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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.

Keywords:
Cytochrome-b
Illegal wildlife trade
Rhino horn
Wildlife forensic science
Standardization
Validation

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) [1]. 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 [1,2]. 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 market in

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consumer countries [3]. 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 [4]. 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 [4] 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 [4,5].

Wildlife forensic science is a sub-discipline of forensic science that can assist authorities in the event of wildlife crimes [6]. 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 [4,7]. 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 [4]. 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 [7]. 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 [7].

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 [4]. 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 [8], infrared spectroscopy [9], odour profiling based on volatile organic compounds [10] and DNA identification utilizing a nested-PCR protocol [11]. 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 [12]. Tobe et al. [13] 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 [11]. Here we present a DNA-based species identification test, designed to exploit the species differences that have previously been established by Hsieh et al. [11], 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 [14]. 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; [4]) (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 [15,16]) 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 ([11], 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 [17] and OLIGO 7 primer analysis software [18], 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. [11]
H15197 (reverse)	CCGATATAAGGGATTGCTGA			
RID_FWD (forward)	AACATCCGTAAATCYCACCCA	55	230	This study
RID_REV (reverse)	GGCAGATRAARAATATGGATGCT			

2.3. Validation

Validation components of the species identification test were undertaken across five laboratories from four different countries. These laboratories were: Australian Centre for Wildlife Genomics (ACWG) (Australian Museum Research Institute, Australia), Flinders University (Australia), Institute of Ecology and Biological Resources (IEBR) (Vietnam), Science and Advice for Scottish Agriculture (SASA) (UK) and the Wildlife Forensic Science Unit (WIFOS) (Department of National Parks, Thailand). Validation was based on the following characteristics: 1) reproducibility, 2) specificity, 3) phylogenetic resolution and 4) sensitivity.

2.3.1. Reproducibility

The primers RID_FWD/RID_REV were tested using PCRs on DNA from all of the reference samples from Section 2.1 (Table S1). Most of the samples were tested multiple times in separate PCRs to ensure the reproducibility of the assay (indicated in Table S1). Additionally, an alternate analyst performed a species identification on three randomly selected high-quality vouchered samples (from different species) in a blind trial (see Table S1).

PCRs were carried out using a range of supplier reagents commonly used by each of the participating laboratories, and can be found in Supplementary data (Appendix II). The amplification took place as follows: initial denaturation step (3–15 min at 94 °C, depending on reagents used), followed by 40 cycles of denaturing at 94 °C for 20 s, annealing at 55 °C for 40 s and 72 °C for 40 s, and a final extension step at 72 °C for 5 min. Note that 40 cycles were used as the PCR protocol was optimized and validated for low-template poor-quality samples; however, using 30 cycles is more appropriate for higher quantity DNA extracts (*e.g.* DNA from rhino tissue). PCR products were purified and sequenced using a range of methods (Supplementary data, Appendix II).

Sequence quality checks and sequence editing was carried out using either SEQUENCHER version 5.2.4 (Gene Codes Corporation) or Geneious (versions 8.1.9, 9.1.6, 10.1.1) [19]. The primer sequence and poor quality sequence was trimmed from the start and end of each sequence. Nucleotide discrepancies between the forward and reverse sequences were corrected if necessary, or changed to 'N' where ambiguous. The aim was to achieve > 90% sequence quality after editing, however lower values were acceptable if the sequences were clearly readable (*i.e.* when there were no discrepancies between the forward and reverse sequences, and/or when the contaminant sequence was easily identified *via* visual inspections of the chromatograms). Phylogenetic analyses were carried out on all generated reference sequences (Table S1) using MRBAYES version 3.2 [20] and sequence similarities were calculated using MEGA version 6.06 [17] to confirm the species identification of the reference material.

A temperature study was conducted on three high-quality samples (*i.e.* plucked hair roots, blood and tissue samples, indicated in Table S1) from white, black and Indian rhino, whereby the PCR annealing temperature was altered by ± 1.5 °C to test if these conditions produced the same results. This was done to ensure the protocol would produce a result even if the PCR machine used is out of calibration.

A 'confirmation test' was constructed to standardize the species identification protocol across multiple laboratories. It entailed a 299 bp region of DNA which was synthesized for each of the five rhino species, consisting of the aforementioned 230 bp cyt-b region, flanked by the RID_FWD and RID_REV primers. Additionally, a 302 bp DNA sequence was synthesized based on *Raphus cucullatus* (Dodo) 12S rRNA as a positive control, following [21], with the flanking primer sequences RID_FWD/RID_REV incorporated. All synthetic DNA sequences are included in a FASTA file in the Supplementary data (Appendix III). The sensitivity of the synthetic DNA samples was tested *via* serial dilutions and qPCR to establish the concentrations suitable for the confirmation test. Gel electrophoresis and sequencing was conducted on non-amplified synthetic DNA to investigate whether false positives could be produced. The confirmation test was trialled at ACWG and Flinders University. The six synthetic DNA samples underwent PCRs using the RID_FWD/RID_REV primers, following the PCR protocol outline above, and following the methods in the Supplementary data (Appendix II). Successful sequences were compared to reference sequences *via* phylogenetic analyses and assessment of sequence similarities to confirm correct species identification.

2.3.2. Specificity and phylogenetic resolution

In order to design the species identification test to exclude the DNA of possible contaminants on seized items, DNA from five human buccal swab samples (Table S1) at varying quantities (8.66–92.4 ng) were tested in a PCRs with the RID_FWD/RID_REV primers. Additionally, the RID_FWD/RID_REV primers were tested against the GenBank database using the NCBI Primer-BLAST software to identify what non-target species the primers may bind to *in silico*. This test was performed to identify whether any common contaminant species may preferentially amplify when conducting a PCR on suspected rhino horn.

We conducted a mixed samples experiment to identify the effect of incorporating multiple DNA sources in the PCR. DNA extracts from white rhino and cow, and white rhino and human (samples indicated in Table S1) were mixed at a ratio of 1:1, 1:5 and 1:20. PCRs were carried out following the protocols in Supplementary data (Appendix II).

Multiple methods were implemented to test the phylogenetic resolution of the 230 bp cyt-b region amplified by RID_FWD/RID_REV to identify if this marker is appropriate to successfully discriminate the five rhino species. Bayesian inference was conducted using MRBAYES version 3.2 [20], and maximum likelihood and neighbor joining inference was conducted using MEGA version 6.06 [17]. The sequences generated in Section 2.3.1 were used in the phylogenetic analyses, with the addition of two Javan rhino GenBank sequences to ensure adequate sample sizes for each species (n ≥ 3). One of the Javan rhino GenBank sequences (accession: FJ905815.1) was generated from a toenail sample from a skeleton at the Oxford University Museum of Natural History (England), received in 1905 (Mark Carnall personal communication; [22]), while the other sequence (accession: AJ245725.1) was generated from a rib sample at the Muséum national d'Histoire naturelle (France), received in the early 19th century (Joséphine Lesur personal communication; [23]). A horse sequence (accession: NC_001640.1) was used as the outgroup as it is a distant relative of the rhino within the order *Perissodactylla*. The Hasegawa-Kishino-Yano (HKY) nucleotide substitution model with gamma distributed rates among sites was chosen using the Bayesian Information Criterion (BIC) values generated using MEGA version 6.06 [17]. The Bayesian analysis was performed using four independent chains run for 100 million generations, and sampled every 1000 generations. Convergence diagnostic was calculated every 1000 generations; convergence was reached when the convergence diagnostic was ≤0.01. The convergence diagnostics from the Bayesian phylogenetic analysis were assessed using TRACER version 1.6 [24], and results were considered when effective sample sizes (the number of effectively independent draws equivalent to the Markov chain) were above 200 (a commonly used threshold). Maximum likelihood analysis was performed using the nearest-neighbor-interchange heuristic method with 1000 bootstrap replicates. Neighbor joining analysis was performed using the Maximum Composite Likelihood model with gamma distributed rates among sites with 1000 bootstrap replicates. Phylogenetic trees were visualized using FIGTREE version 1.4.2 [25].

A matrix comprising the range of sequence similarities between the five rhino species (interspecific similarity), and within each of the species (intraspecific similarity), was constructed to show the sequence divergence at this cyt-b region. Sequence similarity was calculated by dividing the number of identical nucleotides (calculated using MEGA version 6.06 [17]) by the total number of nucleotides (230), and converted to a percentage. In concordance with the phylogenetic analyses, the sequence similarity analyses were carried out using the sequences

generated in Section 2.3.1 (with the addition of the two Javan rhino GenBank sequences).

2.3.3. Sensitivity

Sensitivity of the species identification test was determined by testing three independent serial dilutions from high quality DNA extracts. DNA quality assessment was based on the DNA absorbance at 260 nm (A_{260}) and 280 nm (A_{280}), whereby the A_{260} is between 0.1 and 1.0, and the A_{260}/A_{280} ratio is between 1.8 and 2 [26,27]. These absorbance parameters were measured using a NanoDrop spectrophotometer ND-1000 (NanoDrop technologies). DNA from one white rhino, one black rhino and one Indian rhino (indicated in Table S1) were used to create the serial dilutions from ~10 ng/μL to ~1 pg/μL for this sensitivity study.

2.3.4. Case studies

Casework testing was undertaken to demonstrate the utility of the species identification protocol in relation to actual customs seizures of suspected rhino horns. A total of 52 seized items were tested: 14 horns from multiple Australian Customs seizures, 18 horns from a Vietnamese Customs seizure in August 2015, and 20 horns from a Thailand Customs seizure in March 2017 (Table S2). DNA extractions and PCR amplifications were undertaken for each of the case studies in the countries where the seizures occurred, following the protocols presented in the Supplementary material (Appendix I and Appendix II). Successful sequences were compared to reference sequences *via* phylogenetic analyses and assessment of sequence similarities to confirm correct species identification.

3. Results

3.1. Reproducibility

The RID_FWD/RID_REV primers successfully amplified PCR products in 100% of the white rhino (n = 12), black rhino (n = 13), Indian rhino (n = 4), water buffalo (n = 4) and horse (n = 1) samples, 75% of the Sumatran rhino samples (n = 4), 33% of the Javan rhino samples (n = 3), and 0% of the cow samples (n = 2) (Table S1). All PCR amplicons were sequenced and the species identification was confirmed when compared to reference sequences *via* phylogenetic analyses and assessment of sequence similarities (including the samples that were tested multiple times).

The alternate analyst successfully identified all three rhino samples in the blind test. All samples in the temperature study were successfully amplified and sequenced when the annealing temperature was altered by ± 1.5 °C.

Results from serial dilutions and qPCR demonstrated that the most appropriate synthetic DNA dilution for use in the confirmation test was 10 fg/μL; all testing was conducted with the synthetic DNA at this concentration. When testing for false positives using non-amplified synthetic DNA, no electrophoresis bands were visible and no sequences were generated, indicating that false positives will not be a problem when using the synthetic DNA diluted to ~10 fg/μL. Both laboratories that participated in the rhino species identification confirmation test successfully amplified and sequenced all five of the rhino synthetic DNA samples and the positive control Dodo synthetic DNA sample. All sequences provided a correct identification when compared to reference sequences *via* phylogenetic analyses and assessment of sequence similarities.

3.2. Specificity and phylogenetic resolution

No amplicon was produced in PCRs with human DNA using the RID_FWD/RID_REV primers (Table S1). The top 100 hits in GenBank using the NCBI Primer-BLAST software included 8 rhinos (3 species), 79 bats (29 species), 6 mongooses (2 species), 3 tapirs (1 species), 3 pigs (3 species) and 1 zebra; however, the authors acknowledge the top 100 hits are largely dependent on the number of sequences represented by closely matching species in the database. The average number of mismatches between the primer sequences and reference sequences for each of the groups above, and the associated standard error (SE), was 0 (SE = 0), 1.28 (SE = 0.08), 2 (SE = 0), 1 (SE = 0), 2 (SE = 0) and 1 (SE = 0) respectively. The range of sequence similarities (as a percentage) between the 8 rhino sequences and the 92 non-target species sequences identified in this analysis was 73.91-88.70%, and the average was 80.06% (SE = 0.079). This *in silico* test must be qualified as it is dependent on the GenBank database, which may lack appropriate reference data, hence these results only provide an indication of potential contaminant species the primers may amplify. Further, the average number of mismatches between the primers and the five water buffalo and three human GenBank sequences utilized in primer design (Table S1) was 3 (SE = 0) and 9.33 (SE = 0.33) respectively.

In the mixed samples study, a single PCR product at the expected size was obtained for all six amplifications (three dilutions for both the rhino-cow and rhino-human mixes). Marginally brighter bands are visible with lesser mass of bovine and human DNA. DNA sequence data from the 20:1 cow to white rhino mix showed only the presence of white rhino DNA, indicating that the primers are specific to rhino in the presence of large amounts of bovine DNA.

The phylogenetic trees constructed for the 230 bp cyt-b region amplified by the RID_FWD/RID_REV primers clustered the five rhino species into highly distinct lineages using Bayesian, maximum likelihood and neighbor joining methods. Results from all three analyses are shown in Fig. 1. There was weak support at some of the basal nodes, however all posterior probabilities and bootstrap support at the species level were > 0.95.

The interspecific sequence similarity matrix demonstrated sufficient resolution between the five rhino species at this cyt-b region to conduct robust identifications *via* the sequence similarity method (Table 2). As expected, the intraspecific matrices demonstrated significantly less variation. The lowest intraspecific sequence similarity (98.7%) was considerably larger than the highest pairwise interspecific sequence similarity (94.78%).

3.3. Sensitivity

The results from the serial dilutions showed successful amplification down to 20 pg of input DNA for all replicate dilutions for white, black and Indian rhino samples (Fig. S1). The white and Indian rhino dilutions were all successful at 2 pg of input DNA, and 2/3 of the black rhino dilutions were successful at 2 pg. This means that the test is reliable down to a lower limit of 20 pg input DNA for these three species.

3.4. Case studies

In the casework testing, 47 out of 52 horn samples were successfully identified using the species identification protocol (Table S2). Of the 47 identified horns, 32 were from white rhino, 11 were from black rhino, 2 were from an Indian rhino and 2 were from water buffalo. No amplicons were generated for five of samples. Phylogenetic trees for each of the seizures are presented in Fig. S2.

4. Discussion

We are currently amidst a rhino poaching crisis driven by high demand for rhino horn [4]. For most non-range countries, in the event that suspected rhino horn is seized, species identification is the most important, and often the only component to initiate a criminal investigation. A forensic test should be conducted to determine whether the suspected rhino horn is actually from a rhino, and if so, determining the species from which the horn was derived. This paper presents an internationally standardized rhino species identification test, based on a

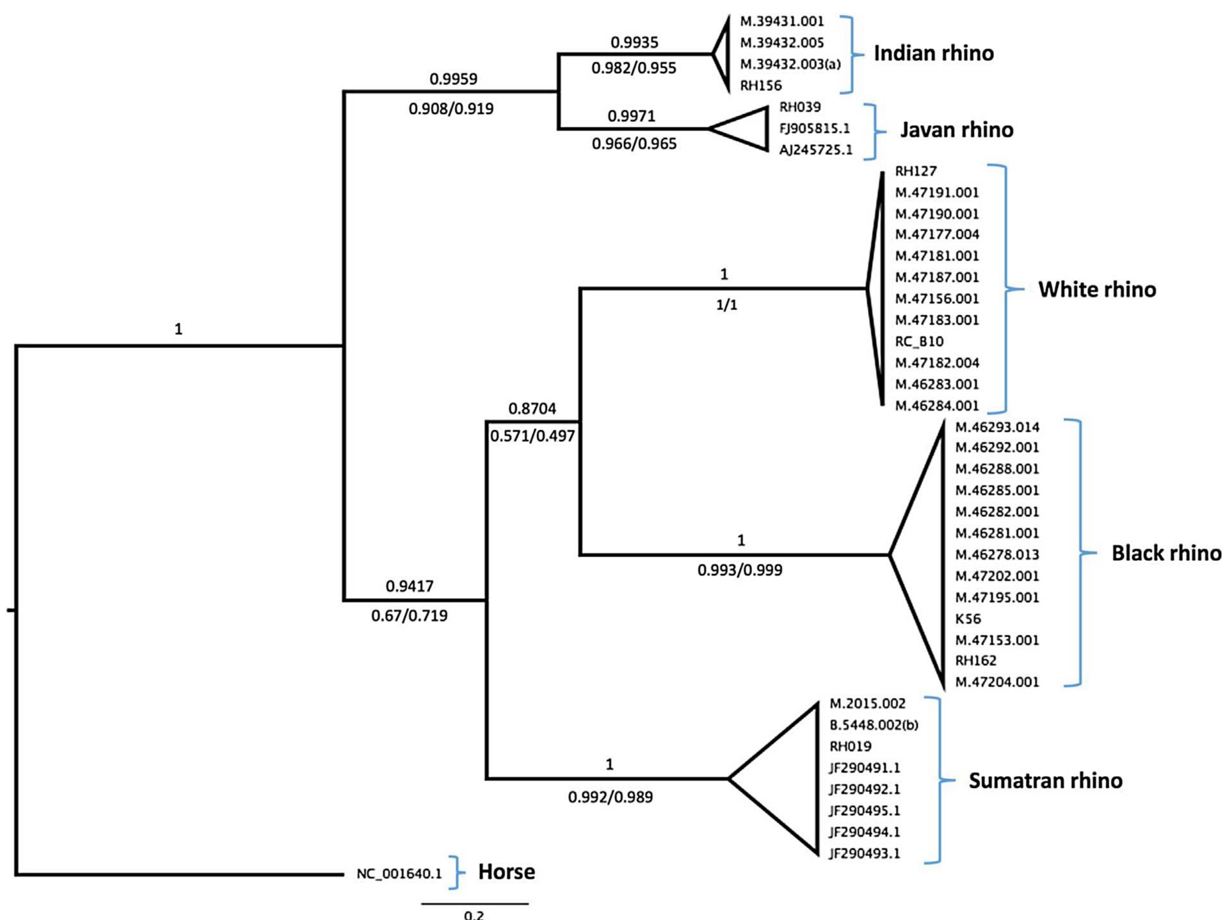

Fig. 1. Bayesian phylogenetic analysis of the 230 bp cytochrome-b fragment amplified by the RID_FWD/RID_REV primers, using the HKY + G site distribution evolutionary model. Bayesian posterior probabilities are above the branches, and the corresponding bootstrap support are below the branches for the maximum likelihood analysis/neighbor joining analysis.

230 bp cyt-b region. The test was validated across five different laboratories in both rhino horn consumer and transit countries to ensure its integrity as forensic evidence in the criminal justice systems worldwide [28]. The development of this species identification test was one of the key recommendations outlined in the proceedings from the Collaborative Action Planning workshop based on rhino horn forensics [7].

The 'universal rhino primers', RID_FWD/RID_REV, were designed to amplify DNA from all five rhino species and water buffalo, while simultaneously excluding any human contaminant DNA. In a single-step PCR, the L14696/H15197 primers designed by Hsieh et al. [11] had limited success in amplifying rhino horn samples (Table S3). Further, amplification using these primers often produced spurious secondary PCR products, and human DNA was amplified (Table S3). However, the L14696/H15197 primers were designed for use in a nested PCR protocol which may explain why they did not perform optimally in a single-step PCR. The RID_FWD/RID_REV primers (Table 1) amplify a relatively short genomic region of cyt-b (230 bp). Species identification for 33 of the 36 rhino samples from museum and zoo collections was successfully verified using the RID_FWD/RID_REV primers in a single-step PCR, including all of the vouchered samples and all horn samples (Table S1). The test successfully verified the species for numerous bones and horns more than 100 years old, and multiple faecal samples, demonstrating the utility of identifying the species of degraded rhino samples. Three references samples were not able to be verified using these methods (one Sumatran and two Javan rhino samples). This was likely due to the fact that samples were taken from museum specimens, and only minimal samples of these rare and historic specimens were provided for testing. We surmise therefore that it is likely that the DNA extracted was highly degraded and/or the quantity of DNA was below the limits of detection of the test (< 20 pg). Due to the successful identification of numerous other degraded rhino materials (Table S1), we expect the test to successfully identify horn derivatives that may be encountered in the trade (*e.g.* libation cups or dagger handles), provided adequate samples can be taken.

In Vietnam, a large proportion of fraudulent rhino horns are thought

Table 2
A matrix containing the range of pairwise interspecific sequence similarities (as a percentage) for (230 bp) reference sequences from each rhino species, and the range of intraspecific sequence similarities (on the diagonal, bold).

Species		(1)	(2)	(3)	(4)	(5)
(1)	White rhino	**100–100**				
(2)	Black rhino	89.13–90	**99.13–100**			
(3)	Sumatran rhino	89.13–90	90.43–91.30	**99.13–100**		
(4)	Indian rhino	89.13–89.13	85.65–86.09	87.83–88.70	**100–100**	
(5)	Javan rhino	87.39–88.26	86.09–87.39	87.83–89.57	93.91–94.78	**98.70–99.57**

to be made from water buffalo horn [4]. The species identification test was used to successfully identify all four of the water buffalo samples and the horse sample, hence can be used to identify fake horns manufactured from these species in an investigation. Conversely, the test was not able to identify either of the two cow samples. Other materials are known to be manufactured and sold as fake rhino horn, such as caseins, resins, wood (*e.g. Dalbergia cochinchinensis*), hair and plastics (Edgard Espinoza personal communication; [4]). Therefore, if no amplicon is produced when carrying out this test, additional testing may be required to identify what the suspected rhino horn is manufactured from.

The successful and consistent results obtained when testing known specimens in different laboratories using different reagents, and the blind test conducted by an alternate analyst, demonstrates the reproducibility of the rhino species identification test. Additionally, the results obtained in the temperature study validate this protocol over a relatively broad range of annealing temperatures (53.5 °C–56.5 °C). A species identification using the described protocol can consequently be conducted using a range of reagents, and even when a PCR machine is slightly out of calibration.

Successful results from the confirmation tests further demonstrates the reproducibility of this species identification test. Synthetic DNA was used in this test to standardize the PCR protocol, and not the DNA extraction methods, as the equipment and techniques used in DNA extractions varies widely between laboratories and between sample types (Supplementary data, Appendix I). Additionally, synthetic DNA is much easier to export/import than real rhino samples, owing to the permits required to move samples from species listed in CITES Appendix I. The Dodo synthetic DNA sample provides a positive control that can identify if the test is working to specification, whilst removing the risk of cross-contamination between casework samples (or research samples) and control samples, as it is highly unlikely that an extinct bird will be encountered in the laboratory [21]. The positive control Dodo synthetic DNA can be used in subsequent casework involving suspected rhino horn. We intend to expand this confirmation test, and recommend its use as an international proficiency test *via* the Society of Wildlife Forensic Science (SWFS). This standardized proficiency testing will extend the international standardization of the rhino species identification presented in this paper to other labs, and provide a resource for labs to comply with SWFS guidelines [29], ISFG guidelines [30] and/or lab accreditation standards (*e.g.* ISO/IEC 17025 accreditation). All synthetic DNA sequences are included in a FASTA file (Supplementary data, Appendix III) for synthesis if required.

The specificity of the primers was tested to ensure contaminants are not preferentially amplified in a PCR. No amplicons were produced when testing human samples with the RID_FWD/RID_REV primers in a PCR. Further, in the mixed samples experiment, there was no inhibition when mixing rhino with cow or human, as we were able to successfully generate a rhino sequence even with a 1:20 mix (rhino to human or cow). When testing the primers on the NCBI database *in silico*, no notable species were identified that may potentially contaminate the assay, such as human and bacteria. All non-target putative amplicons identified *in silico* showed < 89% sequence similarity to the rhino amplicons. Our expectation is that non-target DNA should not affect the resulting amplification when using this species identification test. This is a sequence-based species identification protocol, with all PCR products sequenced; therefore, even if non-target DNA was amplified, this would not affect the results of the test as it is extremely unlikely that the resultant sequence would match rhino.

The 230 bp cyt-b region amplified by the RID_FWD/RID_REV primers successfully resolved the four rhino genera (the Indian rhino and Javan rhino are both from the genus *Rhinoceros*) in the phylogenetic analyses (Fig. 1). The weak statistical support at the more basal nodes of the phylogenetic trees is consistent with previous rhino mtDNA phylogenetic studies [22]. However, the phylogenetic tree at the species level provides strong support to differentiate the five species (Fig. 1). The sequence similarity matrix (Table 2) demonstrates clear differentiation between the five species. Although the relatively small sample sizes do not represent the full phylogeographic variation of each of the species, the sequence similarities provide a clear indication that there is no overlap between the intraspecific and interspecific variation. These results demonstrate the validity of using phylogenetic analyses and/or sequence similarity to report a species identification for this test. The lack of appropriate reference data and the reliance on GenBank sequences to make a species level identification can lead to unreliable or incorrect identifications [14]. Using vouchered reference sequences for sequence comparisons in a wildlife forensic investigation is crucial, and one of the ISFG recommendations [30]. We have provided sequence data from vouchered specimens for four species of rhino in the Supplementary material (Appendix IV) to ensure laboratories meet this requirement. Javan rhino voucher material was not available for testing in this study. However, given there are fewer than 100 individuals remaining, it is highly unlikely that Javan rhino horns will be encountered in the rhino horn market [31].

The utility of this species identification test in active casework was demonstrated in the field testing. Ninety percent of seized horn specimens were successfully identified in the three case studies (Table S2). There are numerous reasons why five horns were reported as 'no result'; for example, they could have been fake or substitute horns other than water buffalo horn or horse, the test failed due to human error, or the extracted DNA was below the limits of detection of the test (< 20 pg). Additional testing, utilizing universal mammal primers and/or universal plant primers, could be carried out to identify whether the five failed samples were manufactured from other biological materials such as cow or wood. The results from this field testing provide important data in regards to which species are being poached and currently part of the illegal trade. Horns from white, black and Indian rhino were identified, with white rhino horns constituting 68% of the horns identified, supporting trends that currently white rhinos are the main poaching targets [4]. Interestingly, there were also two water buffalo horns identified, demonstrating the need for its incorporation into this test and supporting the findings in the Milliken and Shaw [4] study, that water buffalo horn is a common substitute in the rhino horn trade.

We outline the first published fully validated species identification test developed for application in crimes involving the illegal trade in rhino parts, primarily horns. This test, along with the generation of vouchered references samples for comparison, and development of a rhino specific confirmation test, provides a comprehensive forensic toolkit for any lab carrying out identification of rhino products. This test involved a collaboration between five different labs in both rhino horn consumer and transit countries to ensure comprehensive validation and standardization, making it the most effective and appropriate method to identify the species of a seizure specimen in a rhino horn trafficking investigation currently available. This study serves as a model for how wildlife forensic species identification tests should be standardized and disseminated for use as forensic evidence. The test has already been transitioned into the criminal justice system, demonstrated by three case studies. It is anticipated that the implementation of this new protocol will improve the enforcement of rhino horn trafficking crimes and subsequent prosecution outcomes, ultimately acting as a deterrent for individuals that wish to enter the rhino horn trade.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2017.10.003.

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