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Research paper

An internationally standardized species identification test for use on suspected seized rhinoceros horn in the illegal wildlife trade

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We would like to dedicate this work to the late Sam Kudeweh whose passion for the rhinos and her tireless efforts on the management of the southern white rhino program will be missed by all. Sam was instrumental in facilitating the collection of samples from the regional rhinos for our work and in doing so has made a lasting contribution to rhino conservation.

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ABSTRACT

Rhinoceros (rhino) numbers have dwindled substantially over the past century. As a result, three of the five species are now considered to be critically endangered, one species is vulnerable and one species is near-threatened. Poaching has increased dramatically over the past decade due to a growing demand for rhino horn products, primarily in Asia. Improved wildlife forensic techniques, such as validated tests for species identification of seized horns, are critical to aid current enforcement and prosecution efforts and provide a deterrent to future rhino horn trafficking. Here, we present an internationally standardized species identification test based on a 230 base pair cytochrome-b region. This test improves on previous nested PCR protocols and can be used for the discrimination of samples with < 20 pg of template DNA, thus suitable for DNA extracted from horn products. The assay was designed to amplify water buffalo samples, a common 'rhino horn' substitute, but to exclude human DNA, a common contaminant. Phylogenetic analyses using this partial cytochrome-b region resolved the five extant rhino species. Testing successfully returned a sequence and correct identification for all of the known rhino horn samples and vouchered rhino samples from museum and zoo collections, and provided species level identification for 47 out of 52 unknown samples from seizures. Validation and standardization was carried out across five different laboratories, in four different countries, demonstrating it to be an effective and reproducible test, robust to inter laboratory variation in equipment and consumables (such as PCR reagents). This is one of the first species identification tests to be internationally standardized to produce data for evidential proceedings and the first published validated test for rhinos, one of the flagship species groups of the illegal wildlife trade and for which forensic tools are urgently required. This study serves as a model for how species identification tests should be standardized and disseminated for wildlife forensic testing.

1. Introduction

The rhinoceros (rhino) is an iconic mega-herbivore from the family *Rhinocerotidae*. Currently there are five extant rhino species native to Africa and Asia. The two African species are the white rhino (*Ceratotherium simum*) and black rhino (*Diceros bicornis*), and the three Asian species include the Indian rhino (*Rhinoceros unicornis*), Sumatran rhino (*Dicerorhinus sumatrensis*) and Javan rhino (*Rhinoceros sondaicus*). By 1977, all five rhino species were listed under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) as Appendix I (and II in the case of *C. s. simum* South African and Swaziland populations) [30]. CITES is enforced *via* legislation of signatory countries and prohibits the commercial trade of rhinos or their parts between countries to ensure that the wildlife trade does not further threaten their survival [30,1]. We are, however, currently amidst a rhino poaching crisis that has been driven by a dramatic increase in demand for rhino horn. Rhino horn commands prices in the tens of thousands of dollars (US) per kilogram on the illegal black

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market in consumer countries [2]. The majority of rhino horn demand is currently from Vietnam, where an economic boom and relatively young population has seen horn purchased and consumed as a symbol of status [3]. Rhino horn has been used in traditional medicines for decades, but more recently it has been used by patients with life-threatening diseases such as cancer [3] even though rhino horn is comprised of keratin, the same substance in hair and fingernails, and has no scientific evidence of medicinal properties. Levels of poaching are buoyed by a perceived leniency in the prosecution of offenders and high monetary reward from rhino horn trafficking, making it attractive for individuals to operate illegally [3,4].

Wildlife forensic science is a sub-discipline of forensic science that can assist authorities in the event of wildlife crimes [5]. Developing and enhancing wildlife forensic tools to improve enforcement of rhino horn trafficking crimes is recognized as an essential aspect to combat the current rhino poaching crisis [3,6]. Enforcement action for alleged rhino horn trafficking crime requires robust species identification testing. To exploit the high market value of rhino parts and products there has reportedly been a proliferation of fraudulent/substitute rhino horn products on the market, such as water buffalo (*Bubalus bubalis*) horn [3]. Species identification tests to determine counterfeit from real horn is the first and most important step in an investigation in order to determine if a criminal act has occurred and enforce legislation within that jurisdiction (in most cases implementing CITES), particularly for non-range states (*i.e.* nations that are not naturally inhabited by rhinos), in which individual identification through DNA profiling tends not to provide any extra evidential value to a prosecution [6]. Additionally, it is important to consistently identify the species of seized horn, not only to provide legal evidence, but also to monitor the market trends of rhino horn trafficking in range states and destination countries. For these reasons, the wildlife forensic community has identified the need to develop an internationally standardized and validated species identification test for all rhino species [6].

Horns can be difficult to morphologically distinguish at the species level, and can also be sold as powders, small fragments or worked products such as sculptures [3]. A number of rhino horn species identification methods have been developed that do not rely on external identifying characteristics, such as element and isotope fingerprinting [7], infrared spectroscopy [8], odour profiling based on volatile organic compounds [9] and DNA identification utilizing a nested-PCR protocol [10]. However, none of these methods are validated for use as forensic evidence in court.

DNA-based species identification of wildlife is often carried out on sequence differences of mitochondrial genes, typically the cytochrome-b (cyt-b) gene and/or the cytochrome c oxidase I (CO1) gene [33]. Tobe et al. [11] demonstrated that the cyt-b gene is more suitable to carry out species identification mammalian species, and previous work carried out on rhinos identified a 402 base pair (bp) region within cyt-b, amplified using a nested Polymerase Chain Reaction (PCR) protocol, was appropriate to use for species identification of rhino horns [10]. Here we present a DNA-based species identification test, designed to exploit the species differences that have previously been established by Hsieh et al. [10], but to amplify a shorter, yet still phylogenetically informative cyt-b region for all five rhino species and water buffalo (a common substitute) in a one-step PCR. Targeting a shorter cyt-b region improves success of amplifying DNA from low-template samples (*i.e.* from horn and/or horn derivatives). The test was standardized and validated across five laboratories from four different rhino horn consumer and/or transit countries. To complement the test, using synthetic DNA we have developed a rhino species identification 'confirmation test' and a DNA positive control sample. Additionally, for those laboratories without access to reliable reference material to make species level identification, we also provide sequence data from vouchered rhino specimens to avoid the use of erroneous reference sequences and/or unreliable reference sample information which may cause misidentifications [12]. Finally, the species identification test was applied to demonstrate its effectiveness in real life seizures. We have provided a complete and validated toolkit to assist any laboratories carrying out species identification for rhino horn products and derivatives. We anticipate that the enhancement of enforcement capabilities will act as a deterrent to individuals participating in the lucrative horn trade.

2. Materials and methods

2.1. Sample acquisition and DNA extraction

Reference samples comprised of tissue, hair, horn, bone, faeces and skin from deceased and live animals from 12 white rhinos, 13 black rhinos, 4 Indian rhinos, 4 Sumatran rhinos and 3 Javan rhinos in this study (Supplementary Table S1). Five Sumatran rhino sequences were also made available for this study. Of these 41 reference rhino samples, 26 were from specimens considered to be voucher specimens (*i.e.* a reference specimen of known provenance) (Table S1). Additionally, four blood samples from water buffalo, two samples from domestic cow (one tissue and one horn), and one tissue sample from a horse were tested, as horns/hooves from these species are known to be sold fraudulently as rhino horn (Edgard Espinoza personal communication; [3]) (Table S1). Five buccal swab samples from human were also tested to represent likely contaminants. All DNA extraction protocols in this study can be found in Supplementary material (Appendix I).

2.2. Design of a species identification test suitable for a degraded product

'Universal rhino primers' RID_FWD and RID_REV (RID: rhino identification) (Table 1) were designed to amplify the 14774–15003 cyt-b region (coordinates based on the revised Cambridge Reference Sequence for the human mitochondrial genome [13,14]) for all five rhino species as well as water buffalo, but to exclude human DNA. In order to develop these primers, sequences from the cyt-b gene were generated *via* PCR using previously published primers L14696 and H15197 ([10], Table 1) from blood, tissue and horn samples from six black rhinos and two white rhinos (Table S1). These sequences were aligned with rhino sequences from GenBank in order to identify regions that were informative both at the intra-specific and inter-specific level and suitable for primer design. Water buffalo and human DNA sequences from GenBank were also included in the primer design. In total, 30 sequences were used for primer design, including all rhino species, water buffalo and human (Table S1). Primers were designed using MEGA version 6.06 [15] and OLIGO 7 primer analysis software [16], with annealing temperatures confirmed *via* a series of gradient PCRs.

Table 1
PCR primers used to amplify regions of cytochrome-b.

Primer Name	Primer Sequences (5′–3′)	Annealing temperature (°C)	Amplicon length (bp)	References
L14696 (forward)	TCTCACATGGACTTCAACCA	50	500	Hsieh et al. [10]
H15197 (reverse)	CCGATATAAGGGATTGCTGA			
RID_FWD (forward)	AACATCCGTAAATCYCACCCA	55	230	This study
RID_REV (reverse)	GGCAGATRAARAATATGGATGCT			