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RESEARCH ARTICLE

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Early fetal sexing in the rhinoceros by detection of male-specific genes in maternal serum

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Genetic sexing of animals with long gestation time benefits the management of captive populations. Here, X and Y chromosome-specific primers, based on equine gene sequencing data, were developed and tested on captive rhinoceroses (10 males, 20 females) representing four species (*Diceros bicornis*, *Certaotherium simum simum*, *Rhinoceros unicornis*, and *Dicerorhinus sumatrensis*). The Y chromosome-specific primer set targeted *SRY* (Sex-determining region Y), and amplified a 177-bp product following PCR of DNA extracted from males, but not females, of all species. A primer set based on the equine *AMEL* (Amelogenin) gene resulted in a 232-bp product following PCR of all rhinoceros species. These gene-specific primer sets were then evaluated for their ability to determine gender in cell-free DNA from rhinoceros serum. Modifications to the original extraction and PCR protocols were required to obtain sufficient DNA quantities from serum, and both DNA yield and PCR amplification were substantially reduced or absent following multiple freeze-thaw cycles of serum. When fresh serum from 14 pregnant rhinoceroses (ultimately bearing seven male and seven female calves), representing four species at different stages of gestation (Days 61–490), were probed in a PCR-based assay, an accuracy of 71% was achieved for male-specific gene detection of *SRY*, which improved to 100% by including a reamplification step into the protocol. Such early sex determination should be a valuable tool for current management practices as well as future assisted reproduction of rhinoceroses.

KEYWORDS
Amelogenin, PCR, rhinoceros, serum, *SRY*

1 | INTRODUCTION

Captive breeding programs serve an important role in the strategy to conserve endangered and threatened species. Of the five extant rhinoceros species (*Diceros bicornis*, *Ceratotherium simum simum*, *Dicerorhinus sumatrensis*, *Rhinoceros unicornis*, and *Rhinoceros sondaicus*), four have been maintained and managed in captivity. Yet, challenges in the reproductive management of captive rhinoceroses severely limit long-term sustainability.

The biology of species that exhibit long gestation lengths (≥16 months) and inter-calving intervals inherently constrain reproductive potential. Knowing the sex of fetuses while still in utero would provide zoological institutions and personnel managing these populations more lead time to plan for appropriate housing requirements and for subsequent breeding recommendations. Such planning is especially important for populations with harem or herd social structures, which increase demand for females and challenges placing males. Both African white and Indian rhinoceroses are most successfully managed in multi-female, single-male herds in large spaces (Metrione & Eyres, 2014), yet captive rhinoceros populations have experienced skewed

Abbreviations: AMEL, Amelogenin; SRY, sex-determining region Y.

natal sex ratios (Dennis et al., 2007; Edwards et al., 2015; Foose & Wiese, 2006). Accurate determination of fetal gender could also be important for species like the Indian rhinoceros, in which stillbirths account for a high percentage (17.4%) of overall births, and 67% of these stillbirths are male (Kennedy, 2016). Knowing if a female is carrying a male would allow Indian rhinoceros managers and veterinary personnel substantial lead time to plan and prepare for a potentially higher-risk pregnancy outcome.

Determining fetal sex using sex-specific DNA sequences in maternal serum by PCR is successfully performed in species with varied types of placentation and hence different degrees of maternal fetal contact—including horses (de Leon et al., 2012; Kadivar et al., 2016), cattle (da Cruz et al., 2012; Davoudi et al., 2012; Lemos et al., 2011; Wang et al., 2010), sheep (Asadpour, Asadi, Jafari-Joozani, & Hamidian, 2015; Kadivar et al., 2013), humans (Lo et al., 1997), and primates (Jimenez & Tarantal, 2003; Yasmin, Takano, Nagai, Otsuki, & Sandai, 2015). *SRY* (Sex-determining region Y) is the most commonly used gene for sex typing in mammals, particularly when an internal control is included to detect a gene present on the X chromosome as validation of the amplification process. The *AMEL* (Amelogenin) gene, present on both the X (*AMELX*) and Y (*AMELY*) chromosomes, has been used as an amplification control for cattle (Ennis & Gallager, 1994), sheep, red and sika deer (Kadivar et al., 2013; Pfeiffer & Brenig, 2005; Saberivand & Ahsan, 2016; Yamauchi, Hamasaki, Miyazaki, Kikusui, & Mori, 2000), goats (Phua, Abdullah, & Mohamed, 2003), bears (Yamamoto et al., 2002), and horses (Hasegawa, Sato, Ishida, Fukushima, & Mukoyama, 2000). Furthermore, subtle base-pair differences in many species exist between *AMELX* and *AMELY* that can be visualized by gel electrophoresis (Hasegawa et al., 2000; Salido, Yen, Koprivnikar, Yu, & Shapiro, 1992).

Developing a method to successfully detect fetal gender early in gestation would provide a potentially powerful management tool for institutions currently propagating rhinoceroses, and could help guide the translation of similar methods to other species in which sex ratio in captivity is a concern. Over the last decade tremendous strides have been made in establishing successful assisted reproductive techniques for both African and Asian rhinoceros species (Hermes et al., 2009; Hildebrandt et al., 2007; Stoops et al., 2016). Technologies regularly utilized in the livestock industry to influence the sex ratio of offspring prior to implantation, such as cytometric sexing of spermatozoa and embryo sexing, may soon play a part in the genetic and demographic management of captive rhinoceros populations (Behr et al., 2008, 2009; O'Brien et al., 2011, 2015). Therefore, establishment of a validated molecular sexing method using very small quantities of rhinoceros template DNA will be of utility as in vitro fertilization, embryo maturation, and embryo transfer become established in the different rhinoceros species. The work performed herein achieve such a method by (i) identifying X and Y chromosome-specific primers for the rhinoceros; (ii) establishing PCR protocols for whole blood and serum from African and Asian rhinoceroses; and (iii) testing the accuracy of the PCR test for sexing the fetuses in pregnant rhinoceroses using fresh and frozen-thawed serum obtained during gestation.

2 | RESULTS

2.1 | *SRY* primers

Genomic DNA from a domestic gelding (*Equus caballus*) and male Indian rhinoceros subjected to PCR using published equine *SRY* primers (ESRY) (Hasegawa et al., 2000) amplified a 429-bp product for the former but not the latter species. This failure of cross-species amplification was likely due to sequence differences in the orthologous region in the rhinoceros *SRY* gene, so a different region of the *E. caballus* gene was chosen for amplification. Rhinoceros-specific primers (RSRYF and RSRYR) against this alternative region (base pairs 281–458 of the *E. caballus SRY*) consistently produced a 177-bp product following PCR of genomic DNA from male Indian, African black, and African white rhinoceroses (at the time of testing, no whole-blood sample had been obtained from a male Sumatran rhinoceros). The intensity of the band increased as DNA concentration in the reaction increased. No product was amplified following PCR of genomic DNA from female Indian, Sumatran, African black, and African white rhinoceroses (data not shown).

Sequencing of the amplified region, followed by basic local alignment search (BLAST), indicated that the nucleotide sequence encoded by the African black, African white, Indian, and Sumatran rhinoceros *SRY* gene matched 94%, 92%, 96%, and 85%, respectively, of *E. caballus SRY*. A total of six nucleotide replacements were common to all rhinoceros species (Figure 1).

2.2 | *AMEL* primers

Genomic DNA from a mare, a gelding, and both sexes of all rhinoceros species produced a single 184-bp *AMEL* PCR amplicon using published equine primers (Hasegawa et al., 2000), when resolved on a 1.6% agarose gel; two distinct bands at 184 and 160 bp became evident for the gelding when resolved on a 3% agarose gel, whereas only a single 184-bp band was observed for male rhinoceroses (data not shown). Given that the PCR amplicon produced by the equine *AMEL* primers was only 7 bp more than the 177-bp rhinoceros *SRY* gene fragment (Section 2.1), we decided to test additional *AMEL* primer sets. All five novel primer sets resulted in amplicons of the expected size for the X chromosome when tested on DNA from the Indian rhinoceros; however, only three of these primer sets worked for the Sumatran and African black rhinoceros (data not shown). The three pan-rhinoceros primer sets produced products of 232, 340, and 390 bp from both sexes (Table 1).

2.3 | Fresh serum pregnancy samples

Fresh serum was collected during 14 full-term pregnancies (Indian [$n = 3$]; Sumatran [$n = 2$]; African black [$n = 2$], African white [$n = 7$]) that resulted in the birth of seven female and seven male calves. Serum was obtained an average of 217.64 ± 38.93 days of gestation (female fetus range: 61–490 days; male fetus range: 72–450 days); the average day that serum was obtained from pregnant rhinoceroses carrying male

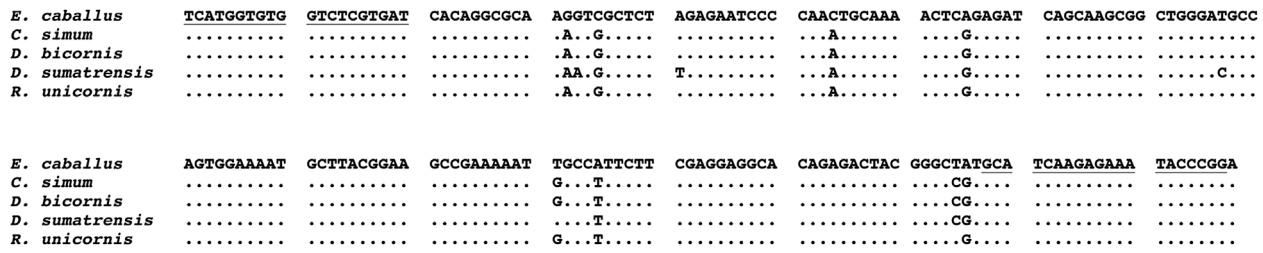

FIGURE 1 Alignment of the *E. caballus SRY* nucleotide sequence with those of *SRY* PCR products from the four rhinoceros species. The region amplified using primer pairs RSRYF and RSRYR (oligonucleotides are underlined) relates to positions 281–458 of the *E. caballus* sequence (GenBank accession number AB004572). Identical nucleotides are represented by dots

calves was lower (168 ± 59.64 days) than those carrying female calves (254.88 ± 50.58 days). DNA isolated from these fresh serum samples were then used for fetal sexing.

Combining the results of the single-round PCR amplification analysis for *SRY* and *AMEL* on these pregnancies, the overall test accuracy for sex determination using fresh serum samples was 86–71% (5/7) for pregnancies that bore known males and 100% (7/7) for pregnancies that bore known females. Given the inconclusive *SRY* amplification obtained in test runs on several male rhinoceros frozen-thawed serum samples following a single round of PCR, which were made conclusive by a second round of re-amplification using the same pair of primers (Figure 2a), a second round of amplification was applied to the 14 pregnancy cases. PCR re-amplification of *SRY* boosted the overall accuracy of the test to 100% for both sexes (Figure 2b).

3 | DISCUSSION

Ultrasonography has been the only method applied to date to determine fetal sex in captive rhinoceros species (Radcliffe, Czekala, & Osofsky, 1997; Radcliffe, Eyres, Patton, Czekala, & Emslie, 2001; Roth et al., 2001; Stoops et al., 2016). This approach requires that animals be trained for rectal ultrasound exams, and that operators with extensive experience conducted the analysis at a specific time of gestation to ensure accurate results (Curran & Ginther, 1989; Mari, Castagnetti, & Belluzzi, 2002). The current study sought a faster, more accurate molecular approach to fetal sexing based on the hypothesized presence of fetal DNA in maternal serum of the rhinoceros.

Rhinoceros X and Y primer sets developed herein were based on existing gene sequencing data from the domestic horse, which are closely related to rhinoceroses (Steiner & Ryder, 2011). Whole blood samples obtained from male and female captive Indian, African white, African black, and Sumatran rhinoceroses were used to develop and validate a primer set specific for *SRY*, a Y-chromosome-specific gene, that resulted in a 177-bp amplification product. The product of *SRY* is responsible for the initiating male sex determination in most placental mammals, and has been widely used for sex identification from a variety of biological sources (Sinclair et al., 1990). As a control for DNA integrity and PCR amplification, fragments of the *AMEL* gene were also amplified. *AMEL* is a highly conserved vertebrate gene, located on the sex chromosomes, that encodes extracellular matrix proteins of dental enamel (Salido et al., 1992). In most species, the coding region of

TABLE 1 Primer sequences for rhinoceros genes

Primer set	Sequence	Amplicon size (X)	Amplicon size (Y)
RSRYF **RSRYR**	5′-TCATGGTGTGGTCTCGTGAT 5′- CCGGGTATTTCTCTTGATGC	n/a	177 bp
AMEL47F AMEL48R	5′-GTGGATGGCTGCACCACCAA 5′-CCCGCTTGGTCTTGTCTGTTGC	390	366[a]
AMEL47F AMELR[b]	5′-GTGGATGGCTGCACCACCAA 5′-AGCATAGGGGGCAAGGGCTGCAAGGGGAAT	340	316[a]
AMELF[b] **AMEL48R**	5′-CCAACCCAACACCACCAGCCAAACCTCCCT 5′-CCCGCTTGGTCTTGTCTGTTGC	232	208[a]
AMELHF AMEL48R	5′-CAGCCAAACCTCCCTCCAC 5′-CCCGCTTGGTCTTGTCTGTTGC	217	193[a]
AMELF[b] AMELR[b]	5′-CCAACCCAACACCACCAGCCAAACCTCCCT 5′-AGCATAGGGGGCAAGGGCTGCAAGGGGAAT	184	160[a]
AMELHF AMELR[b]	5′-CAGCCAAACCTCCCTCCAC 5′-AGCATAGGGGGCAAGGGCTGCAAGGGGAAT	167	143[a]

The primer sets chosen for assessment of fetal sex across all rhinoceroses is in bold.

[a]Expected product size.

[b]Hasegawa et al. (2000).

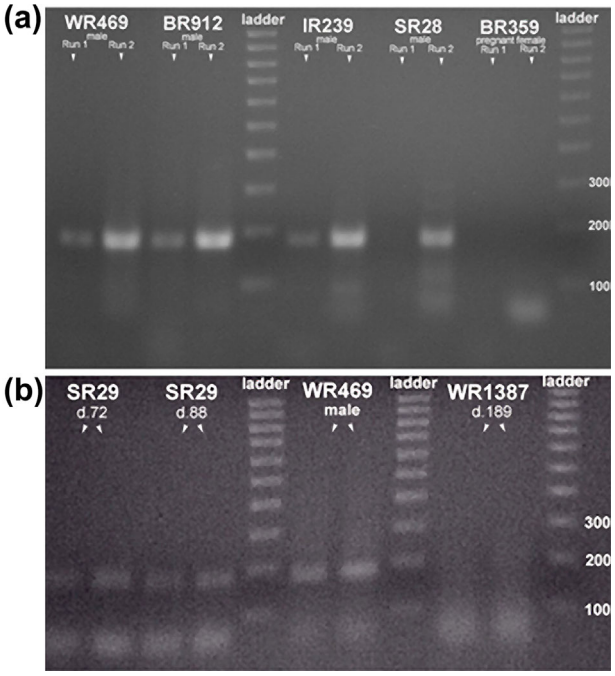

FIGURE 2 Gel electrophoresis of *SRY* gene product amplified by PCR from rhinoceros serum. (a) First and second round PCR products from cell-free DNA extracted from frozen-thawed rhinoceros serum, separated on a 1.6% agarose gel. A male calf was spontaneously aborted 2 days following the serum collection in a female African black rhinoceros (BR359). (b) PCR products amplified from fresh serum of pregnant female Sumatran (SR29) and African white (WR1387) rhinoceroses at different stages of gestation, for which predictions of male and female pregnancies, respectively, were correctly made: a male calf was born to SR29 at 479 days of gestation whereas a female calf was born to WR1387 at 476 days of gestation. Frozen-thawed serum from a male African white rhinoceros (WR469) was used as a control. Numbers on the right correspond to the ladder positions, in base pairs

AMELY is at least 20 bp shorter than *AMELX*, resulting in two DNA products in males following PCR of the appropriate region (Hasegawa et al., 2000; Nakahori, Hamano, Iwaya, & Nakagome, 1991; Salido et al., 1992). Of six *AMEL* primers pairs tested in rhinoceros, four were validated for both sexes in four species; the one finally chosen produced amplicons that clearly differed in size compared to the *SRY* amplification product, but was still relatively small (232 vs. 340 and 390 bp) since previous research has shown circulating DNA in serum and plasma is of smaller size (Chan et al., 2004; Xu et al., 2016; Yu et al., 2014). In contrast to the primer set developed to detect *AMELX* and *AMELY* in the horse, which differ by 24 bp (Hasegawa et al., 2000), the rhinoceros primer pair amplified only one PCR product of a size consistent with the expected number of base pairs on the X chromosome when separated on 1.6% or 3% agarose gels (data not shown). Therefore, less variation may exist in the coding region of rhinoceros *AMELX* and *AMELY* compared to the horse.

The ability of the chosen primer sets to amplify cell-free DNA isolated from male and female rhinoceros serum was tested next. The white cells in whole blood provide high quantities of extractable genomic DNA, primarily from the mother, whereas serum contains cell-free DNA of generally low concentration (Kaufman, Wu, Donghern, & Cseke, 1995). In addition, cell-free DNA concentrations in serum can vary substantially among individuals, and fluctuates when measured over both long and short periods (Stroun et al., 1989; Zhong et al., 2000). Among rhinoceroses, cell-free DNA yield and PCR amplification quality varied by individual and by species; indeed, overall PCR product quality was low or absent from Sumatran rhinoceros compared to the other African and Asian male rhinoceroses examined. One source of this low DNA abundance could be the diffuse epithelial-chorial placenta, without invasion of the endometrium, utilized by rhinoceroses (Benirschke & Lowenstine, 1995). Additionally, we discovered that the Sumatran rhinoceros has more variation in the *SRY* gene sequence at the primer annealing sites compared to the other rhinoceros species, based on the variable amplification quality from this species.

DNA yield and PCR product were further reduced or absent following multiple freeze-thaw cycles of serum—a phenomenon that was especially pronounced for *AMEL*. In one study, DNA degradation of 30% per year of storage at −80 °C was documented for human plasma samples (Sozzi et al., 2005), whereas another study found no significant change after repeated freeze-thaw cycles of serum/plasma (Chan, Yeung, Lui, Rainer, & Lo, 2005). This discrepancy was attributed to differences in pre-analytical factors, either prior to or after cryostorage of the samples (Bronkhorst, Aucamp, & Pretorius, 2015; Holdenrieder, Von Pawel, Nagel, & Stieber, 2010). The frozen serum samples tested in our study came from multiple institutions that undoubtedly used different protocols to collect, process, handle, and store the blood. In addition, many of the samples tested had been frozen-thawed on multiple occasions for other research purposes prior to our use. The observed failure to amplify product of either gene in some frozen-thawed serum samples collected at >200 days of gestation from four rhinoceros species (Sumatran [1 male, 1 female fetus], African black [3 male fetuses]) occurred (Figure 1a) after two rounds of PCR suggested poor quality or degradation of the isolated DNA. Therefore, total DNA concentrations and integrity in the samples used for initial validation were likely depleted by the freeze-thaw process (Chan et al., 2005).

Human fetal derived DNA in maternal plasma is of smaller size (Chan et al., 2004), accounts for 4% to 30% of the total DNA in maternal plasma, and is influenced by both maternal and fetal characteristics (Ashoor, Syngelaki, Poon, Rezende, & Nicolaides, 2013; Hudecova et al., 2014; Lo et al., 1998; Shi, Zhang, Cram, & Liu, 2015). Whether similar or lower concentrations of fetal DNA are present in the maternal plasma/serum of the non-primate species for which this technique has also proven successful and where there is no direct contact between the placenta and maternal bloodstream is unknown. Expecting that similarly low fetal DNA concentrations would exist in pregnant rhinoceroses, and that freeze-thaw would further degrade the DNA integrity, only freshly collected samples were used for fetal sexing, and a reamplification step was incorporated into the PCR protocol for the *SRY* gene to enhance the sensitivity of product detection.

Serum samples collected from pregnant rhinoceroses for the sexing analysis were either extracted the same day they were obtained

at our institution or were shipped overnight on cold packs from animals at other zoological institutions within the USA and extracted immediately upon arrival (Hindestrand et al., 2012). As extreme temperature variation during shipment can negatively affect cell-free DNA yield from serum (Chan et al., 2005), samples collected from pregnant rhinoceroses overseas were extracted in-country, and the resulting DNA was transported to us since isolated DNA appears more resistant to fragmentation than DNA in serum/plasma (Chan et al., 2005).

Serum from 14 rhinoceroses pregnant with 7 male and 7 female fetuses during 61–490 days of gestation were analyzed by PCR-based sexing, and corroborated the sex of each delivered calf following a reamplification step for *SRY*. Similarly high levels of accuracy were documented for other PCR-based assays in maternal serum from bovine (da Cruz et al., 2012; Davoudi et al., 2012; Lemos et al., 2011; Wang et al., 2010), ovine (Kadivar et al., 2013; Saberivand & Ahsan, 2016), and equine (de Leon et al., 2012; Kadivar et al., 2016). The earliest that fetal sex determination was made was 61 (female fetus) and 72 (male fetus) days of gestation, which is the timeframe when fetal sex can be determined via rectal ultrasound in all rhinoceros species (Radcliffe et al., 1997, 2001; Roth et al., 2001; Stoops et al., 2016). Although we did not test anything earlier, our results are consistent with the earliest period when (56 days) fetal sexing can be determined in equine maternal serum using a nested PCR (Kadivar et al., 2016). Unfortunately, we were unable to establish a correlation on assay accuracy and stage of gestation, given the opportunistic nature of the pregnancies that occurred during the study, the long gestation for rhinoceros species, and the low number of animals enrolled. Nevertheless, the confidence in the assay results should improve with later collection windows since quantitative changes in maternal serum documented throughout gestation in a number of species indicate an accumulation of free fetal DNA as pregnancy increases, including humans (Lo et al., 1998) and sheep (Kadivar et al., 2013).

In conclusion, this study demonstrated the first application of fetal sex determination using circulating cell-free fetal DNA from the maternal serum of captive African and Asian rhinoceroses, which are non-domestic *Perissodactyla* species. The accuracy rate for detecting male rhinoceros calves early in utero was 100% when a reamplification step was included to detect faint levels of fetal *SRY*. This sex determination assay has the potential to help guide future management decisions for captive rhinoceros populations, and only requires collecting a single serum sample from a female ≥72 days into gestation. This versatile approach will also be a useful when applications such as in vitro oocyte maturation, fertilization, and embryo transfer are established in these species.

4 | MATERIAL AND METHODS

4.1 | Animals

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Cincinnati Zoo and Botanical Garden (protocols 04–052, 07–072, 10–095). A total of eight Indian (4 males [SB147, SB239, SB087, SB049] and 4 females [SB238, SB189, SB274, SB230]), four Sumatran (2 males [SB28, SB44] and 2 females [SB29, SB43]), nine African black (*Diceros bicornis michaeli*, 3 females [SB225, SB397, SB359]; *Diceros bicornis minor*, 3 females [SB411, SB574, SB405] and 3 males [SB378, SB523, SB912]), and nine African white (1 male [SB469] and 8 females [SB1224, SB1020, SB1218, SB1387, SB1386, SB1358, SB1357, SB1353]) rhinoceroses maintained across 11 Zoological institutions in North America and New Zealand were enrolled in the study. Privately owned domestic horses (1 non-pregnant mare and 1 gelding) were also sampled. Breeding or artificial insemination dates were obtained for all pregnant rhinoceroses in the study.

4.2 | Sample collection and preparation

The restraining device used at the Cincinnati Zoo and Botanical Garden for obtaining blood samples is a chute designed for conducting ultrasound and providing routine veterinary checks. Participating institutions also had customized chutes for their rhinoceroses. Samples obtained from San Diego Global were collected during anesthetic events associated with pre-transport examinations. Blood was collected by venipuncture into a leg, ear, or tail vein. Whole-blood samples were collected into a common anti-coagulant, and kept at 4 °C until DNA extraction was performed. Serum samples were either used immediately for DNA extraction or stored at −80 °C until extraction.

Fresh serum samples collected at distant facilities within North America were shipped overnight on cold packs, and extracted immediately upon arrival. The fresh serum samples obtained from rhinoceroses in New Zealand were extracted in an in-country lab using the same protocol as at the Cincinnati Zoo and Botanical Garden, and the resulting DNA in TE buffer (10 mM Tris, 0.1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.3) buffer was shipped to us for PCR analysis.

4.3 | DNA extraction from whole blood

DNA was extracted from fresh blood following a previously published protocol (Kaufman et al., 1995). Briefly, whole blood was diluted (1:2, v/v) with sterile double-distilled water in a 50-ml Falcon tube, and centrifuged at 2,554*g* for 10 min at room temperature using a Mistral 3,000i centrifuge with rotor 43,117–605 (Sanyo Gallenkamp, Itasca, IL). The supernatant was decanted, the pellet was resuspended with an equivalent volume of sterile double-distilled water, and centrifuged again. After decanting the supernatant, the pellet was resuspended in cell lysis buffer (0.5 M EDTA, 1 M Tris-HCl, 0.5% sodium dodecyl sulfate [SDS], 10 mg/ml RNase) using a volume equal to the original blood volume, and incubated at 37 °C for 1 hr. Sample tubes were cooled to room temperature, an equal volume of phenol-chloroform (item P2069; Sigma–Aldrich, St. Louis, MO) was added, and the samples were centrifuged at 5,108*g* for 15 min. The viscous aqueous phase was transferred to a fresh tube, and the phenol-chloroform extraction step was repeated to remove proteins and cellular debris. Fifteen percent volume (0.15 units) of 7.5 M $NH_4CH_3CO_2$ was added to the aqueous phase resulting from the last centrifugation, and

then this mixture was poured into a fresh beaker containing 2.5 volumes of 100% chilled ethanol to precipitate the DNA. A sterile glass hook was used to transfer the DNA to a microfuge tube. Tubes containing DNA were air-dried for 15 min before resuspending in 200 µl TE buffer. The amount and quality of DNA were determined by spectrophotometer. Only DNA of sufficient purity (a 260/280 nm absorbance ratio of 1.2–1.93) was used for PCR analysis.

4.4 | DNA extraction from serum

A 1- to 1.5-ml volume of serum and an equal volume 1X SDS-proteinase K solution (1% SDS,500 µg/ml proteinase K) (Promega, Madison, WI) in TE buffer were mixed well in a 50-ml Falcon tube, and digested overnight in a 55 °C water bath. The following morning, 3 ml of equilibrated phenol-chloroform was added to two sets of serum separator tubes (SST) labeled "A" and "B." The digested solution was decanted into SST "A," vortexed 30 s, and centrifuged at 699*g* for 10 min (rotor 43,124–708). The supernatant was transferred to SST "B," and the process was repeated. After centrifugation, the supernatant was poured into a fresh 50-ml Falcon tube, and 3 µl of glycogen (item 901 393) (Roche, Penzberg, Germany), 1 ml of 7.5 M $NH_4CH_3CO_2$, and 8 ml 100% ethanol were added. The sample was mixed gently followed by centrifugation at 5,108*g* for 60 min. As the supernatant was decanted, care was taken to ensure the pellet remained on the side or bottom of the tube. A 10 ml volume of 70% ethanol was added to the tube, centrifuged at 5,108*g* for 10 min, the ethanol was discarded, and the tube was spun briefly before the remaining fluid was removed with a pipette. The DNA in each tube was left to air dry in a sterile hood for 10 min. DNA was resuspended in 200 µl TE buffer, transferred to a 1.5-ml sterile microfuge tube, and stored at 5 °C until analysis.

4.5 | Oligonucleotide sequences

Equine primers for *SRY* and *AMEL* were previously published (Hasegawa et al., 2000). Novel primers for rhinoceros were designed using Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on existing gene sequence data from *E. caballus* and synthesized by Qiagen (Alameda, CA).

The oligonucleotide sequence of the primers used to test detection of *SRY* in the rhinoceros were (ESRYF/R) (Hasegawa et al., 2000) and (RSRYF/R) (Table 1). The ESRY and RSRY primer sets were designed to amplify a single fragment of 429 and 177 bp on the Y chromosome, respectively. The primers tested for the *AMEL* gene were designed to amplify two fragments of varying sizes on the X and Y chromosome (Table 1). Upon arrival, oligonucleotide sequences were diluted to 100 µM. The *SRY* (RSRYF, RSRYR) and *AMEL* (AMELF (Hasegawa et al., 2000), AMEL48R) primers ultimately chosen to test serum of pregnant rhinoceroses yielded 177 and 232 bp products, respectively.

4.6 | PCR

The master mix solution used for PCR reaction of the *SRY* gene fragment using DNA isolated from whole blood consisted of 100 µl 5X buffer (2.5 mM $MgCl_2$), 20 µl 2.5 mM dNTPs, 6 µl Taq DNA polymerase (Takara Bio, Otsu, Japan), 10 µl 0.1 M $MgCl_2$, and 164 µl sterile water. The master mix solution for PCR reaction of the *AMEL* gene fragment was the same as above, except the 10 µl 0.1 M $MgCl_2$ was replaced with sterile water. Amplification reactions were set up in a volume of 30 µl consisting of 6 µl template DNA (5, 10, 20, 30 µg/ml), 18 µl master mix, and 3 µl 10 µM each primer evenly split between two capillary tubes. The $MgCl_2$ concentration of each master mix solutions was increased twofold when using cell-free DNA from serum. Amplification reactions consisted of 9 µl master mix, 15 µl extracted DNA from serum, and 3 µl 10 µM each primer evenly split between two capillary tubes. The second round of PCR for the *SRY* gene fragment was performed as above using the same primers and primer volume, 5 µl of first round PCR amplicons as templates, and 10 µl TE buffer per capillary tube.

PCR was performed using a RapidCycler (Idaho Technology, Salt Lake City, UT). Y-specific amplicons were obtained using an initial denaturation at 94 °C for 1 min, followed by 30 cycles at 94 °C for 0 s, 60 °C for 15 s, and 72 °C for 30 s. A final elongation step at 72 °C for 2 min was performed. Reaction conditions were as described above for the second round of PCR. The thermocycling protocol for *AMEL* PCR reactions had the same initial denaturation and final elongation parameters, but differed in that the 30 cycles were carried out at 94 °C for 5 s, 62 °C for 5 s, 72 °C for 30 s.

4.7 | Electrophoresis

PCR product (30 µl) was electrophoresed (Apogee Electrophoresis, Baltimore, MD) through a 1.6% agarose gel and stained with ethidium bromide (2 µl/ml). Each gel contained at least one well loaded with 100-bp low ladder (item P1473) (Sigma–Aldrich). The products of each gel were viewed using ultraviolet illumination, and documented via digital photography. A 3% agarose gel was used to test existing equine *AMEL* primers and to assess <20-bp difference in X and Y chromosome products.

4.8 | Sequencing

SRY amplicons were purified using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany), according the manufacturer's protocol. Samples were submitted for sequencing at the University of Cincinnati, DNA Core Facility. Primers were diluted to 20 ng/ml. Sequencing reactions were electrophoresed on an ABI-Prism v3.3 3100 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany), according to manufacturer's instructions.

4.9 | Determination of fetus sex

Our sexing method was based on the presence of (male fetus) or absence (female fetus) of the Y-chromosome in the serum samples of pregnant rhinoceroses. The sex of the fetus resulting from each of the pregnancies was recorded at birth. Standard descriptive statistics were

used to calculate mean ± standard error on the day of gestation for serum samples analyzed in the study.

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