene, and the primers 12Sa/12So [18] to amplify a 151 bp fragment of 12S rRNA mitochondrial (12S) gene as an internal control.

DNA reference samples from two males and two females of each species (*P. onca* and *P. concolor*) were used to validate the protocol. These samples were obtained from animals studied during genetic research projects conducted by the Pontifical Catholic University of Rio Grande do Sul (PUCRS), Brazil. DNA samples were quantified with Qubit™ dsDNA HS or BR kits in a Qubit® 2.0 fluorometer (Thermo Fisher Scientific).

The target fragments were co-amplified in 25 µl PCR reactions containing 2U of AmpliTaq Gold® (Applied Biosystems), 1.5 mM of MgCl2 (Applied Biosystems),

0.2 mM of dNTPs (Promega), 0.4 µM of primers SRY (IDT), 0.12 µM of primers 12S (IDT), and 5 ng total DNA. A negative control (ultrapure water) was included to check for contamination. The cycling parameters were as follows: 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. SYBR® Green (Bio-Rad) stained amplification products, with a 100 bp DNA ladder, were checked under UV light after electrophoresis in 2% agarose gels.

Amplification products from one male and one female of each species were sequenced to confirm the identity of the amplified fragments. They were purified with Nucleosap (Cellco) and sequenced for both strands using the Big Dye™ Terminator v1.1 sequencing kit (Applied Biosystems). After EDTA/ethanol precipitation, 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 [19]. Consensus sequences were queried with BLAST in GenBank [20] to check their identity.

After validation, the protocol was applied to five unsexed *P. onca* and *P. concolor* DNA samples available at the Brazilian Federal Police DNA Laboratory. These samples originated from the bone collection of the Brazilian Federal Police Forensic Zoomorphology Laboratory or from previous forensic casework (Tab. 01). DNA extraction from all items was carried out using the same protocol, although at different dates. After surface cleaning by abrasion and pulverization of 0.5 cm³ fragments in a cryogenic mill (Freezer Mill), DNA from 50 mg of the resulting powder was extracted with PrepFiler™ BTA extraction kit in an AutoMate Express™ system (Applied Biosystems). Following the extraction, DNA samples were kept at -20ºC until use.

Table 1. Source of DNA samples sexed with the protocol (all samples from the bone collection of the Brazilian Federal Police Forensic Zoomorphology Laboratory or from previous forensic casework).

Item	Species	Origin	DNA extraction (year)
Tooth	*P. onca*	Airport seizure in 2024	2024
Skull	*P. onca*	Bone collection*	2024
Skull	*P. onca*	Bone collection*	2024
Skull	*P. concolor*	Bone collection*	2024
Bone (rib)	*P. onca*	Dry bone from a 2020 killing site	2020
Bone (fibula)	*P. onca*	Dry bone from a 2020 killing site	2020

*Collection year uncertain. At least 4 years old. Possibly boiled to be cleaned.

3. RESULTS AND DISCUSSION

When applied to the reference samples, the protocol used here yielded consistent and unambiguous results, with two clear bands within the expected size for males and a single band for females (Fig. 01 and 02). The sequencing of amplified fragments from selected samples was successful and matched sequences from the expected regions. When queried in GeneBank, sequenced fragments from females resulted in 100% identity with 12S sequences from the expected species (*P. onca* or *P. concolor*). Sequences from males resulted in 100% identity with SRY and 12S sequences, also from the expected species. The obtained sequences are available at GenBank (PX102575, PX102576 and PX103752-PX103755). The results confirm the efficiency of the SRY region for sexing mammals and the applicability of the method described in [16] for jaguars and pumas.

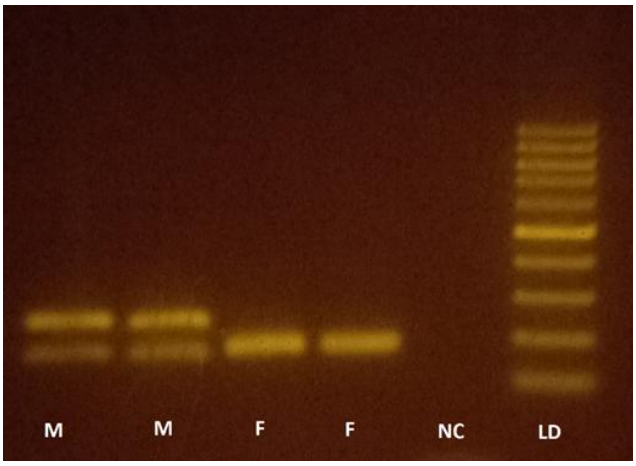

Figure 01. Molecular sexing of DNA reference samples from *P. onca* (M = male; F = female; NC = negative control and LD = 100 bp ladder).

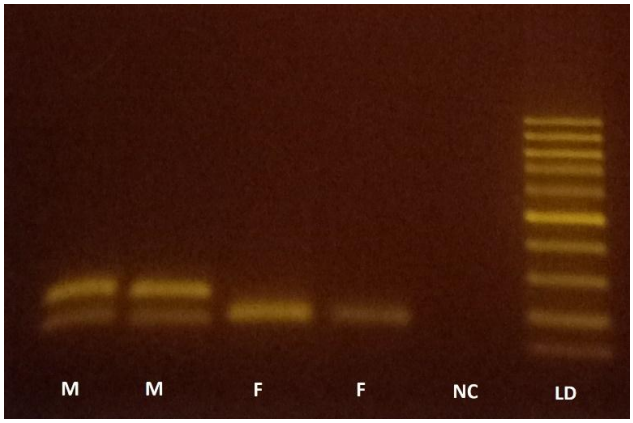

Figure 02. Molecular sexing of DNA reference samples from *P. concolor* (M = male; F = female; NC = negative control and LD = 100 bp ladder).

Although only a small number of specimens were tested here, it was observed that the protocol also presented good results when applied to DNA samples derived from poorly preserved tooth and bones. This kind

of material often presents low-quality DNA and represents most samples received by the Laboratory. One of the skulls of *P. onca*, the skull of *P. concolor* and the tooth sample came from males, while the other samples came from females (Fig. 03). It is worth mentioning that, although visible, the SRY band from the *P. concolor* skull sample was fainter than the SRY bands from other samples. This result could be explained by DNA degradation, a process that reduces the amount of template available for amplification, affecting PCR efficiency and the intensity of bands in agarose gels [21]. Also, it is known that, in degraded samples, the higher number of copies per cell favors mitochondrial DNA amplification when compared to the nuclear DNA [22], which explains the clear 12S band from the same sample. Considering this information, in situations of extreme sample degradation, there would be a possibility to observe false females, which are males whose SRY band amplification failed. In fact, some molecular sexing protocols use fragments of single-copy nuclear genes as an internal control to have a better assessment of the quality of samples [15.16]. This same approach could be used in a future improvement of the protocol presented here.

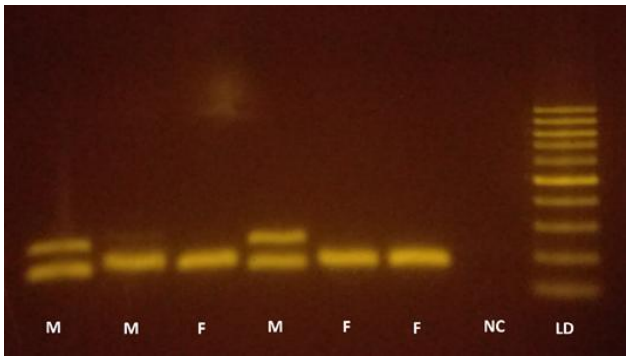

Figure 03. Molecular sexing of bone and tooth DNA samples, from left to right *P. onca* skull; *P. concolor* skull; *P. onca* skull; *P. onca* tooth; *P. onca* fibula; and *P. onca* rib (M = male; F = female; NC = negative control and LD = 100 bp ladder).

4. CONCLUSION

The results confirm the efficiency of the SRY region for sexing mammals and the applicability of the proposed methodology to jaguars and pumas. In addition to a wide range of other applications, molecular sexing techniques are crucial in forensics, where they can be used to determine the sex of animal remains or products. This kind of information is very important to clarify many aspects of wildlife crime investigations, including illegal hunting and trafficking of animal parts, and have the potential to influence the penalties applied to the offenders. The protocol tested here presented good results, although future studies with a larger number of forensic samples preserved under different conditions would be desirable. Besides, the use of a fragment of a single-copy nuclear gene as an internal control would represent a methodological improvement, making the molecular sex determination in extremely degraded samples more accurate.

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DECLARATION

Brazilian laws do not require a permit or approval by an ethics committee for animal studies carried out by official forensic laboratories, such as the Brazilian Federal Police DNA Laboratory, which are legally required to use biological material from animals in the context of criminal investigations or to develop protocols to be used in these situations. Blood samples, source of DNA reference samples, were collected under the permits IBAMA 002/1998, IBAMA 12/2003, IBAMA 12/200, SISBIO 21447-4, IMAP 011/2002, IBAMA 251/2001 and IBAMA 277/2005.

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