Sex determination of the Pampas deer (Ozotoceros bezoarticus) via high-resolution melting analysis

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Research Article

Keywords: Amelogenin, KY1/KY2 Cervidae, Neotropical, HRM

Posted Date: March 31st, 2025

DOI: https://doi.org/10.21203/rs.3.rs-6264248/v1

Version of Record: A version of this preprint was published at Mammal Research on July 3rd, 2025. See the published version at https://doi.org/10.1007/s13364-025-00803-3.

Abstract

We present a fast and reliable genetic method for sexing Pampas deer (*Ozotoceros bezoarticus*) via high-resolution melting (HRM) analysis of amplicons obtained through real-time polymerase chain reaction (PCR-RT). This method employs the KY1/KY2 primer set to amplify an informative fragment of the *Amelogenin* gene that accurately determines the sex of Pampas deer. This approach is effective for analyzing noninvasive samples with low DNA quality and quantity. Additionally, this protocol can be easily adapted for sexing noninvasive samples from other deer and ungulate species.

Introduction

Pampas deer (*Ozotoceros bezoarticus* L. 1792) is a Neotropical species that was once abundant across the grasslands and savannas of South America, including Uruguay. Over the past two centuries, it has undergone a dramatic decline, primarily due to habitat loss and poaching (González et al. 1994, 1998, 2010). In Uruguay, two endemic subspecies, *O. b. uruguayensis* and *O. b. arerunguaensis*, are recognized. The species is currently classified as Critically Endangered (CR) in the country and Near Threatened (NT) across its entire range, according to the IUCN Red List™ of Threatened Species (González et al. 2016). Several studies have been conducted to estimate the population structure of Pampas deer in Uruguay and other regions of South America (Merino et al. 2011; Semeñiuk 2013; Cosse and González 2013). With only a few wild populations remaining, monitoring and conservation efforts are essential.

Sex determination is crucial for understanding population dynamics, exploring ecological variables such as parasite persistence or dietary differences, and predicting population viability (Lacy 2000). Recent advancements, such as real-time polymerase chain reaction (PCR-RT), have significantly improved the accuracy and efficiency of sex determination, especially for degraded or low-quality DNA samples commonly used in noninvasive studies (Beja-Pereira et al. 2009; Zemanova 2010; González et al. 2015). Additionally, the incorporation of high-resolution melting (HRM) analysis into PCR-RT allows for the differentiation of PCR products with greater speed and reliability. This eliminates post-PCR processes, such as agarose gel electrophoresis and, in some cases, sequencing, thereby reducing the risk of contamination and the time required to obtain results.

A widely utilized approach for sex determination relies on the *Amelogenin* gene (AMEL), which encodes a protein present in the tooth enamel of certain vertebrates and is highly conserved across mammals (Gurgul et al. 2010). The gene exists in two variants located on the sex chromosomes, AMEL-X and AMEL-Y, with a length difference between these sequences enabling sex determination through polymerase chain reaction (PCR) followed by agarose gel electrophoresis. This method offers clear advantages over SRY gene amplification, particularly when dealing with degraded samples, as it coamplifies an internal control to ensure proper PCR performance (Yamauchi et al. 2000). The AMEL gene is especially useful for sex determination in placental mammals such as humans, cattle, and wild ruminants (Akane et al. 1991; Nakahori et al. 1991; Ennis and Gallagher 1994; Yamauchi et al. 2000; Pajares et al. 2007; Brinkman and Hundertmark 2009; Oliveira and Duarte 2013; Zenke et al. 2022).

In previous studies, the use of various primers to amplify small fragments (approximately 140–280 bp) of the AMEL gene, such as SE47/48 and KY1/KY2 (Yamauchi et al. 2000; Brinkman and Hundertmark 2009), has proven effective for sex determination in cattle, wild ruminants, Holartic deer, and red brocket deer (Oliveira and Duarte 2013). However, until now, the effectiveness of these markers for sex determination in Pampas deer has not been tested. In this study, we aimed to evaluate the use of KY1/KY2 primers on Pampas deer (*Ozotoceros bezoarticus*) samples. Additionally, we designed a PCR-RT protocol to improve the efficiency of sex determination across samples of varying origins and DNA qualities, offering a potentially useful tool for the conservation of this endangered species.

Materials and methods

Samples and DNA extraction procedures

Various sample types collected from living animals (blood, hair and faeces) and from dead animals (tissues from muscle, skin and bones) over a 20-year period and deposited at the Genetics Tissue and DNA Bank of the Department of Biodiversity were evaluated. (See Online resource Tables 1 and 2). DNA extraction was performed via the QIAamp Fast DNA Stool Mini Kit for feces, the DNeasy Blood & Tissue Kit by QIAGEN for tissue and blood, and the protocol developed by González et al. (2015) for the other types of samples.

Table 1
Detailed number and type of DNA samples from the two Uruguayan subspecies of *Ozotoceros bezoarticus* amplified by PCR end point and tested on 2% agarose Gel.

Subspecies	Type	N	Male	Female
Ozotoceros bezoarticus arerunguaensis	Muscle	1	0	1
	Tissue	1	0	1
	Hair	6	2	4
	Skin Tissue	15	10	5
	Blood	10	7	3
	Faeces	2	2	0
Ozotoceros bezoarticus uruguayensis	Blood	2	0	2
	Tissue	3	2	1
Total		**40**	**23**	**17**

Table 2
Detailed numbers of different type of samples amplified by RT-PCR from the two *Ozotoceros bezoarticus* subspecies in Uruguay are provided. The average melting peak temperatures for the X and Y linked products are reported along with their standard deviations, indicated using ±. ND: No Data.

Supspecies	Type	N	Male	Female	X linked product peak (°C)	Y linked product peak (°C)
Ozotoceros bezoarticus arerunguaensis	Skin Tissue	33	20	13	84.1 ± 1.5	88.2 ± 0.5
	Bones and skin tissue	1	0	1	83.1	ND
	Faeces	16	10	6	83.70 ± 0.9	88.46 ± 0.6
	Hair	1	0	1	83.6	ND
Total samples and mean values		**51**	**30**	**21**	**83.74 ± 1.6**	**88.42 ± 0.7**
Ozotoceros bezoarticus uruguayensis	Muscle	2	1	1	84.75 ± 1.3	87.7
	Skin tissue	2	0	2	84.79 ± 0.4	ND
Total samples and mean values		**4**	**1**	**3**	**84.77 ± 0.9**	**ND**
Total		**55**	**31**	**24**	**84.04 ± 1.3**	**88.3 ± 0.55**

PCR protocols

We used the KY1/KY2 primers described by Yamauchi et al. (2000) to amplify a fragment of the *amelogenin* gene. The PCRs included 1 µl of DNA template (60–120 ng for end-point PCR and 25–35 ng for PCR-RT), 5 µl of ImmoMix reaction mix (for endpoint PCR) and SensiFAST™ (for PCR-RT), 0.2 µl of 10 mM of each primer (KY1 and KY2), and Milli-Q water to a final volume of 10 µl.

We developed two distinct PCR protocols. For endpoint PCR, we included an initial denaturation step at 95°C for 3 minutes, followed by cycling at 95°C for 60 seconds, 68°C for 30 seconds, and 72°C for 60 seconds. A final extension step was performed at 72°C for 15 minutes. For PCR-RT, we used a similar protocol, performed in a Mic qPCR Cycler (Bio Molecular Systems), adjusting the annealing temperature to 65°C and adding a final high-resolution melting (HRM) analysis melting curve step with a 72–95°C temperature ramp, increasing by 0.3°C per step. The cycling performance and HRM melting analysis results were inspected via MicPCR v2.12.6, and the average melting peaks with the corresponding standard deviations were calculated.

Agarose gels for visualization of the PCR products were prepared with 50 ml of TBE buffer and 1 g of Sigma agarose (2%) and stained with GoodView™. We obtained results similar to those of Brinkman and Hundertmark (2009) via agarose gel electrophoresis. Individual gel bands from two individuals of each

sex were excised via a sterile scalpel blade and purified with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research). The final products were sequenced via the Macrogen service in Seoul, Korea.

Results and Discussion

The PCR end point protocol, which uses the KY1/KY2 primers, unambiguously assigns the sex of the control samples (Table 1). The amplicons obtained consistently presented a single band (> 200 bp) for females and a two-band pattern (> 200 bp and < 200 bp) for males (Online Resource Fig. 1). We successfully amplified only two samples from 40 individuals belonging to the two Uruguayan subspecies from faecal DNA. These results align with those reported in previous studies on other mammals via conventional PCR (Brinkman and Hundertmark 2009). The sequences of the X- and Y-linked products obtained in this study were deposited in GenBank under the following accession numbers: PV130447, PV130448, PV130449, PV166110 and PV166111. The complete comparison of SNPs (Single Nucleotide Polymorphisms) between the individuals from Brinkman and Hundertmark (2009) and our sequences is provided in Table 3 of the online resource. The alignment analysis revealed a difference of 46 bp between the X- and Y-linked products due to the presence of InDels in the Y chromosome (Online resource Fig. 2). This is attributed to a tandem-repeat motif (“tgcagcccy”), which has already been detected in previous studies on caribou, mountain goats, and Sitka black-tailed deer (Brinkman and Hundertmark 2009).

The RT—PCR protocol effectively amplified various sample types from the two *Ozotoceros bezoarticus* subspecies in Uruguay. The HRM protocol efficiently determined the sex of each sample and showed greater effectiveness in amplifying low-quality DNA, such as that extracted from faeces, bones or small amounts of hair. In total, we amplified 4 samples of *O. b. uruguayensis* and 51 samples of *O. b. arerunguaensis*, 16 of which were from faecal DNA. These samples could not be amplified with conventional PCR. As expected, the HRM analysis revealed a pattern of two melting curves in the case of known-sex males and a single curve in the case of known-sex females in all the samples tested (Fig. 1; Table 2).

The average melting temperature for the X allele was 84.04 ± 1.34°C, whereas that for the Y allele was 88.3 ± 0.55°C (Online Resource Table 2). As previously shown, the differences in the dissociation curve patterns are attributed to variations in the base composition and InDels of the amplicons.

The advantage of this method is that it provides a fast (under 2.5 hours) and efficient way to unambiguously determine sex from different DNA sources, saving considerable amounts of time and laboratory consumables and increasing the efficiency of processing samples with degraded or low-quality DNA. Additionally, HRMA is a useful tool for performing population genetic surveys in wildlife areas via noninvasive sampling methods that focus on faecal DNA for sex determination of Neotropical deer (Beja-Pereira et al. 2009).

This molecular sexing method represents a powerful tool for monitoring and conserving threatened species such as the pampa’s deer. By enabling accurate sex determination from faecal DNA, it facilitates

the study of population dynamics and provides critical insights into the long-term viability of emerging populations within their geographic range (Lacy 2000). This approach not only minimizes disturbance to individuals but also enhances our capacity to implement effective conservation strategies in previously unexplored areas.

Declarations

Statements and declarations

The authors declare that they have no competing interests.

Acknowledgments

Special thanks to landowners from Salto and Rocha and personnel from Estación de Cria de Fauna Autóctona (ECFA).

Compliance with ethical standards

Sample collection was performed with DINABISE authorization (03/23) and the CEUA protocol (02/23).

Disclosures and declarations

The research followed ethical standards.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Funding

This research was supported by Agencia Nacional de Investigación e Innovación (ANII), Programa de Desarrollo de Ciencias Básicas PEDECIBA, Comisión Sectorial de Investigación Científica de la Universidad de la República, (CSIC-UdelaR), and Environmental Minister DINAMA (Resolution 644/2019) from Uruguay and the Continuation Whitley Fund for Nature from the United Kingdom.

Author contributions

All authors contributed to the conception or design of the work or the acquisition, analysis, or interpretation of data for the work and final approval of the version to be published. Susana González was responsible for funding the research.

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Figures

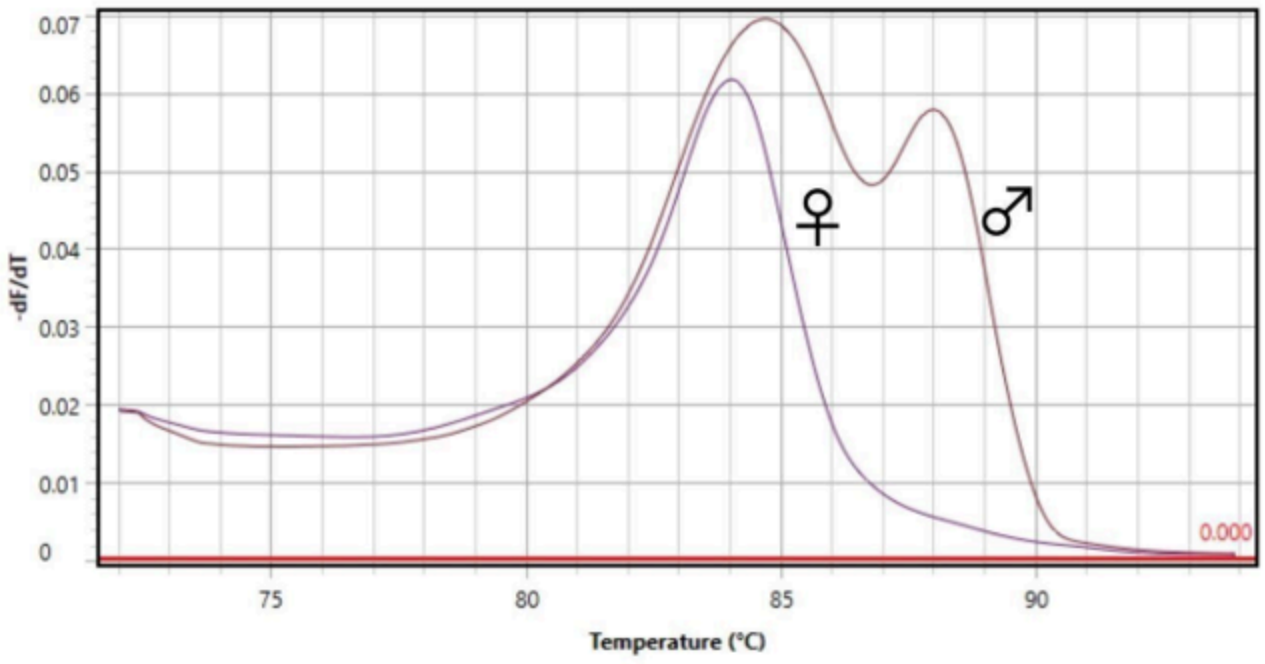

Figure 1

High-resolution melting curves for X- and Y-linked alleles on the amelogenin gene of *Ozotoceros bezoarticus* using the KY1/KY2 primer set. The female sample of SG 320 (“”) presented a single peak

corresponding to the X-linked allele, whereas the male sample of SG 397 (“”) presented two peaks corresponding to the alleles from both the X and Y chromosomes.

Supplementary Files

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