Noninvasive genetic census of greater one-horned rhinoceros *Rhinoceros unicornis* in Gorumara National Park, India: a pilot study for population estimation

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SUPPLEMENTARY MATERIAL Technical details of the genetic analysis.

DNA extraction

DNA was extracted from dung samples using the guanidine isothiocyanate−silica-based protocol (Boom et al., 1990), with the following modifications: (1) 500 µl of DETs buffer containing the faecal sample was added to 1,000 µl of L6 lysis solution (5 M guanidine isothiocyanate; 100 mM Tris, pH 6.4; 20 mM EDTA, pH 8.0; and 1.3% Triton X-100) in a clean 1.5 ml microcentrifuge tube and incubated overnight at room temperature, with intermittent vortexing. (2) The mixture was centrifuged at 8,000 rpm for 1 minute and the supernatant was transferred to a clean 1.5 ml microcentrifuge tube containing 100 µl of 10% polyvinyl polypyrrolidone solution. After mixing by gentle inversion the suspension was incubated at room temperature for 30 minutes and then centrifuged at 12,000 rpm for 2 minutes. (3) The supernatant was transferred to a clean 1.5 ml microcentrifuge tube and mixed with 50 µl of 6% silica solution. After incubation at room temperature for 30 minutes the silica matrix was pelleted through centrifugation at 12,000 rpm for 1 minute. (4) Silica was washed twice with 500 µl of L2 solution (5 M guanidine isothiocyanate; 100 mM Tris, pH 6.4; and 20 mM EDTA, pH 8.0) and 500 µl of ethanol wash buffer (100 mM Tris, pH 7.5; 100 mM sodium chloride; 1 mM EDTA, pH 8.0; and 60% ethanol) and then once with 500 µl of ice-cold 80% ethanol and 500 µl of ice-cold acetone. (5) The washed pellets were dried in a heating block at 55°C and DNA was eluted at 55 °C with 75 µl of TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0). All DNA extractions were performed in a room dedicated for low-quality DNA work. DNA extractions from reference tissues and samples were performed using the DNeasy Blood & Tissue kKit (QIAGENiagen, Hilden, Germany), following standard kit protocols.

Selection of polymorphic microsatellite markers

Seventeen microsatellite loci, nine from greater one-horned rhinoceros (Zschokke et al., 2003) and eight from Sumatran rhinoceros *Dicerorhinus sumatrensis* (Scott et al., 2004), were first screened on 10 reference samples from greater one-horned rhinoceros to determine the level of polymorphism (×Table 1). The forward primers of each microsatellite marker were labelled at the 5´ end with one of the fluorescent dyes 6–FAM (blue), PET (red), VIC (green) or NED (yellow). Polymerase chain reaction (PCR) products generated after amplification of DNA from dung samples were loaded in the capillary electrophoresis-based ABI 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, USA). The genotype data obtained from these samples were used in selecting a panel of microsatellite loci, based on (1) polymorphism information content and expected heterozygosity, with no linkage disequilibrium, (2) mean PCR success from dung samples, and (3) multiplexing compatibilities. The selected markers were screened on a subset of DNA from 10 dung samples from the same study area to demonstrate the power of individual identification as well as standardization of multiplexing PCR reactions.

Individual rhinoceros identification based on dung samples

The selected polymorphic loci were used to genotype the samples of rhinoceros dung. Multiplex PCRs were performed in four panels, with three different loci in a single PCR reaction (×Table 2), and each locus was labelled with a fluorescent label. Multiplexing was carried out using the QIAGEN Multiplex PCR Kit (QIAGEN, Hilden, Germany), following standard kit protocols for reagent concentration, with 0.2 µM of each primer and 2.5 µl template DNA in a 10 µl PCR

reaction. Thermal cycling was performed with initial denaturation/activation at 95°C for 15 minutes, followed by 40 cycles of 94°C for 60 s, multiplex panel specific annealing temperature for 45 s, and 72°C for 45 s, followed by a final extension step of 72°C for 15 minutes.

Genotype quality control and assessment of errors

Microsatellite genotyping errors principally arise as a result of allelic dropout or false alleles. We used a multiple tube approach (Taberlet et al., 1996), assigning quality indices to the genotype data, following Miquel et al. (2006). Each sample and locus was typed at least three times, depending on the genotypic discrepancy, and consensus genotypes were created from these repeat results. To select a final set of samples for individual identification, a quality index value was assigned to each genotype, with a cut-off value of 0.667 (Miquel et al., 2006). Samples that revealed the same genotype in all three repetitions had a quality index of one, and samples that yielded the same genotype in two of the three amplifications had a quality index of 0.667. To minimize electropherogram stutter patterns and prevent allelic dropout, dedicated microsatellite PCR kits (Multiplex PCR Kit, QIAGEN, Germany) were employed throughout for genotyping. Negative controls with reagent blanks were used in all batches of PCR to monitor contamination during PCR handling. Moreover, all the work was carried out in a room dedicated for low copy number DNA analysis, and aerosol-barrier tips were used to prevent sample-to-sample contamination.

Genetic data analysis

Allele sizes were determined from the raw electropherogram data by manual inspection and using the allele-calling software GENEMAPPER v. 3.7 (Applied Biosystems, Carlsbad, USA). From the repeat genotype data, consensus genotypes were created, and genotyping error rates were estimated using *Gimlet v. 1.3.3* (Valière, 2002). From the final genotype data we used the same software to calculate observed and expected heterozygosity, probability of identity, and probability of identity among siblings. We tested for linkage disequilibrium and Hardy–Weinberg equilibrium using *Arlequin v. 3.0* (Excoffier et al., 2005). The unique multilocus microsatellite genotypes (i.e. individual rhinoceros) were identified using the identity analysis module of *Cervus v. 3.0* (Marshall et al., 1998; Kalinowski et al., 2007). As stringent data generation and selection criteria were applied, unique multilocus genotypes based on zero and single locus mismatch were compared for individual identification.

Sex identification of rhinoceros

We used Y-chromosome-specific SRY (sex-determining region of the Y chromosome) primers designed from horse SRY sequences, namely SRYHorseR: 52-TCATGGTGTGGTCTCGTGAT-32 and SRYHorseR: 52-CCGGGTATTTCTCTTGATGC-32 (primers were designed at the Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo & Botanical Garden, USA). Microsatellite locus SR_IIIA (108–116 bp) was used as nuclear control in a multiplex reaction with the SRY (165 bp) marker to distinguish between PCR failures and female samples in a single-tube PCR followed by visualization in agarose gel. Male samples were identified by the presence of both SRY and SRIIIA PCR products and female samples were identified by the presence of only SRIIIA products, visualized on 2% agarose gel with a 100 bp DNA ladder (GeneRuler, Fermentas, Lithuania). The amplification was performed in a 10 μl PCR using the QIAGEN Multiplex PCR Kit, following standard kit protocols for reagent concentration, with 0.5 μM of each SRY primer, 0.2 μM of each SRIIIA primer and 3.2 μl of template DNA. Thermal cycling was performed with initial denaturation/activation at 95°C for 15 minutes, followed by 40 cycles of 94°C for 45 s, 58°C for 30 s and 72°C for 45 s, followed by a final extension step at 72°C for 10 minutes. This multiplex marker system was initially validated on reference tissue samples from four male and six female rhinoceros, and used on DNA extracts from dung samples for gender identification.