

Detecting White Rhinoceros DNA From Footprints

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Article

Keywords:

Posted Date: September 18th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10512728/v1>

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Additional Declarations: No competing interests reported.

Abstract

This study develops non-invasive DNA sampling methods for identification of white rhinoceros (*Ceratotherium simum*), with a particular focus on detection from footprints. Footprint samples in addition to scat and drinking water were collected from southern white rhinoceros (SWR) in Dublin Zoo, Ireland. A SYBR green quantitative (q)PCR protocol was designed and optimised to detect SWR mtDNA originating from footprint samples, representing the first species specific assay designed to detect this species. Species specificity was further confirmed by testing the assay on samples from black (BR) (*Diceros bicornis*) and greater one horned rhinoceros (IR) (*Rhinoceros unicornis*). In addition, the mtDNA sequences returned from scat revealed that two of the four remaining SWR haplotypes are present in the rhinoceros population at Dublin Zoo. This assay represents a novel tool that could be used in the wild to detect relict populations of white rhinos, particularly in difficult to survey habitats that could contain the last northern white rhinoceros (NWR). These findings highlight the potential of eDNA, particularly footprint-derived eDNA as a valuable tool for management and conservation of this species, enabling the detection of elusive or remnant rhinoceros populations where traditional survey methods are not viable.

INTRODUCTION

The emergence of modern humans and their subsequent spread out of Africa coincided with the extinction of megafaunal species worldwide, with continental impacts on Earth's ecosystems ¹. This trend continues today with c. sixty percent of the world's largest species being threatened with extinction ². Such catastrophic trends are indicative of a sixth mass extinction event, unless effective conservation strategies are widely and quickly implemented ³. Rhinoceroses constitute one of the largest remaining megafaunal groups on land, and represent keystone species where they still exist in Africa and Asia ^{4,5}. However, three of the five extant species of rhinoceros are listed as Critically Endangered under the IUCN Red List of Threatened Species; black (BR) (*Diceros bicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran (*Dicerorhinus sumatrensis*), while white rhinoceros (*Ceratotherium simum*) and greater one horned rhinoceros (IR) (*Rhinoceros unicornis*) are listed as Near Threatened and Vulnerable respectively. Despite their range stretching throughout Asia and Africa, almost all rhinoceroses are now entirely restricted to conservation areas ⁶. Historically rhinoceroses were extirpated from their native habitat through a combination of habitat loss and over hunting, with poaching of rhinoceros horn now being the greatest modern threat facing all species ⁷.

Despite an international ban on the trade of rhinoceros horn beginning in 1977, an increasing demand for horn products in South-East Asia since 2008 has created a global acceleration of illegal rhinoceros poaching ⁸. Owing to its relatively high abundance, the white rhinoceros has taken the brunt of these losses, with estimates suggesting that the white rhinoceros population in Kruger National Park could be reduced by 35% by the year 2030 ⁹⁻¹¹. While poaching continues to drive rhinoceros deaths, habitat loss and degradation have affected many species of rhinoceros in the last two hundred years ^{12,13}.

The white rhinoceros is the most abundant of the five extant rhinoceros species. It consists of two sub-species: the southern white rhinoceros (SWR) (*C. simum simum*) and the northern white rhinoceros (NWR) (*C. simum cottoni*). The southern sub-species suffered significant population declines in the early 1900s, bringing the species to the brink of extinction with estimates of only 20–50 individuals remaining in the wild ^{14,15}. Through extensive conservation and protection efforts the southern sub-species has made a notable recovery to approximately 16,000 individuals; however, poaching still continues to persist ¹⁶. Their northern counterpart had a population size of 2,360 individuals at the beginning of the 1960s, but as a result of human activities such as poaching, armed conflict and bush meat hunting this population shrunk to just fifteen animals in the 1980s with the sub-species now considered functionally extinct, as only two post-reproductive females remain ^{17,18,16}.

An important tool in the conservation and monitoring of any species is genetic analysis as it allows for the identification of individuals ¹⁹, population structure assessments ²⁰ and the detection of elusive or declining populations ²¹. Traditionally, genetic analysis is performed using DNA obtained via tissue samples collected during the capture of target individuals ^{22,23}. However, studies involving the capture and handling of animals are invasive, causing stress, disturbance, injury or even mortality to the target individual. In addition, invasive studies require more permits, especially when working with endangered species and are potentially dangerous for the researchers involved. These techniques are costly, logistically difficult, labour intensive and result in proportionally small sample sizes which limits population-wide inferences; these constraints are only enhanced when studying elusive or rare species ²⁴. To address these issues, non-invasive methods have been devised to obtain DNA from wild animals without having to contact the target individuals. They were first used to study wild brown bears more than thirty years ago ²⁵ and since then these methods have continued to improve our understanding of molecular ecology, conservation genetics and related disciplines, such as forensics and epidemiology. In a wildlife management setting, they can provide information on abundance, genetic diversity, relatedness, phylogeography, sex and geography ²⁶. These methods enable biologists to conduct genetic studies without contacting, interfering with or even seeing the target individuals ²⁷. A wide set of sampling methods have been developed which can be used to conduct non-invasive genetic analysis of environmental (e)DNA. These methods range from the most common techniques of preserving scat ^{28–32}, hair ^{33,34} and water ³⁵ to less common methods such as sequencing DNA from non-invasively acquired blood ³⁶, feathers ³⁷, and even footprints in snow ^{38–41}, soil ⁴² and sand ^{43,44}. Recent studies have increasingly focused on the recovery of trace DNA from environmental substrates such as footprints or disturbed snow, soil or sand. These approaches are at the forefront of non-invasive sampling allowing for the detection of terrestrial species without the need for physical contact, visual sightings or biological material such as scat, hair or blood. The DNA shed from epithelial cells can persist within substrates following deposition and may prove especially valuable for monitoring rare and low-density fauna where traditional surveying approaches are logistically challenging.

For optimum detection of a target species, the sampling of numerous potential sources of eDNA alongside traditional methods is ideal ⁴⁵. Low population sizes can make species difficult to locate and monitor ^{46,47}, but by utilising eDNA samples and traditional methods such as camera trapping the range of methods available to track these animals is greatly increased. This study aimed to develop a qPCR assay that detects white rhinoceros DNA from footprints, scat and drinking water, which can be used in the field to detect and monitor wild and semi-wild rhinoceroses. The tools developed here can also be adapted to survey other species of rare or elusive mammals.

RESULTS

DNA AMPLIFICATION AND ANALYSIS

The amplification of nDNA was based on the presence of a band at the expected molecular weight following gel electrophoresis. All three microsatellite loci (SW35, BR6 and DB1) amplified from scat produced viable sequences for each white rhinoceros individual. The mtDNA control region fragment was successfully amplified from all scat and water samples for all white rhinoceros individuals; however, footprint samples failed to amplify following nested PCR. BR, IR and Asian elephant also failed to amplify when viewed on a 1.5% agarose gel.

For white rhinoceros, three of the four scat samples and three of the four water samples subjected to commercial Sanger sequencing resulted in a clear non-ambiguous DNA sequence. The alignment of forward and reverse strands resulted in sequence segments of 248 bp for each individual. For individual A, the generated sequence matched 100% to six sequences available on Genbank (Accession numbers: FJ004917.1, OP762460.1, FJ004918.1, AF140274.1, OP762457.1 and OP762453.1). The aligned sequence for animal B matched 100% to the same isolates as those for animal C. They both matched 100% to nine sequences available on Genbank (Accession numbers: OP762451.1, OP762455.1, OP762459.1, NC001808.1, AF187836.1, FJ004916.1, OP762452.1, FJ004915.1, and AY742828.1) sharing a haplotype previously identified in rhinoceroses originating from South Africa's Kruger National park and Umfolozi game reserve ^{48,49}.

QUANTITATIVE PCR FOOTPRINT AMPLIFICATION

DNA was positively detected from scat, water and footprint samples from white rhinoceros using universal SYBR™ Green staining. Footprint samples had a mean CT of 31.41 and a mean standard deviation of 0.511. BR scat samples were also cross-amplified by our primers; however, they exhibited weaker amplification (CT mean – 36.485, SD CT mean – 0.334) than white rhinoceros footprint samples. IR water samples were not successfully amplified while 2 out of three scat samples were amplified with a CT difference of 6.336, which does not meet our criteria for successful amplification (Fig. 2.).

DISCUSSION

As we appear to be entering into a sixth mass extinction event, preserving vulnerable species requires a range of conservation techniques, ranging from habitat protection and breeding programmes to the genetic monitoring of wild populations. In particular, genetic data can inform on population structure and size, evolutionary relationships and distribution^{19–21}. However, obtaining the genetic material of vulnerable species has traditionally been constrained by the need to minimise disturbance to the target individual highlighting the value of non-invasive sampling methods. For elusive or rare species, non-invasive genetic approaches offer a method of detection which was previously challenging when relying on traditional techniques. In the context of this study, developing methods capable of non-invasive identification provides a promising avenue for the identification of unknown rhinoceros populations where traditional survey methods are limited.

There is a growing need for effective and reliable monitoring practices as pressures such as poaching, climate change, land use change and disease continue to impact all rhinoceros species^{53–55}. As populations decline and sightings of rare species become less frequent, methods which allow researchers to detect and obtain DNA for species non-invasively become more valuable, particularly when working with dangerous or legally protected species such as rhinoceroses. Here, we demonstrate for the first time that white rhinoceros mtDNA can be successfully detected from footprint samples via a SYBR Green qPCR assay. In combination with scat and water sampling, this research adds to the growing toolkit available which allows for the detection and monitoring of vulnerable and rare species.

Previous studies have demonstrated that footprint impressions can yield target DNA; however, these studies have focused primarily on obtaining DNA deposited in snow, which inherently slows the eDNA degradation rate, as it occurs at cold temperatures³⁹. The work here extends this approach to a different ecological context by applying footprint-derived eDNA to megafauna found in arid, savanna-like ecosystems. To the best of the authors knowledge, this study appears to be the first demonstration of footprint eDNA detection from ungulates. Rhinoceros footprints represent a novel and largely unexplored source of environmental DNA. Unlike scat and water, footprints are deposited continuously across the landscape as animals move. Rangers and field staff already routinely track rhinoceros as part of anti-poaching and monitoring patrols, allowing for the integration of footprint sampling into already existing workflows. This technique could be particularly relevant in contexts such as remote or heavily poached populations, where establishing species presence is highly important. Despite successful amplification via qPCR, footprint samples did not return viable mtDNA sequences, likely due to the low concentration of target DNA. Alternative extraction techniques to the DNeasy PowerMax soil kit could be used to increase the throughput of sediment and ultimately increase the concentration of DNA. The slurry method was shown by Gallard-Gongora et al., (2022) to outperform the PowerMax Soil Kit and could be used to increase yield in subsequent studies focused on detection from footprint sediment. Other methodological improvements, such as the adoption of probe-based techniques should be prioritised to increase sensitivity and reliability. The qPCR assay used in this study weakly amplified BR scat with a mean CT of 36.485; as a result of this we propose a limit of detection to be placed at CT 33;

amplification beyond this threshold should be considered unreliable, as it is unclear whether the product represents true target DNA or non-specific amplification.

By 1895, the SWR had been reduced to 20–50 individuals found only in the iMfolozi Game Reserve, South Africa ¹⁵ and as a result of this severe bottleneck, the genetic diversity of this sub-species was greatly reduced. Dublin Zoo possessed seven rhinoceroses at the time of this study with three different individuals analysed, resulting in two haplotypes. By utilising scat allows researchers to opportunistically analyse genetic diversity in rhinoceros populations as well as assess diet and parasite load and identify individuals via microsatellites or SNPs ^{57,58}. Additionally, surface water is considered the limiting factor in arid and semi-arid environments, making watering holes (both natural and artificial) common agglomeration locations for many species ⁵⁹. Watering holes therefore serve as ideal sampling sites for monitoring not only rhinoceros but also a wide range of biodiversity ⁶⁰.

This study demonstrates a proof of concept for the detection of white rhinoceros from scat, drinking water and in particular footprint samples in a controlled environment. The footprint-based detection assay developed in this study is a powerful tool in this context of NWR, where sporadic unconfirmed sightings of the subspecies have been reported in South Sudan's Southern National Park, where systematic surveys are limited due to the areas remoteness and political instability. Challenges inherent to field sampling, such as environmental degradation and greater inhibitor loads have yet to be addressed, and we recommend that future studies scale this approach both spatially and temporally, applying these methods to wild rhinoceros populations across a range of habitat types and seasons. The integration of footprint, scat and water eDNA sampling into routine conservation and anti-poaching workflows has the potential to substantially improve our capacity to monitor and protect rhinoceros populations.

MATERIALS AND METHODS

SAMPLE COLLECTION

SWR scat samples were collected in Dublin Zoo, Phoenix Park, Ireland. Fourteen scat samples were taken in total from three unrelated, adult individuals. Five from animal A (male), 5 from animal B (female) and four from animal C (female). A sterile cotton swab (Deltalab) was rubbed on the outer surface of the scat, targeting areas with surface mucous until visibly coated with faecal material. After the samples were taken, the tip of each wooden swab was cut off and placed into a 2-ml O-ring tube (Eppendorf). Approximately five sterile silica beads (2.5–6.0 mm) (Fisher Scientific) were added to the tube to preserve the scat. The samples were immediately brought back to the laboratory at University College Dublin (UCD) and kept at -20°C until further analyses. SWR water samples were collected from a communal self-refilling trough located inside the SWR house. A total of 2 litres were collected and immediately stored out of sunlight at ~ 4°C until filtration occurred at UCD on the same day. For SWR footprint samples, approximately five kilograms of fine, commercially purchased sand was spread over

an area of $\sim 0.5\text{m}^2$ in the outdoor paddock. Before this, the sand had no interaction with any animals. A SWR calf and mother were then allowed to enter the habitat, where both individuals walked over the sample area. Where they walked and the tracks they left on the sand were observed and noted. Once the rhinoceros had left the enclosure, approximately 10 ml of sand was taken for each sample. These samples were then held at $\sim 4^\circ\text{C}$ and immediately transported to UCD, where they were stored at -20°C until DNA extraction. Eight footprint samples were collected in total. Four samples were taken from the footprint of each individual as well as a field blank taken after deposition of the sand but before coming into contact with the rhinoceros to assess the background concentration of SWR DNA in the outdoor enclosure.

DNA EXTRACTION

As CTAB has been shown to bind to the polysaccharides found in the faeces of herbivores a modified CTAB extraction protocol was carried out on all scat samples⁶¹. In brief, swabs containing scat samples were placed in a lysis mixture of CTAB (600 μL) (2%) and proteinase K (10 μL) (20 mg/ml) and incubated overnight at 56°C . Following this 3 μL of RNase A was added, and the mixture further incubated at 37°C for 30 minutes. After incubation, the swabs were removed, placed in an Eppendorf tube and stored at -20°C . DNA isolation was performed by adding an equal volume of chloroform–Isoamyl alcohol (24:1) and mixed thoroughly for one minute, followed by centrifugation at $7,500 \times g$ for five minutes; $\sim 250 \mu\text{L}$ of the upper aqueous phase was transferred to a new tube and then precipitated by adding an equal volume of ice-cold isopropanol. Following precipitation, the pellet was washed with 70% ethanol and eluted in 50 μL of ddH₂O.

Water samples were filtered through sterile 1.5 μm Whatman glass microfiber filter papers (Fisher Scientific) attached to a vacuum pump until saturation. A volume of 360 ml SWR drinking water was passed through the filter, which was then divided into two pieces and each half placed in a 1.5 ml-O-ring tube (Eppendorf). For extraction, the same CTAB protocol was employed as for the scat samples, with the replacement of the scat swabs with the glass fibre filters, the only modification being that the filter and liquid were added to a QIAshredder Mini Spin Column (Qiagen) following the final incubation and centrifuged at $5,000 \times g$ for five minutes to separate the liquid from the filter.

A DNeasy PowerMax Soil Kit (Qiagen cat.no. 12988-10) was used to extract DNA from all footprint samples. The manufacturer's instructions were followed barring the final stage, where 2 ml of sterile C6 solution was used to elute the DNA as opposed to the recommended 5 ml as previously described in Koda et al., 2023. All extracted DNA was stored at -20°C until amplification. Following extraction, a volume of 1 μL DNA from all samples was used to measure its respective DNA concentration with a BioDrop μLITE spectrophotometer. After extraction, samples with a concentration of $> 100 \text{ ng/ml}$ total DNA were diluted by a factor of 1/10.

MICROSATELLITE AMPLIFICATION

To examine whether nuclear DNA (nDNA) could be amplified from white rhinoceros scat, three sets of primers were used to amplify the SW35, BR6 and DB1 loci (Table 3) ^{62–64}. A 20µL PCR master mixture was prepared in a UV sterilised fume hood, each containing 2.0 µL of 10X buffer (Kapa Biosystems), 0.4 µL mM dNTP (Invitrogen), 0.08 µL Taq polymerase (Kapa Biosystems), 0.8 µL of each primer (10µM) (Integrated DNA Technologies), 14.92 µL water and 1.0 µL DNA template per sample. The PCR conditions were as follows: initiation at 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 54°C for 30 s and 72°C for 30 s. The final elongation stage was 75°C for 2 minutes. Amplification for all samples occurred in a separate room to where they were extracted to minimise cross-contamination risk. Microsatellite PCR products were then stained with a 6X gel loading buffer and run on a 1.5% agarose gel with a GelPilot 100 bp ladder. DNA-free, sterilised water was used as a negative control. Samples which presented bands at the expected molecular weight were sent for commercial Sanger sequencing (Macrogen).

Table 1

Description of the three microsatellites amplified from white rhinoceros scat samples. Loci obtained from Rohrer et al. 1994 (SW), Cunningham et al. 1999 (BR), Brown & Houlden, 1999 (DB) and. F, forward primer; R reverse primer.

Locus	Number of Alleles	Size range of alleles (bp)	Primer Sequence (5' – 3')
SW35 F	5	129–137	TCA AGT TGG AGA GTC TGA GGC
SW35 R			AAG ACT GCC CAC CAA ATG AG
BR6 F	10	126–158	TCA TTT CTT TGT TCC CCA TAG GAC
BR6 R			AGC AAT ATC CAC GAT ATG TGA AGG
DB1 F	4	121–127	AGA TAA TAA TAG GAC CCT GCT CCC
DB1 R			GAG GGT TTA TTG TGA ATG AGG

MITOCHONDRIAL AMPLIFICATION

White rhinoceros primers were developed using primer-blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The primers chosen, F (5'-ACCCTCACATGTAACACACCA-3') and R (5'-ACTTGGATGGGGTATGTCCG-3'), were used to amplify a 249 base pair segment of the mtDNA control region. These developed primers were tested in silica using nucleotide BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The primers were also tested in vitro where they were used to amplify SWR DNA alongside black BR, IR, common hippopotamus (*Hippopotamus amphibius*) and Asian elephant (*Elephas maximus*). PCR products was subsequently visualised on a 1.5% agarose gel stained with a 6X gel loading buffer and a GelPilot 100 bp ladder. Separate primers, mt155996L (5' – TCCACCATCAGCACCCAAAGC-3') and mt16502H (5'-TTTGATGGCCCTGAAGTAAGAACCA-3') ⁴⁸ were used to amplify a 532 bp fragment of the mtDNA control region of both BR and SWR DNA to act as a positive control and confirm the presence of mtDNA

following extraction. The resulting PCR product was then subject to commercial Sanger sequencing for both BR and SWR.

The same 20 µL PCR master mix was used as for the amplification of nDNA with the only alteration being the addition of 0.4 µL Bovine serum albumin 10 mg/ml (Thermo Fisher Scientific). A ProFlex PCR system (Thermo Fisher Scientific) was used to run the PCR; the conditions are as per those used for microsatellite amplification. For each PCR reaction, DNA-free, sterilised water was used as a negative control. PCR products were then stained with a 6X gel loading buffer and run on a 1.5% agarose gel (SafeView gel stain) with a GelPilot 100 bp ladder (Qiagen). Footprint samples underwent the same PCR amplification as scat and water samples but failed to produce bands of the correct molecular weight following gel electrophoresis. Thus, a nested PCR was performed using the same protocol, with the only alteration being the use of 1 µL of PCR product from the original footprint PCR amplification instead of template DNA.

Samples that presented clear bands at the right molecular weight following gel electrophoresis, implying successful amplification, were chosen for commercial Sanger sequencing (Macrogen). The obtained sequence data were analysed using Geneious Prime Version 2023.0.4⁶⁵. Forward and reverse strands were aligned and then checked through BLAST nucleotide search⁶⁶. Corresponding sequences for wild individuals of both species were downloaded from Genbank (<https://www.ncbi.nlm.nih.gov/genbank/>) and a maximum likelihood phylogenetic tree was constructed (Fig. 1).

QUANTITATIVE PCR

SWR scat, water and footprint DNA samples alongside IR scat and water and BR scat DNA samples underwent quantitative (q)PCR amplification using universal SYBR™ Green staining. Each sample reaction contained SYBR™ green (10 µL), forward and reverse primers (10 mM) (0.6 µL each), dH2O (5.8 µL) and template DNA (3 µL). Amplification was carried out on a QuantStudio 7 Flex Real-Time PCR thermocycler. The protocol consisted of initial denaturation at 95°C for 20s, followed by 45 cycles of 95°C for 1s and 60°C for 20 s for annealing and extension. Melt curve analysis was performed at the end of the reaction, which consisted of 95°C for 15 s, 60°C for 1 min and final increase to 95°C for 15 s. DNA extracted from SWR scat samples that had previously produced viable genetic sequences after commercial Sanger sequencing (Macrogen) was used to generate the standard curve using seven 10:1 serial dilutions. The concentrations of the serial dilutions ranged from 29 µg/ml to 2.9×10^{-6} µg/ml of total DNA (quantified with a BioDrop µLITE spectrophotometer). All standard-curve samples, field samples, and field and laboratory controls were quantified in triplicate. Positive detection was specified as being within the range of the standard curve and when at least two out of three of the replicates were shown to amplify DNA within a cycle threshold (CT) not exceeding 0.5.

Declarations

FUNDING

This study was funded by the School of Biology & Environmental Science, University College Dublin.

Author Contribution

MC, JB, JC and A.J.T concept development. MC, JC, A.J.T preparation of figures and produced draft and revised manuscripts. MC, SH, J.EL.C and OGV performed sample collection, lab work and data analysis. MC wrote the manuscript. All authors approved the manuscript.

Acknowledgement

MC was supported by the Environmental Protection Agency (Project code; 2022-NE1154.). A.J.T was supported by Revive and Restore under the Catalyst Science Fund (CSF/2022-035). The authors would also like to thank the teams at Dublin Zoo, Fota Wildlife Park and Chester Zoo for providing access and samples for this study.

Data Availability

Sequence data generated have been uploaded to Zenodo (<https://doi.org/10.5281/zenodo.21809066>).

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Figures

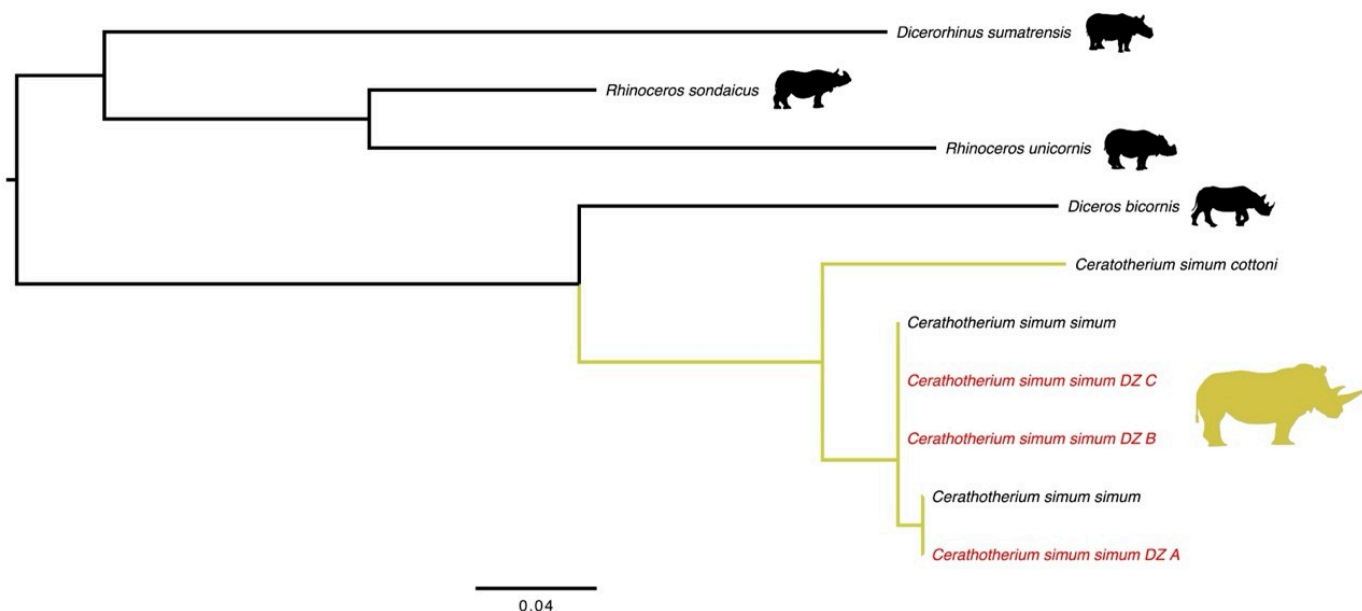


Figure 1

Maximum-likelihood phylogenetic tree of *Rhinocerotidae*. The tree was constructed using aligned mitochondrial control region sequences including individuals sequenced from Dublin Zoo (DZ). Tree construction was performed via IQ-TREE V3.1.2.⁵⁰ under the HKY+F+G4 model selected from model finder according to the Bayesian Information Criterion⁵¹, with node support values calculated using a 1000 bootstrap replicates. The final tree was visualised using FigTree v1.4.4.⁵²



Figure 2

Boxplot of the CT values for successfully amplified samples following SYBR Green qPCR. The limit of detection of CT 33 can be seen by the red dotted line.