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Research paper

Optimisation and application of a forensic microsatellite panel to combat Greater-one horned rhinoceros (*Rhinoceros unicornis*) poaching in India

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ABSTRACT

The Greater one-horned (GoH) rhinoceros is one of the most charismatic endemic megaherbivores of the Indian subcontinent. Threatened by poaching, habitat loss and disease, the species is found only in small areas of its historical distribution. Increasing demands for rhino horns in chinese traditional medicine has put the existing population under continuing threat, and large profits and low conviction rates make poaching difficult to contain. DNA forensics such as the RhoDIS-Africa program has helped in combating illegal rhino horn trade, but the approach is yet to be optimised for Indian GoH rhinoceros. Here we followed the International Society for Forensic Genetics (ISFG) guidelines to establish a 14 dinucleotide microsatellite panel for Indian GoH rhinoceros DNA profiling. Selected from a large initial pool (n = 34), the microsatellite markers showed high polymorphism, stable peak characteristics, consistent allele calls and produced precise, reproducible genotypes from different types of rhino samples. The panel also showed low genotyping error and produced high statistical power during individual identification (P_{IDsibs} value of $1.2*10^{-4}$). As part of the official RhoDIS-India program, we used this panel to match poached rhino carcass with seized contraband as scientific evidence in court procedure. This program now moves to generate detailed allele-frequency maps of all GoH rhinoceros populations in India and Nepal for development of a genetic database and identification of poaching hotspots and trade routes across the subcontinent and beyond.

1. Introduction

The Greater one-horned (GoH) rhinoceros (*Rhinoceros unicornis*) is one of the most iconic, obligate grassland-dwelling megaherbivores endemic to India and Nepal [1]. Once distributed throughout the northern part of the Indian subcontinent covering all the major river basins from Pakistan to Indo-Myanmar borders during the 1600s, the species is currently found in 12 fragmented, protected regions occupying >2000 km^2 area in India and Nepal [1]. With a global population size of ~3700 individuals, the species is categorized as 'Vulnerable' by IUCN [1] and is listed in Appendix I of CITES and Schedule I of the Wildlife Protection act of India (1972) [1]. The species was on the brink of extinction around the early 1900s but strong conservation measures have resulted in population recovery of Indian GoH rhinoceros population [2,3]. All extant GoH rhino populations are currently found exclusively within protected areas [1]. The major threats for the species are poaching, decline in habitat quality, disease risks, resource depletion and occasional human-animal conflicts [1,4–7].

Like all other rhino species, one of the most alarming conservation challenges for GoH rhinoceros populations is poaching for their body parts, especially horns [1,7]. Poaching threats on the existing rhino populations are continuously increasing due to high demands of their horns for traditional chinese medicine and other purposes in the illegal wildlife markets globally [8,9]. Recent reports from India corroborate this information as 239 rhino poaching incidents (~10 % of the current population size) have been recorded between 2001–2016 [10]. The large profits and low conviction rates in illegal wildlife trade has further hastened the threats of poaching by organized syndicates. One way of tackling this problem is to focus on preventing poaching at the source, where its containment would be most disruptive to these criminal networks. It is also important to understand the extent, form and dynamics of this trade at the supply end and focus on the poaching hotspots in source countries as well as on potential transit routes between source and transit countries [11–13]. Given the trend of increasing poaching pressures for rhino horn [3], DNA-based forensic tools are critical to counter this threat as such tools have shown great potential to address

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poaching and trade issues at national as well as international scales [11–13]. The Veterinary Genetics Laboratory, University of Pretoria has effectively employed the Rhino DNA Indexing System (RhoDIS) across South Africa and other African rhino range countries to help in rhino crime case convictions [13]. This program is one of the rare and exceptional examples of collaboration between forest authorities, scientific organizations and legal authorities to provide scientific evidences in cases of alleged poaching events [13]. While this system is promising in the context of GoH rhinoceros conservation and managing the threats of poaching, the system has not been tested or optimised in India. For GoH rhinoceros, species identification protocols are well tested and standardized for forensic use [9,14], but the microsatellite markers have not been optimised in the line of the African-RhoDIS program and has only been used in population genetic research [15–18].

In this paper, we addressed key methodological issues related to optimisation of microsatellite markers for rhino forensic use. We followed International Society for Forensic Genetics (ISFG) guidelines [19,20] to systematically screen dinucleotide microsatellite markers for forensic use in Indian GoH rhinoceros (RhoDIS-India). While there is a general concern regarding use of dinucleotide markers in forensic research, they have been widely used in wildlife forensics [19,20], including rhinos [21,22]. In addition, we tested DNA extraction protocols from rhino horns based on the protocols described in earlier studies [9,14,21]. Finally, we used the microsatellite panel to match poached carcass samples with seized rhino body parts as scientific evidence in court proceedings. We believe that this approach can be used as a model to tackle wildlife crime issues in other trade-target species.

2. Materials and methods

2.1. Permission and ethical considerations

The RhoDIS-India program is a Ministry of Environment, Forests and Climate Change, Government of India led rhino conservation project with a goal to control rhino poaching at national and regional scale. All relevant permissions to implement field sampling have been received from Government of India (Letter No. 4-22/2015/WL). All tissue sampling from naturally dead or poached rhinos were conducted during post-mortem examinations performed by trained officials of the respective State Forest Departments of Assam, West Bengal and Uttar Pradesh and did not require any other ethical clearances.

2.2. Sample collection

All optimisation of laboratory protocols was performed with field-collected wild Indian GoH rhinoceros tissue samples. A total of 96 reference rhino tissue samples (6 blood samples and 90 muscle tissues) were provided to us from forest department authorities of Assam (n = 86), West Bengal (n = 6) and Uttar Pradesh (n = 4). These samples were collected from naturally dead rhinos (n = 92) during post-mortem examinations as well as during rhino translocation programs (n = 4). All field-sampling was conducted by trained persons of the respective forest departments. Once brought to the laboratory, the samples were catalogued and stored in −20 °C freezer until further processing. In addition, we have also used four reference rhino horn samples for initial laboratory protocol optimisation. These samples were part of Wildlife Institute of India's (WII) national repository of Wildlife Forensics and Conservation Genetics Cell. About 200 mg of small horn pieces were cut using sterile blades and stored in −20 °C freezer for protocol optimisation in the laboratory.

2.3. DNA extraction

DNA was extracted from blood samples using QIAamp DNA Tissue Kit (QIAGEN Inc., Hilden, Germany) following the standard protocol for blood samples. To extract DNA from various quality rhino tissue samples from the field we used the protocol described in Mondol et al. [24]. In brief, about 20 mg of tissue was macerated with sterile blade and digested with 30 μL of Proteinase K (20 mg/mL) and 300 μL of ATL buffer (Qiagen Inc., Hilden, Germany) overnight at 56 °C, followed by Qiagen DNeasy tissue DNA kit extraction protocol. DNA was eluted twice in 100 μL preheated (70 °C) 1X TE buffer. For every set of samples (n = 11), one extraction negative was included to monitor possible contaminations.

For implementation of RhoDIS-India program we needed to optimise the DNA extraction protocol from rhino horns as the DNA quality from the horns can be variable due to presence of keratin, which acts as a PCR inhibitor [23]. Problems with downstream processing of rhino horn DNA can further be compounded due to small quantities of starting material (e.g. chopped pieces and chips or powders) received for casework. We tested two different DNA extraction protocols that use Qiagen DNeasy tissue DNA kit (QIAGEN Inc., Hilden, Germany) on four rhino horn samples from WII tissue repository and compared the DNA yield and amplification success for microsatellite markers. The first protocol was exactly the same used for rhino tissue samples (see above) whereas in the second method we used a dekeratinizing agent DTT (Dithiothreitol) during initial lysis [9,14,21,23]. The macerated rhino horn base tissue (~20 mg weight) were lysed for 16 h at 56 °C with 30 μL of 1 M DTT, 30 μL of Proteinase K (20 mg/ ml) and 300 μL of ATL buffer (Qiagen Inc., Hilden, Germany) with intermittent vortexing. Post-lysis remaining steps were followed as described in Qiagen DNeasy tissue DNA kit. DNA was eluted twice in 100 μL preheated (70 °C) 1X TE buffer [24]. For each extraction, extraction negatives were included to monitor possible contaminations. PCR amplifications are performed as described below.

2.4. Primer selection

The RhoDIS program (both in South Africa [13] and the targeted Indian program) aimed to provide legal aid for rhino poaching and trade cases at a global scale. Achieving this goal is only possible through a same panel of microsatellite markers along with reference samples to ensure data uniformity across laboratories. The Veterinary Genetics Laboratory, University of Pretoria has successfully standardized a panel of 23 microsatellite markers for use in forensic case work of African rhinos [21]. Some of these 23 markers (n = 18) have been tested on a few Indian GoH rhinoceros [25], (n = 18) but the entire panel has never been assessed for use in forensic casework for the species in India. Thus, we optimised the same markers for the RhoDIS-India program to select the best ones based on the criteria including i) marker polymorphism and heterozygosity; ii) amplification success; iii) ease in allele calling and iv) stable allele characteristics [19,20,26,27]. In addition, we have also added 11 loci earlier used on Indian GoH rhinoceros [16]. This was done anticipating that some African rhino markers might not amplify or produce erroneous results with the Indian rhinos [25,28]. Initially we tested all 34 markers on 30 field-collected rhino tissue samples from different rhino-bearing areas in India (Assam = 21, West Bengal = 5 and Uttar Pradesh = 4 samples, respectively), and the final panel of markers were used for all remaining samples (n = 66). The primer selection process involved the following steps:

2.4.1. Optimisation of annealing temperatures and DNA concentrations

Before annealing temperature optimisation, we quantified DNA concentrations for all 30 samples in Epoch Microplate Spectrophotometer and found highly variable concentrations (ranging from 10 ng/μL – 745 ng/μL). Further we selected six samples representing a gradient of DNA concentrations (~10, 80, 150, 300, 500 and 700 ng/μL) for further optimisations. We serially diluted these DNA concentrations to 1X, 1/10X, 1/25X and 1/50X, representing input DNA concentration ranging from 0.2 to 700 ng/μL. To optimise the annealing temperature, PCR reactions were performed for all 34 primers (See Table 1) in 10 μL reactions containing 4 μL of 2X Qiagen multiplex PCR buffer mix

Table 1
Details of the 34 microsatellite markers used for GoH rhinos in this study. The selected markers are listed according to their informative content.

Locus ID	Repeat type	Dye	Ta	Amplification success (%)	No. of allele	Allelic size range	Ho	He	Cumulative P_{ID} (unbiased)	Cumulative P_{ID} (sibs)	ADO	FA	NA	Between run precision (S.D)	Selection criteria	Multiplex PCR sets
RH10	Di	HEX	60	98.7	7	14	0.67	0.79	7.21E-02	3.79E-01	0.02	0.01	0.06	0.1	Selected	MPI
RH4	Di	6-FAM	57	96.7	6	24	0.72	0.74	7.36E-03	1.55E-01	0.02	0	0.01	0.18	Selected	MPIV
RH5	Di	HEX	60	99.6	5	12	0.56	0.72	9.06E-04	6.55E-02	0	0.02	0.08	0.07	Selected	MPII
SR63	Di	6-FAM	60	99.6	4	12	0.64	0.72	1.12E-04	2.78E-02	0.02	0.01	0.05	0.04	Selected	MPII
RH3	Di	NED	60	100	3	34	0.59	0.66	2.08E-05	1.30E-02	0.03	0.01	0.04	0.04	Selected	MPI
RH1	Di	6-FAM	60	98.4	3	4	0.59	0.63	4.38E-06	6.36E-03	0.04	0	0.03	0.09	Selected	MPI
RH11	Di	HEX	57	94.7	4	12	0.62	0.60	1.03E-06	3.27E-03	0.03	0.02	0.01	0.15	Selected	MPIV
IR12	Di	HEX	60	95.1	2	4	0.19	0.49	3.57E-07	1.96E-03	0	0.01	0.20	0.05	Selected	MPIII
SR281	Di	6-FAM	60	97.1	2	2	0.45	0.49	1.34E-07	1.18E-03	0	0.01	0.03	0.04	Selected	MPI
12F	Di	HEX	60	90	4	6	0.39	0.47	5.07E-08	7.22E-04	0.01	0	0.06	0.05	Selected	MPIII
7B	Di	FAM	60	93.1	3	6	0.43	0.45	1.91E-08	4.43E-04	0.03	0	0.00	0.04	Selected	MPIII
RH9	Di	PET	57	96.3	3	24	0.37	0.45	7.25E-09	2.77E-04	0.03	0.08	0.06	0.13	Selected	MPIV
RH7	Di	NED	60	99.6	2	2	0.47	0.44	2.94E-09	1.75E-04	0.02	0	0.00	0.08	Selected	MPII
IR10	Di	NED	60	99.6	2	8	0.31	0.34	1.42E-09	1.23E-04	0.08	0	0.03	0.09	Selected	MPII
SR262	Di	6-FAM	60	–	–	–	–	–	–	–	–	–	–	2.3	Stutter related inconsistency (rejected)	–
7C	Di	6-FAM	60	–	–	–	–	–	–	–	–	–	–	1.5	Stutter related inconsistency (rejected)	–
DB66	Di	6-FAM	60	–	–	–	–	–	–	–	–	–	–	1.7	Stutter related inconsistency (rejected)	–
Rh6	Di	PET	60	–	–	–	–	–	–	–	–	–	–	1	Stutter related inconsistency (rejected)	–
Rh8	Di	PET	60	–	–	–	–	–	–	–	–	–	–	1.7	Stutter related inconsistency (rejected)	–
32F	Di	VIC	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
B1Rh37D	Di	NED	57	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
SR74	Di	NED	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
DB44	Di	VIC	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
DB23	Di	VIC	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
BR6	Di	NED	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
IR22	Di	VIC	57	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
Rh12	Di	VIC	57	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
DB1	Di	PET	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
32A	Di	6-FAM	60	–	–	–	–	–	–	–	–	–	–	–	Low RFU (rejected)	–
B1Rh1B	Di	NED	60	–	–	–	–	–	–	–	–	–	–	–	Multiple peak (rejected)	–
SR268	Di	VIC	60	–	–	–	–	–	–	–	–	–	–	–	Low RFU (rejected)	–
Rh2	Di	6-FAM	–	–	–	–	–	–	–	–	–	–	–	–	Did not amplify (rejected)	–
DB52	Di	PET	–	–	–	–	–	–	–	–	–	–	–	–	Did not amplify (rejected)	–
B1Rh1C	Di	PET	–	–	–	–	–	–	–	–	–	–	–	–	Did not amplify (rejected)	–

Ta- Annealing temperature, Ho and He- observed and expected heterozygosity, ADO- allele drop out, FA- false alleles, NA- null alleles.

(QIAGEN Inc., Hilden, Germany), 0.2 μM labelled forward primer, 0.2 μM unlabelled reverse primer, 4 μM BSA (4 mg/mL) and 2 μL of rhino DNA at respective concentrations calculated above. PCR conditions included an initial denaturation (95 °C for 15 min); 35 cycles of denaturation (95 °C for 30 s), annealing (50−60 °C gradient for 40 s) and extension (72 °C for 40 s); followed by a final extension (72 °C for 20 min). During each reaction set, PCR and extraction negatives were included to monitor contamination. Amplified products were visualized with 2 % agarose gel and further genotyped using HiDi formamide (Applied Biosystems, California, United States) and LIZ 500 size standard (Applied Biosystems, California, United States) in an ABI 3500XL Genetic Analyser (Applied Biosystems, California, United States). The fragment lengths were scored manually using the program GENEMARKER (Softgenetics Inc., Pennsylvania, United States) and allele call bins were created for all the loci. The entire process was repeated three times in two different PCR machines (Eppendorf flexlid and ABI Veriti) and results were analyzed to determine optimal annealing temperatures and DNA concentration and to identify primers that were not amplifying in Indian GoH rhinoceros. One of the tissue samples was selected as a 'genotyping reference sample' and has been independently genotyped seven times to confirm the alleles. This sample was used as a standard to reduce genotyping errors from allele shifts among multiple runs in all subsequent genotyping analyses for Indian GoH rhinoceros and designated as reference sample for RhoDIS-India program.

2.4.2. Peak characteristics, allele calling and marker polymorphism

Following temperature optimisation of all primers and selection of the optimum input DNA concentration (see result section) with six samples, we amplified the next set of samples (n = 24) three independent times to select primers based on allele characteristics (multiple peaks and stutters, peak ratio etc). While allele calling we identified primers with low 'relative fluorescence units (RFU)' (less than 100) and multiple peaks. These primers were rejected from further analyses as they can lead to erroneous allele calls [26,27]. Subsequently, we prepared consensus genotypes for all 30 samples using a 'Quality index' approach involving all three independent results [29]. To check polymorphism in all the markers, we amplified them with remaining tissue samples (n = 66). All monomorphic markers were then excluded from downstream analyses.

2.4.3. Stutter-related inconsistencies and multiplexing of primers

To assess stutter-related inconsistencies (particularly in dinucleotide loci) in our selected loci, we calculated peak RFU ratios of heterozygote (ratio between short and long allele) and homozygote (ratio between -2R stutter and its homozygote peak) alleles for all loci [30]. This approach helped us to select loci with stable stutter character after quantifying the inter and intra sample variations in peak ratio. The ratios were independently calculated for all three replicates of all polymorphic loci for 30 selected samples. Subsequently, we calculated the standard deviation in the ratios, and the loci with high standard deviation values were rejected as they can be prone to stochastic errors leading to inconsistent allele calling [30].

The selected markers were multiplexed based on allele size and dye colour, and then each multiplex panel was checked for data accuracy by replicating the same PCR conditions mentioned above with different number of amplification cycles (20, 25, 30 and 40). Finally all sample (n = 96) were genotyped (for final multiplex panel) in three replicates to ensure consistent results by comparing them with previously generated genotypes.

2.5. Data analyses

We quantified allelic dropout and false allele (FA) rates manually as error rates per locus including the replicate data. Allelic drop out was quantified as ratio between number of amplifications with loss of one allele and total number of positive heterozygous individual amplification. The false allele was calculated for both homozygotes and heterozygotes as proportion of number of false allele amplification versus total number of amplifications [27,31]. Further, we used the program FreeNA [32] to determine the frequency of null alleles (NA), which estimates the NA frequency using the EM algorithm [33]. Program GIMLET [34] was used to calculate the $P_{ID(sibs)}$ for all the individuals. Following this, any allele having less than 10 % frequency across all amplified loci was rechecked for allele confirmation. ARLEQUIN [35] was used to calculate overall summary statistics and determine Hardy Weinberg equilibrium and linkage disequilibrium for all the loci.

2.6. Forensic use of the RhoDIS-India marker panel

We received 16 rhino poaching cases from various legal authorities in India. The case properties were transferred to our laboratory following strict 'Chain of custody' protocols. Of these, 13 samples were from poached rhino carcass and three samples were of seized rhino horn contraband. Some of the case properties from rhino carcasses had multiple samples for individual matching (n = 7). Following photographic and administrative documentations at the forensic laboratory, DNA was extracted, quantified and species identification was performed by partial cytochrome *b* and 12S rRNA sequences as described in Jha et al. (2017). This species identification protocol was chosen over earlier described ones (for example, see [9]) due to the requirement of India's leagl procedure requirements, where ascertainment of species is required to be matched with our country's sequence database. PCR reactions were performed in 10 μl reaction volumes containing 4 μl of 2X Qiagen Hotstart mastermix buffer (QIAGEN Inc., Hilden, Germany), 0.3 μM of each forward and reverse primer, 4 μM BSA (4 mg/mL) and 1 ng of extracted DNA. PCR conditions included an initial denaturation (95 °C for 15 min); 35 cycles of denaturation (95 °C for 30 s), annealing (55 °C gradient for 40 s) and extension (72 °C for 40 s); followed by a final extension (72 °C for 10 min). The amplified products were electrophoresed, cleaned up with Exo-SAP mixture, sequenced bidirectionally and matched against our Indian forensic database as well as with the Genebank for species confirmation. Confirmed rhino samples were then genotyped three independent times using the final multiplex panels as determined via the optimisation process outlined above. Finally, individual genotypes for the entire panel were matched against the poached rhino carcass data.

3. Results

3.1. Selection of rhino marker panel

We conducted systematic exclusions of non-suitable markers from the initial 34 rhino microsatellites based on amplification success, marker characteristics and polymorphism (see methods for details). During optimisation of the markers we tried 24 different combinations of input DNA concentrations ranging 0.2−700 ng/μl and found that minimum 1 ng/μl DNA is required for reliable microsatellite data generation. We used this DNA concentration for all subsequent PCR amplifications.

During the first stage of marker amplification with the reference set of samples (n = 6) we found that three of the 34 initial markers (Rh2, DB52 and B1Rh1C) failed to show any amplification (see Table 1). These primers were rejected from further processing. In the next step we assessed the marker suitability based on peak characteristics (tested with 30 samples) and polymorphism (with 96 rhino samples). When looking for peak characteristics, we found that two loci (32A and SR268) consistently produced very low RFU and one locus (B1Rh1B) showed multiple peaks (Table 1). These primers were eliminated from further testing. The remaining loci (n = 28) were tested for polymorphism and we identified nine monomorphic loci (Table 1) which were removed from the panel. Finally, data from all the polymorphic loci (n = 19) were

analysed for stutter-related inconsistencies across multiple genotyping runs (tested with 30 samples). We found that five loci (SR262, 7C, DB66, Rh6, Rh8) showed high standard deviation values ranging from ±1 to ±2.3, which may lead to inconsistent allele calling in dinucleotide loci. The remaining 14 loci showed low standard deviation values (±0.04 to ±0.18) (see Table 1), and could be multiplexed into four panels. During testing these panels with different amplification cycles we found that PCR reactions with 20 and 25 cycles consistently produced low RFU values for all the loci, whereas reactions with 30 cycles produced low RFU value for larger alleles. While 35 and 40 cycles produced almost identical results, we decided to continue with 35 cycles for multiplex PCRs in the line of the singleplex reactions. All samples (n = 96) produced identical data with these multiplex panels when compared with the data from singleplex reactions (See Supplementary Fig. 1 for representative electropherograms).

3.2. Summary statistics for the RhoDIS-India marker panel

None of the selected 14 loci in this panel showed signatures of large-scale allelic dropout. The mean allelic dropout and false allele rates were 0.02 and 0.01, respectively. Average frequency of null allele was 0.05, indicating this panel has low genotyping error rates. Amplification success ranged between 90 %–99.6 % from different tissue types (blood and tissue, n = 96). These loci showed high to medium levels of polymorphism (Table 1). The mean observed heterozygosity was found to be 0.50 ± 0.15 and none of the loci deviated from Hardy-Weinberg equilibrium or showed any evidence for strong linkage disequilibrium between any pair of loci. The cumulative PID_{sibs} and $PID_{unbiased}$ values were found to be 1.2×10^{-4} and 1.4×10^{-9}, respectively. Given that the rhino population size across India is ~3000 [1], this panel provides strong statistical support for unambiguous individual identification in forensic use. All summary statistic measures of polymorphism (number of alleles, allelic size range, heterozygosity etc.) for all loci are presented in Table 1.

3.3. DNA extraction protocol from rhino horns

We compared the efficiency of two DNA extraction protocols and found that with DTT protocol yielded 16 ng/μl- 102 ng/μl, whereas without DTT yielded 0.3 ng/μl- 1 ng/μl from the same samples. Further, DTT-extracted samples amplified all 14 markers in the panel compared to none with the other protocol. Given the stark contrast in amplification success, we used the DTT protocol for all subsequent rhino horn DNA extractions.

3.4. Individual matching of rhino contraband

Sequencing-based species identification assay confirmed two of the horn chips as Indian GoH rhinoceros, whereas the third sample was identified as water buffalo (*Bubalus bubalis*). We generated complete 14 loci panel data for all 15 poaching cases (13 carcass and two horn chips) received as part of the RhoDIS-India program. The genotypes from the horn chips matched with two rhino carcasses from West Bengal and Assam, respectively, showing the efficacy of this approach to match seized contraband to their carcass origins. These results were submitted as scientific evidence to concerned authorities for legal proceedings. We have also matched genotypes of decomposed rhino carcasses with other evidence such as blood-stained soil, bullets recovered from crime scenes etc. These results showcase the power of such DNA-based approaches in wildlife crime investigations.

4. Discussion

Our major aim in this study was to optimise a panel of microsatellites for forensic use in Indian GoH rhinoceros. To the best of our knowledge, this is the only study apart from the African rhinos [13,21,22] where a forensic microsatellite marker panel has been standardized to deal with rhino-related crimes. In the study we followed the ISFG recommendations during initial testing and optimisation of the markers such as use of voucher reference samples, various types of rhino samples (blood, tissue, horn chips representing different input DNA concentrations), temperature and PCR cycle optimisation, etc., that is generally lacking in other published GoH rhino population genetic work [16–18]. We have conducted rigorous testing of a large number of microsatellite loci (n = 34) with field-collected samples before selecting the best ones to develop the panel. Out of the 34 markers initially selected (23 from the African RhoDIS panel [21] and 11 tested on Indian GoH rhinoceros [16], we finally optimised 14 dinucleotide markers into four multiplex reactions as a time-saving and economic option. Most of the RhoDIS-Africa markers did not amplify in Indian GoH rhinoceros samples, possibly due to null alleles resulting from evolutionary differences among genus. Such non-amplification of cross-species microsatellites has also been reported earlier in rhinos [25,28]. Further, it is important to point out that there are general concerns regarding use of dinucleotide markers in forensic studies [27,30] but ISFG recommends them if they are widely used in population genetic research and have stable heterozygote balance [19]. The final selected panel in this study fulfil these criteria and showed very low genotyping error rates (2 % allelic dropout, 1 % false alleles and 5 % null alleles) and produce a statistically significant PID_{sibs} value of 1.2×10^{-4}. However, addition of an allelic ladder [19,36] and further development of a single multiplex reaction (such as the *MeowPlex* for felids [37], *UrsaPlex* for ursids [38], *DogFiler* for canids [39], *SkydancerPlex* for hen harrier [40] etc.) will greatly benefit inter-laboratory data generation and comparisons in rhino forensics. In addition, further validation work in terms of sequencing of the alleles for all the loci in the final panel, cross-species amplification testing, allele nomenclature designation etc. (for example, see [22] for black rhinoceros) are required for wide use of this optimised panel in forensic case work. In future, we plan to conduct these tests rigorously with extensive sampling of all Indian GoH rhinoceros populations for the RhoDIS-India program.

While using the optimised microsatellite panel to identify the origin of seized horns we performed individual genotype matching instead of commonly used random match probability approach for genotype comparisons [41]. We decided to use this approach as the random match probability requires in-depth understanding of population substructure data (also known as Φ value) [20,41]. There are seven isolated Indian GoH rhinoceros populations (four in Assam, two in West Bengal and one in Uttar Pradesh) and no population structure data or population-specific allele frequency assessments are available. Moreover, within each of the population the allele frequencies can differ significantly due to variations in population size and histories (in some cases the entire population has been reintroduced using founders from different sources), resulting in possible erroneous interpretations of our results [41]. We believe that until we generate adequate population-specific information complete genotype matching provides sufficient support in forensic use. This can be replaced by random match probability once significant individual level information from all subpopulations is available. Current advances in non-invasive tools (for example, faeces) make it easier to develop such a database.

5. Conclusion

In conclusion, we optimised a microsatellite panel for Indian GoH rhinoceros and used it to generate rhino DNA profiles and matched with carcasses as scientific evidences in alleged rhino crimes in India. This work is the first step towards generating an allele frequency map of GoH rhinoceros across its distribution in India. In future, this data would be used to determine origins of Indian GoH rhinoceros contraband and identifying poaching hotspots [11–13]. Currently, global rhino populations face major poaching threats as the demands and prices of rhino horn derivatives are at the highest level. To reduce poaching pressure

there have been arguments on legalizing rhino horn sales from existing stockpiles, but recent studies suggested against it [42] and recommended strict law enforcement at the source and consumer regions. The Indian GoH rhinoceros populations are at continuous risk from poaching, primarily due to geographic proximity to the major rhino horn consumer countries (Vietnam and China) and transit country (Myanmar) [7]. We hope that this work, being a government-endorsed program of the Ministry of Forest, Environment and Climate Change, Government of India would improve the court prosecution rate, reduce the time required in prosecutions and in turn will work as a deterrent towards rhino poaching incidences. Long-term accumulation of such information would also provide useful data to identify rhino poaching hotspots and trade routes, thereby help in breaking the wildlife trade chains. Finally, similar approach can also be used for other trade-target species globally.

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CRediT authorship contribution statement

Tista Ghosh: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Amit Sharma:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing - original draft, Writing - review & editing. **Samrat Mondol:** Conceptualization, Methodology, Resources, Writing - original draft, Project administration, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fsigen.2021.102472.

References

[1] S. Ellis, B.K. Talukdar, *Rhinoceros unicornis*: The IUCN Red List of Threatened Species, e.T19496A18494149, 2019, https://doi.org/10.2305/IUCN.UK.2019-3.RLTS.T19496A18494149.en.

[2] V. Menon, Under Siege: Poaching and Protection of Greater One-Horned Rhinoceroses in India, Available at, TRAFFIC India, 1996, https://portals.iucn.org/library/fr/node/7008.

[3] K. Rookmaker, A. Sharma, J. Bose, K. Thapa, D. Dutta, B. Jeffries, A.C. Williams, D. Ghose, M. Gupta, S. Tornikoski, The Greater One-Horned Rhino: Past, Present and Future, WWF, Gland, Switzerland, WWF, 2016.

[4] E. Dinerstein, The Return of the Unicornis-The Natural History and Conservation of the Greater One-horned Rhinoceros, Biology and Resource Management Series, World Wildlife Fund, Washington DC, 2003, pp. 320.

[5] W.J. Ripple, T.M. Newsome, C. Wolf, R. Dirzo, K.T. Everatt, M. Galetti, M. W. Hayward, G.I.H. Kerley, T. Levi, P.A. Lindsey, D.W. Macdonald, Y. Malhi, L. E. Painter, C.J. Sandom, J. Terborgh, B. Van Valkenburgh, Collapse of the world's largest herbivores, Adv. Sci. 1 (2015), e1400103, https://doi.org/10.1126/sciadv.1400103.

[6] G. Cédric, P. Neha, P. Roshan, S. Uttam, G. Rajendra, Assessing and managing the rising rhino population in Kaziranga (India), Ecol. Indic. 66 (2016) 55–64, https://doi.org/10.1016/j.ecolind.2016.01.023.

[7] R.H. Emslie, T. Milliken, B. Talukdar, S. Ellis, K. Adcock, M.H. Hofreiter, African and Asian Rhinoceroses – Status, vol. 5, Conservation and Trade, IUCN Species Survival Commission, 2016, p. 22.

[8] T. Milliken, J. Shaw, The South Africa-Viet Nam Rhino Horn Trade Nexus: a Deadly Combination of Institutional Lapses, Corrupt Wildlife Industry Professionals, and Asian Crime Syndicates, TRAFFIC, Johannesburg, South Africa, 2012.

[9] K.M. Ewart, G.J. Frankham, R. McEwing, L.M.I. Webster, S.A. Ciavaglia, A.M. T. Linacre, D.T. The, K. Ovouthan, R.N. Johnson, An internationally standardized species identification test for use on suspected seized rhinoceros horn in the illegal wildlife trade, Forensic Sci. Int. Genet. 32 (2018) 33–39, https://doi.org/10.1016/j.fsigen.2017.10.003.

[10] B.J. Das, The pre-existing and current status of Indian rhinoceros in Assam (India): a mini review, Int. J. Curr. Res. 11 (2019) 40–43, https://doi.org/10.24941/ijcr.33764.01.2019.

[11] S. Mondol, V. Sridhar, P. Yadav, S. Gubbi, U. Ramakrishnan, Tracing the geographic origin of traded leopard body parts in the Indian subcontinent with DNA-based assignment tests, Conserv. Biol. 29 (2014) 556–564, https://doi.org/10.1111/cobi.12393.

[12] S.K. Wasser, L. Brown, C. Mailand, S. Mondol, W. Clark, C. Laurie, B.S. Weir, Genetic assignment of large seizures of elephant ivory reveals Africa's major poaching hotspots, Science 349 (2015) 84–87, https://doi.org/10.1126/science.aaa2457.

[13] C. Harper, A. Ludwig, A. Clarke, K. Makgopela, A. Yurchenko, A. Guthrie, P. Dobrynin, G. Tamazian, R. Emslie, M. van Heerden, M. Hofmeyr, R. Potter, J. Roets, P. Beytell, M. Otiende, L. Kariuki, R. du Toit, N. Anderson, J. Okori, A. Antonik, K.-P. Koepfli, P. Thompson, S.J. O'Brien, Robust forensic matching of confiscated horns to individual poached African rhinoceros, Curr. Biol. 28 (2018) R13–R14.

[14] D.K. Jha, S.K. Gupta, N.T. Kshetry, R. Panday, B.R. Pokharel, A pioneer case study on identification of infant Rhinoceros horn, J. Forensic Res. 08 (2017), https://doi.org/10.4172/2157-7145.1000374.

[15] S. Zschokke, B. Gautschi, B. Baur, Polymorphic microsatellite loci in the endangered Indian rhinoceros, *Rhinoceros unicornis*, Mol. Ecol. Notes 3 (2003) 233–235, https://doi.org/10.1046/j.1471-8286.2003.00400.x.

[16] P.K. Das, U. Borthakur, H.K. Sarma, B.K. Talukdar, Population genetic assessment of extant populations of greater one-horned rhinoceros (*Rhinoceros unicornis*) in India, Eur. J. Wildl. Res. 61 (2015) 841–851, https://doi.org/10.1007/s10344-015-0960-2.

[17] U. Borthakur, P.K. Das, A. Talukdar, B.K. Talukdar, Noninvasive genetic census of greater one-horned rhinoceros *Rhinoceros unicornis* in Gorumara National Park, India: a pilot study for population estimation, Oryx 50 (2016) 489–494, https://doi.org/10.1017/S0030605314001161.

[18] S. Zschokke, Genetic structure of the Wild Populations of the Indian Rhinoceros (*Rhinoceros unicornis*), Indian J. Hist. Sci. 51 (2016) 380–389, https://doi.org/10.16943/ijhs/2016/v51i2.2/48451.

[19] A. Linacre, L. Gusmão, W. Hecht, A.P. Hellmann, W.R. Mayr, W. Parson, M. Prinz, P.M. Schneider, N. Morling, ISFG: recommendations regarding the use of non-human (animal) DNA in forensic genetic investigations, Forensic Sci. Int. Genet. 5 (2011) 501–505, https://doi.org/10.1016/j.fsigen.2010.10.017.

[20] R.N. Johnson, L. Wilson-Wilde, A. Linacre, Current and future directions of DNA in wildlife forensic science, Forensic Sci. Int. Genet. 10 (2014) 1–11, https://doi.org/10.1016/j.fsigen.2013.12.007.

[21] C.K. Harper, G.J. Vermeulen, A.B. Clarke, J.I. de Wet, A.J. Guthrie, Extraction of nuclear DNA from rhinoceros horn and characterization of DNA profiling systems for white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceros, Forensic Sci. Int. Genet. 7 (2013) 428–433, https://doi.org/10.1016/j.fsigen.2013.04.003.

[22] K.L. Dicks, L.M.I. Webster, I. McDowall, S.M. Muya, J. Hopper, P. O'Donoghue, Validation studies on dinucleotide STRs for forensic identification of black rhinoceros *Diceros bicornis*, Forensic Sci. Int. Genet. 26 (2017) e25–e27, https://doi.org/10.1016/j.fsigen.2016.10.016.

[23] P.F. Campos, T.M.P. Gilbert, DNA extraction from keratin and chitin, in: B. Shapiro, M. Hofreiter (Eds.), Ancient DNA: Methods and Protocols, Humana Press, Totowa, NJ, 2012, pp. 43–49, https://doi.org/10.1007/978-1-61779-516-9_6.

[24] S. Mondol, M.W. Bruford, U. Ramakrishnan, Demographic loss, genetic structure and the conservation implications for Indian tigers, Proc. R. Soc. B 280 (2013), 20130496, https://doi.org/10.1098/rspb.2013.0496.
[25] C.A. Scott, Microsatellite Variability in Four Contemporary Rhinoceros Species: Implications for Conservation, MSc Dissertation, Queen's University, Kingston, Ontario, Canada, 2008.
[26] J.I. Hoffman, W. Amos, Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion, Mol. Ecol. 14 (2005) 599–612, https://doi.org/10.1111/j.1365-294X.2004.02419.x.
[27] F. Pompanon, A. Bonin, E. Bellemain, P. Taberlet, Genotyping errors: causes, consequences and solutions, Nat. Rev. Genet. 6 (2005) 847–859, https://doi.org/10.1038/nrg1707.
[28] A.S. Guerier, J.M. Bishop, S.J. Crawford, A. Schmidt-Küntzel, K.J. Stratford, Parentage analysis in a managed free ranging population of southern white rhinoceros: genetic diversity, pedigrees and management, Conserv. Genet. 13 (2012) 811–822, https://doi.org/10.1007/s10592-012-0331-4.
[29] C. Miquel, E. Bellemain, C. Poillot, J. Bessière, A. Durand, P. Taberlet, Quality indexes to assess the reliability of genotypes in studies using noninvasive sampling and multiple-tube approach, Mol. Ecol. Notes 6 (2006) 985–988, https://doi.org/10.1111/j.1471-8286.2006.01413.x.
[30] R. Andreassen, J. Schregel, A. Kopatz, C. Tobiassen, P.M. Knappskog, S.B. Hagen, O. Kleven, M. Schneider, I. Kojola, J. Aspi, A. Rykov, K.F. Tirronen, P.I. Danilov, H. G. Eiken, A forensic DNA profiling system for Northern European brown bears (*Ursus arctos*), Forensic Sci. Int. Genet. 6 (2012) 798–809, https://doi.org/10.1016/j.fsigen.2012.03.002.
[31] T. Broquet, E. Petit, Quantifying genotyping errors in noninvasive population genetics, Mol. Ecol. 13 (2004) 3601–3608, https://doi.org/10.1111/j.1365-294X.2004.02352.x.
[32] M.-P. Chapuis, A. Estoup, Microsatellite null alleles and estimation of population differentiation, Mol. Biol. Evol. 24 (2007) 621–631, https://doi.org/10.1093/molbev/msl191.
[33] A.P. Dempster, N.M. Laird, D.B. Rubin, Maximum likelihood from incomplete data via the em algorithm, J. R. Stat. Soc. Ser. B (Methodol.) 39 (1977) 1–38, https://doi.org/10.1111/j.2517-6161.1977.tb01600.x.
[34] N. Valière, GIMLET: a computer program for analysing genetic individual identification data, Mol. Ecol. Notes 2 (2002) 377–379, https://doi.org/10.1046/j.1471-8278.2002.00228.x.
[35] L. Excoffier, H.E.L. Lischer, Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows, Mol. Ecol. Resour. 10 (2010) 564–567, https://doi.org/10.1111/j.1755-0998.2010.02847.x.
[36] M.K. Moore, I.L. Kornfield, Best practices in wildlife forensic DNA, in: J. E. Huffman, J.R. Wallace (Eds.), Wildlife Forensics, John Wiley & Sons, Ltd, Chichester, UK, 2012, pp. 201–236, https://doi.org/10.1002/9781119953142, ch11.
[37] J.M. Butler, V.A. David, S.J. O'Brien, M. Menotti-Raymond, The MeowPlex: a new DNA test using tetranucleotide STR markers for the domestic cat, Profiles in DNA, 2002, p. 4.
[38] E.P. Meredith, J.K. Adkins, J.A. Rodzen, UrsaPlex: an STR multiplex for forensic identification of North American black bear (*Ursus americanus*), Forensic Sci. Int. Genet. 44 (2020), 102161, https://doi.org/10.1016/j.fsigen.2019.102161.
[39] E. Wictum, T. Kun, C. Lindquist, J. Malvick, D. Vankan, B. Sacks, Developmental validation of DogFiler, a novel multiplex for canine DNA profiling in forensic casework, Forensic Sci. Int. Genet. 7 (2013) 82–91, https://doi.org/10.1016/j.fsigen.2012.07.001.
[40] M.J.C. van Hoppe, M.A.V. Dy, M. Eiden, A. Iyengar, *SkydancerPlex*: a novel STR multiplex validated for forensic use in the hen harrier (*Circus cyaneus*), Forensic Sci. Int. Genet. 22 (2016) 100–109, https://doi.org/10.1016/j.fsigen.2016.02.003.
[41] D.J. Balding, R.A. Nichols, DNA profile match probability calculation: how to allow for population stratification, relatedness, database selection and single bands, Forensic Sci. Int. 64 (1994) 125–140, https://doi.org/10.1016/0379-0738(94)90222-4.
[42] J.A.J. Eikelboom, R.J.M. Nuijten, Y.X.G. Wang, B. Schroder, I.M.A. Heitkönig, W. M. Mooij, F. van Langevelde, H.H.T. Prins, Will legal international rhino horn trade save wild rhino populations? Glob. Ecol. Conserv. 23 (2020), e01145 https://doi.org/10.1016/j.gecco.2020.e01145.