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Integrated and novel survey methods for rhinoceros populations confirm the extinction of *Rhinoceros sondaicus annamiticus* from Vietnam

S.M. Brook [a,*], P. van Coeverden de Groot [b], C. Scott [b], P. Boag [b], B. Long [c], R.E. Ley [d], G.H. Reischer [e], A.C. Williams [f], S.P. Mahood [a], Tran Minh Hien [a], G. Polet [g], N. Cox [h], Bach Thanh Hai [i]

[a] *WWF Vietnam, D13 Thang Long International Village, CauGiay District, Hanoi, Viet Nam*
[b] *Department of Biology, Queens University, 116 Barrie Street, Kingston, Ontario, Canada*
[c] *WWF-US, 1250 24th Street NW, Washington, DC 20037, USA*
[d] *Department of Microbiology, Cornell University, Ithaca, NY 14853, USA*
[e] *Research Group Environmental Microbiology and Molecular Ecology, Institute of Chemical Engineering, Vienna University of Technology, Gumpendorfer Strasse 1a/166-5-2, A-1060 Vienna, Austria*
[f] *WWF AREAS, WWF International, Av. du Mont-Blanc, 1196 Gland, Switzerland*
[g] *WWF Netherlands, Driebergseweg 10, 3708 JB Zeist, The Netherlands*
[h] *WWF Greater Mekong Programme, 53 Saylom Road, Ben Saylom, Vientiane, Laos*
[i] *Cat Tien National Park, Tan Phu District, Dong Nai Province, Viet Nam*

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ABSTRACT

Javan rhinoceros (*Rhinoceros sondaicus*) is among the most threatened large mammal species in the world. Development of rigorous, non-invasive survey techniques is a high priority, to monitor populations and develop informed conservation management strategies. The critically endangered javan rhinoceros until recently survived in two separate populations, one in Vietnam and one in Indonesia, representing distinct subspecies. The range of the *annamiticus* subspecies around Cat Tien National Park (CTNP) has declined significantly since its re-discovery in 1989, and no accurate estimate of population size had ever been obtained. We employed integrated survey techniques and analyses to determine the population status of the javan rhinoceros in Vietnam. We conducted a comprehensive field survey of the Cat Loc sector of CTNP using scat detection dogs to detect javan rhinoceros dung between October 2009 and April 2010. Twenty-two dung samples were collected for microsatellite DNA analysis, seventeen of which were of sufficient quality to be analysed. The genotyping work confirmed that only a single rhinoceros was present at the start of the survey in 2009 and that this was the same individual that was found dead in April 2010. Although far less definitive than host genotyping, stool bacterial diversity assays also supported the hypothesis that all samples collected by the survey were from one individual. This empirical data combined with field survey data indicate the extinction of the javan rhinoceros in Vietnam. We conclude by discussing the developmental progress of these non-invasive survey techniques to monitor other endangered rhinoceros populations.

1. Introduction

Javan rhinoceros (*Rhinoceros sondaicus*) is one of the most threatened large mammal species in the world. Recognised as Critically Endangered by IUCN, the species is threatened by poaching, habitat loss and reduced population viability (Van Strien et al., 2008). *R. sondaicus* is thought to have survived in two populations, in Indonesia and Vietnam, representing the remaining two of the three recognised subspecies (Corbet and Hill, 1992; Fernando et al., 2006; Van Strien et al., 2008). *R. s. inermis* formerly occurred in north-east India, Bangladesh and Myanmar and went extinct in the early 1900s (Rookmaaker, 1980). *R. s. sondaicus* formerly inhabited Thailand, Malaysia, Java and Sumatra but recent camera-trapping surveys identified a minimum of 35 individuals comprising the last population of this subspecies, found in the 123,051 ha Ujung Kulon National Park, Indonesia (Hariyadi et al., 2011; Van Strien et al., 2008). *R. s. annamiticus* formerly occurred in Lao, Cambodia, eastern Thailand and Vietnam (Groves, 1967; Khan, 1989; Van Strien et al., 2008). Due to political reasons, there were no records from Indochina throughout the second half of the 20th century until reports of an individual having been hunted in southern

* Corresponding author. Address: Fauna and Flora International, No. 19, Street 360, Bouengkeng Kong 1, Phnom Penh, Cambodia. Tel.: +855 (0)16704745; fax: +855 (0)23211242.
E-mail address: sarahmbrook@gmail.com (S.M. Brook).

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Vietnam in 1988 (Groves, 1995; Santiapillai, 1992; Santiapillai et al., 1993; Schaller et al., 1990) suggested a relict population persisted in this area.

A survey (observations of tracks and signs and interviews with local community members) conducted in the target area of southern Vietnam in 1989, suggested a maximum of 10–15 individuals remained in approximately 75,000 ha of habitat around Cat Tien National Park (CTNP), (Schaller et al.1990). Although several subsequent surveys have demonstrated a considerable contraction of the area occupied by this rhinoceros population and suggest a decrease in rhinoceros numbers over the last 20 years, an accurate population estimate has yet to be obtained (Fernando et al., 2006; Polet and Ling, 2004).

More specifically, while Schaller's 1989 survey documented the rhinoceros in the Cat Loc and Nam Cat Tien sectors of CTNP, and just across the Dong Nai River from Cat Loc in Song Be Province (Schaller et al., 1990), all subsequent surveys (1991, 1999, 2001–2002, 2005 and 2006) report signs of a declining population within the Cat Loc sector only (Van Thanh and Polet, 2007, Dang and Osborn, 2004a; Dang and Osborn, 2004b; Polet et al., 1999; Santiapillai et al., 1993; Schaller et al., 1990). In 1991 Santiapillai estimated 8–12 individuals survived; with the javan rhinoceros range estimated at 35,000 ha (all of Cat Loc sector) (Santiapillai, 1992; Santiapillai et al., 1993). By 1999, field surveys and measurement of 111 tracks showed that the estimated 7–8 rhinoceros were confined to Cat Loc, inhabiting only 6500 ha known as the 'rhinoceros core area' (Polet et al., 1999). In 2001, field surveys and analysis of new tracks and the re-evaluation of those measured and cast in 1999 suggested that there were at least 3 individuals inhabiting the 'rhinoceros core area', including one sub-adult (Manh, 2001). In 2002, following the same methods, 1–3 javan rhinoceros were posited (Manh, 2002). An analysis of automatic camera trap pictures and footprint measurements obtained during regular monitoring patrols in 2005 and 2006 concluded that 3 or 4 animals could be present and that there was a female (Nguyen and Polet, 2007).Microsatellite analyses of faecal samples collected in 2001 and 2002 suggested there were at least 4–6 individuals present including both sexes (Fernando and Melnick, 2003; Van Coeverden de Groot, unpublished data).

The accuracy of some of the above conclusions can be questioned as the collection of wild faecals in earlier studies was not systematic so no estimate of coverage or capture probability is possible; the discrimination between individual javan rhinoceros based on footprints and automatic camera trap pictures has not been equivocally demonstrated and the faecal microsatellite study (Fernando and Melnick, 2003) had only used a limited set of indian rhinoceros (*Rhinoceros unicornis)* primers which had not been tested on non-faecal javan rhinoceros for polymerase chain reaction (PCR) amplification reliability nor variability. In this study we address these shortcomings with the use of scat detection dogs and improved genetic tools.

To improve javan rhinoceros dung collection coverage and detection rates we employed scat detection dogs. Dogs are more efficient than humans at locating target species scats (Smith et al., 2003) and have been used successfully for surveys of many different species (Gompper et al., 2006; Long et al.2007) including bears in North America (Wasser et al., 2004), tigers in Russia (Kerley, 2004) and, large cats in Cambodia (T. Gray, unpublished data) to name a few. Scat detection dogs are particularly helpful for wide-ranging, elusive or rare species with low population densities inhabiting large remote areas (Browne et al., 2006); they can detect scats over large distances, are an unobtrusive survey and monitoring method compared to alternatives, whilst improving detection probabilities and increasing sample numbers.

To improve population inferences from genetic analyses of faecal samples we attempted to optimise microsatellite primers cloned from javan rhinoceros as well as those cloned from other rhinoceros taxa that are variable in the javan rhinoceros and that amplified javan rhinoceros microsatellites reliably from their faeces (Van Coeverden de Groot et al., 2008). Early results using P^{33} detection – which is more sensitive than influorescence detection - suggested we had optimised primers variable in the javan rhinoceros (Van Coeverden de Groot et al., 2008). We also optimised the genetic sexing of all rhinoceros taxa using previously described methods (Peppin et al., 2009; Van Coeverden de Groot et al., 2008). We expect that our microsatellites and genetic sexing methods will increase the reliability of collecting variable genetic profiles and genetic sex data from javan rhinoceros faecal samples.

More recently, it has been demonstrated that genetically estimated bacterial diversity profiles of faeces may be different among humans, but remain stable in individuals over 1 year (Ley et al., 2006). While these type of genetic assays have not been conducted on a large scale for wild megafauna the potential for census and diet inferences (to name a few) from this faecal data is vast. To potentially increase the accuracy of the census inference from javan rhinoceros faeces collected in Vietnam, the bacterial diversity profiles of all faecal samples collected were assayed.

Although conservation needs for the javan rhinoceros in Vietnam have always been clear: to protect the rhinoceros and their remaining habitat (AsRSG, 2000; Santiapillai, 1992), information on the population size and composition was urgently required, to: (i) identify whether investment further in CTNP for javan rhinoceros conservation was justified, and; (ii) to provide the necessary impetus for the Vietnamese government to endorse more stringent protection and conservation actions, to protect the rhinoceros population and habitat. The use of dogs and increased potential of new faecal genetic tools suggest a comprehensive field survey for javan rhinoceros in Vietnam coupled with systematic collection of faecal samples and their genetic analysis would provide the most accurate estimate of population status.

In this work we present the results of the recent comprehensive survey of the javan rhinoceros population status in Vietnam. Together our results from the field survey and new genetic analyses of collected faeces point to the extirpation of javan rhinoceros from Vietnam. We conclude with an evaluation of these techniques for monitoring endangered rhinoceros populations.

2. Materials and methods

2.1. Study site

Cat Loc sector of CTNP in Lam Dong Province, Vietnam, held the last remaining population of javan rhinoceros in mainland Southeast Asia. Once tropical lowland semi-evergreen forest, Cat Loc now consists mainly of bamboo and mixed bamboo forest, with some hillsides dominated by dense stands of rattan, probably as a consequence of a long history of shifting cultivation by indigenous communities', heavy defoliation during the Vietnam War, and past logging practices (Polet et al., 1999). Cat Loc is characterised by small steep hills, ranging from 300 m to 600 m asl traversed by streams draining into the Dong Nai River; soils are alluvial with heavy clay (Polet et al., 1999) (Fig. 1).

The 6500 ha area known as the 'rhinoceros core area' was the focus of the survey; this is the area where tracks and signs of rhinoceros were frequently found up until 2010. Approximately 5500 ha of the 'wider area' were also surveyed, where tracks and signs have been observed very infrequently since 1993 (Polet et al., 1999). The rhinoceros core area and wider area are bisected by a dirt track running south-northwest, on which motorbikes frequently travel between human settlements and cashew plantations inside the national park, creating disturbance and a

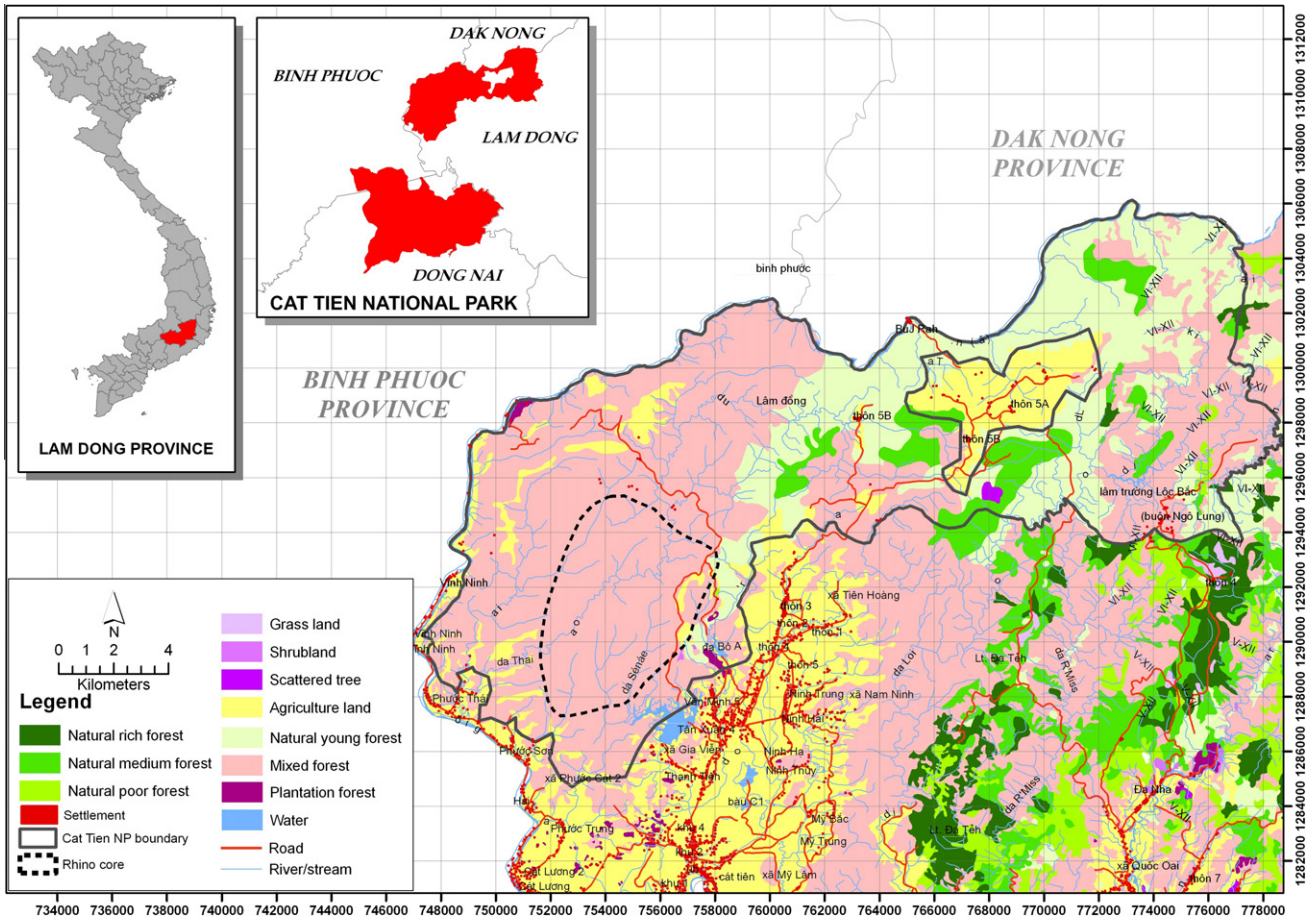

Fig. 1. Location of Cat Tien National Park; Nam Cat Tien (southern area in inset) and Cat Loc sectors (northern area in inset), and habitat and land-use of Cat Loc sector. The area of mixed forest within the park boundary in the west roughly delineates the total survey area and the black dotted line the 'rhinoceros core area'.

potential barrier to rhinoceros movements within Cat Loc (Fig. 1). The rest of Cat Loc was not surveyed because there have been no confirmed records or anecdotal reports of rhinoceros from these parts since reported sightings by forestry staff in 1990 and 1991 (Santiapillai et al., 1993).

2.2. Survey methods

Dung detection dogs were contracted from Packleader LLC, USA. Two dogs were selected and trained in the US to recognise and indicate on rhinoceros dung, which was obtained from captive rhinoceros of all species except javan rhinoceros prior to arrival in Vietnam. The survey team leaders (S.M.B and S.P.M) received three weeks of on site training in detector dog handling. This period allowed the dogs to acclimatise to the local environment and for the trainer to train the dogs on javan rhinoceros dung, collected from Cat Loc 1 month before the survey began.

The survey was conducted from October 27th 2009, to April 8th 2010 during the dry season; the area often becomes inaccessible after heavy rain. The survey was completed in three phases; phase 1 from 27th October to 13th December 2009, phase 2 from 26th January to 25th February, phase 3 from 3rd March to 8th April 2010.

The survey area was divided into 2 km × 2 km (400 ha) grid cells (Fig. 2), based on the estimated home range size of a female javan rhinoceros (500 ha); males potentially wander over larger areas (Van Strien et al., 2008). In total 35 grid cells with suitable habitat were surveyed (approximately 12,000 ha). In the 'rhinoceros core area' all or part of 18 grid cells were surveyed and in the wider area all or part of 17 grid cells were surveyed. 'Rhinoceros core area' grid cells were surveyed in all three phases ($n = 13$) or more peripheral grid cells in 2 phases ($n = 5$). Grid cells in the wider area were surveyed a minimum of once ($n = 15$) with two being surveyed twice (Supplementary Fig. 1–3). Within each cell, 'hotspots' of rhinoceros activity such as swamps, wallows, trails and streams were targeted for searching, to maximise the chances of finding rhinoceros dung. All wallows and swamps were surveyed a minimum of three times if they retained some water during the survey. If rhinoceros footprints were encountered they were followed in both directions for up to 1 km to search for faeces.

The survey was conducted by two teams simultaneously covering adjacent grid cells. Each team consisted of one ecologist/dog handler, one detection dog, one technical staff of CTNP, one Forest Protection Department (FPD) ranger and one local guide. On average, one grid cell per day was surveyed by each team, covering from three to eight kilometres in terms of human distance walked at a speed of $\leqslant$1 km per hour. Three to five kilometres per day is considered to be the optimum daily distance for detector dogs in tropical rainforest conditions (S. Weigley, personal communication). The survey teams were restricted to working for four or five consecutive days, followed by 2 or 3 days off, to ensure the dogs were well rested as fatigue can negatively affect searching ability. It is very difficult to accurately estimate the detection distance of scat detection dogs as this ability depends greatly on local conditions, including temperature, time of day, wind-speed and direction, topography, habitat type and age of dung (Wasser et al.,

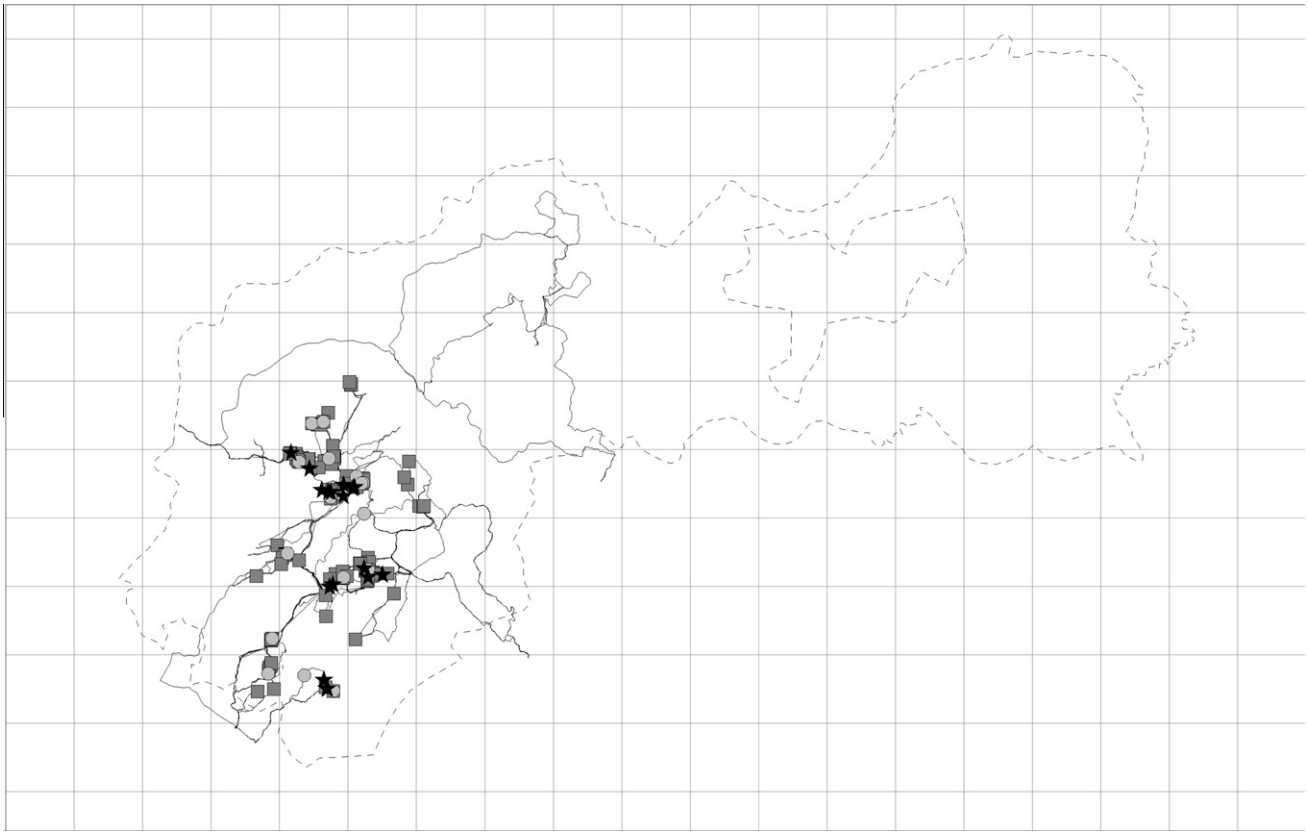

Fig. 2. Map of 2 km grid cells, all survey tracks (lines), location of javan rhinoceros dung (stars), footprints (squares) and wallows (circles).

2004). Studies that have attempted to do so have recorded maximum detection distances of: 38.4 km (mean 4.8 m SD 6.7 m) for kit foxes *Vulpes macrotis mutica* and coyote *Canis latrans* scat (Ralls and Smith, 2004); 62.8 m (mean 13.91 m) for desert tortoises *Gopherus agassizii* in Nevada (Cablk et al., 2008); and 22 m to 1.93 km for boat-based right whale *Eubalaena glacialis* scat surveys (Rolland et al., 2006). A study evaluating scat detection distance of two dogs found that 75% of samples were detected within 10 m of the transect line and detection decreased with increasing distance from the transect (Reed et al., 2011). All of these studies were performed in North America and as far as we are aware there have been no estimates of the detection distance for large ungulate dung by scat detection dogs in moist tropical rainforests.

When rhinoceros faeces were found, three samples were collected from each dung pile, following the MIKE (Monitoring the Illegal Killing of Elephants) collection protocol (Hedges and Lawson, 2006), to investigate the effect of storage type on the genetic analyses. The samples for DNA analysis were stored in 50 mL tubes with EtOH buffer; EDTA buffer; and silica gel sachet. Each sample was heated at 72 °C degrees for 30 min, in accordance with Canadian Food Inspection Agency (CFIA) guidelines. Samples in EtOH and silica gel were stored in the freezer at −20 °C and EDTA buffer samples were stored at room temperature (∼ 32 °C).

The following variables were also recorded with each dung encounter: date, coordinates, habitat type, elevation, bolus intact (y/n), diameter of bolus (if still intact), fungus present in dung (y/n). Each dung pile was marked to ensure they were not sampled more than once during the survey. Locations of rhinoceros footprints were recorded with a GPS to document the distribution of rhinoceros within Cat Loc. GPS tracks were downloaded and mapped in MapInfo Professional 10.0, Pitney Bowes Software Inc, New York, USA.

On 29th April 2010, shortly after the survey was completed, the remains of a dead javan rhinoceros were found in Cat Loc. Two teeth and three tissue samples were taken; the teeth were stored in an airtight container with silica gel and the tissue samples were stored in three different 50 mL tubes one with EtOH; the second EDTA buffer and the last air.

Twenty javan rhinoceros dung samples collected and stored in EtOH by CTNP staff between 2003 and 2006 were also analysed genetically. Only a minimum population estimate for this period could be made from this tissue as the survey and collection of dung was not as comprehensive as this survey.

2.3. DNA extraction and genotyping

The extraction of DNA from javan rhinoceros faecal samples for amplification of microsatellites DNA from target epithelial cells was optimised using a modified protocol for large sample volume for the QIAamp DNA Stool Mini Kit (QIAgen cat# 51504), (see Supplementary methods for details). To confirm that DNA was present in the final eluent, 20 μL of each sample was run on a 0.8% agarose gel.

All potential microsatellite loci – javan and non-javan – were first optimised on a control set of javan rhinoceros samples which comprised seven samples of javan rhinoceros bone and tissue, five of which are from museum specimens over 100 years old and two are from recently deceased rhinos from Vietnam and Indonesia. Whereas we had previously optimised amplification of the all loci using the highly sensitive P^{33} radiation platform, for this work we attempted re-optimisation for high-throughput Licor infrared technology to increase the success of the collection of faecal genotype data in other laboratories.

The 12 microsatellite loci isolated from each of the four non-javan rhinoceros taxa, were optimised for both non-faecal and faecal

(1:100 dilutions of the faecal DNA extraction amplified best in most cases) javan rhinoceros tissue on the Licor platform (Supplementary Table 1). As of yet, eight javan rhinoceros loci optimised for amplification non-faecal javan tissue (Van Coeverden de Groot et al., 2008) are not optimised to amplify DNA from javan rhinoceros faecal samples utilising the Licor platform (see Supplementary methods).

In determining the genotype for a locus X sample cell we followed Borthakur et al. (2010) where three PCR replicates of all faecal extracts from the 2009–2010 survey were completed for each microsatellite locus. A consensus genotype is reported when at least two out of three replicates indicated identical alleles (Supplementary Table 2). If there was no consensus, three additional replicates were attempted and if the same results were obtained, that locus X sample datum was scored as 0:0 and assumed that the target DNA is compromised and no correct product will be obtained. In some locus X sample cases, less than two replicates would amplify after multiple attempts and for that cell 0:0 was entered, suggesting insufficient target DNA.

2.4. Genetic sexing

Sexing primers were evaluated for javan rhinoceros sex determination on both ABI and Licor platforms (Peppin et al., 2009). These primers were chosen because they target short DNA fragments ~95–107 bp, and are therefore best suited for usage with degraded DNA isolated from rhinoceros epithelial cells found in faeces. The primers were assessed using the javan rhinoceros control DNA set (see above) prior to the faecal DNA extracts. These results were compared to those obtained from individuals of known sex for each of the other extant rhinoceros species.

2.5. Faecal bacterial diversity

Bacterial diversity profiles of 104 faecal DNA extracts from the 2003–2006 and the recent 2009–2010 samples sets were collected. The DNA extracts produced for the genotyping and genetic sexing (see above) were PCRed for 16S rRNA genes using barcoded primers 27F-338R for the V1–V2 region of the 16S rRNA gene (Koren et al., 2010).

Sequences were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) software package (Caporaso et al., 2010). Due to low sequence yield 170 sequences were selected from each faecal sample for further analyses (rarefaction). Sequences of a length >200 bases were assigned to operational taxonomic units (OTUs) using UCLUST (Edgar, 2010), with a 97% pairwise identity threshold, and classified taxonomically using the Greengenes database. For tree based analysis, single representative sequences for each OTU were aligned using PyNAST, and the phylogenetic tree used in the UniFrac (Lozupone and Knight, 2005) analysis was built using FastTree (Price et al., 2009). The unweighted UniFrac metric (between sample diversity) was calculated and the resulting matrix was clustered using principle coordinate analysis to visualise the phylogenetic relatedness of the bacterial communities.

3. Results

3.1. Survey results

A total of 118 survey days were conducted by the two field teams, with a minimum of 429 km walked during that time (Fig. 2). During phase 1, 18 cells of the core area and 17 cells of the wider area were surveyed (Supplementary Fig. 2). Phase 2 focused on the rhinoceros core area only, conducting a repeat survey of 14 grid cells (Supplementary Fig. 3). Phase 3 repeat surveyed 16 grid cells of the rhinoceros core area and 10 cells of the wider area (Supplementary Fig. 3).

Eighteen wallows/swamps were recorded, all of which were identified by previous field surveys, with javan rhinoceros signs found during the survey at all but two of these areas (Fig. 2). Footprints were largely concentrated around the wallows and swampy areas but this could be an artefact of the season, with footprints not holding in anything but wet muddy ground during the dry season.

In total, between 27th October 2009 and 4th February 2010, 22 javan rhinoceros dung piles were located, sampled and sent for DNA analysis (Fig. 2). Notably, no new dung piles were found after 4th February, for the last 9 weeks of the survey and no fresh footprints were found after mid February (Supplementary Fig. 3).

3.2. Genotyping

Given the length of time since the collection of the EtOh stored 2003–2006 samples they turned out to have poor amplification success even after serial dilutions and multiple re-extractions. Consequently, genotyping of the 2003–2006 faecal samples was stopped and these extracts were further analysed for bacterial diversity alone (see below). DNA extracted from the EtOH stored 2009–2010 javan rhinoceros faecal samples have greater amplification success than extracts derived from faecal samples stored in Buffer or kept dry (Van Coeverden de Groot et al., 2011). EtOH data are presented below.

Only one javan rhinoceros genotype is present in the 2009–2010 javan rhinoceros faecal samples (Supplementary Table 2). This genotype matches the genotype of the skin samples collected from the dead individual found in CTNP.

The quality of javan rhinoceros epithelial DNA in their faeces is unpredictable and so microsatellite loci have variable amplification success across this tissue. Five samples did not yield any data despite repeated extraction and amplification. These five samples were from four distant locations and appear to be older and more degraded than those that did work. Two were from three dung samples collected at the same time and place at the beginning of the survey in October 2009 (D-4, D-5) and were relatively degraded. The third sample was very degraded (D003); there were no boluses and predominantly fibrous material remained. The fourth sample was found in a swamp (D022) partially submerged in water. The fifth sample was partially degraded, collected in late January 2010 (D024). All of the samples that did not amplify were found close to several other samples, which did amplify.

Considering the data for 17 samples, a given faecal sample had a 57% chance of amplifying the appropriate microsatellite amplicon across all 12 non-javan rhinoceros loci (range 31–85%; Supplementary Table 2). When only samples that amplified at 50% or more loci where considered (dropped six samples) this probability increased to 69%. Similarly not all loci were as reliable as others as on average each locus amplified in 53% of samples (range: 18–82%; Supplementary Table 2). When four loci were removed - IR12, WR32A, WR32F and WR35A - this increased to 67%.

3.3. Genetic sexing

According to the genetic sexing methods of Peppin et al. (2009), the female sex is indicated by one or both of the lower two bands 95/99 while the presence of the male includes these two and an additional band of 107 bp. In all of the six control set samples for javan rhinoceros, two products of 95/99 were generated. This was the same pattern seen in the females of the black (*Diceros bicornis)*, Indian (*Rhinoceros unicornis)*, Sumatran (*Dicerorhinus sumatrensis)* and white rhinoceros (*Ceratotherium simum)* (Van Coeverden de Groot et al., 2011). No upper band of 107 as seen

in the males of the other species was detected in the javan rhinoceros control samples suggesting the control javan rhinoceros were female. From the faecal extracts that did amplify, each showed the female band only as did DNA samples from the recently deceased rhino. These data suggest the last Vietnamese javan rhinoceros was female.

3.4. Faecal bacterial diversity

The bacterial diversity profiles in the samples collected at different dates were consistent with a single javan rhinoceros being sampled in 2009–2010 and two rhinoceros were sampled in 2003–2006 (Fig. 3). Most of the unique 2003–2006 samples cluster together, indicating that they have similar bacterial diversity, except for three samples (Fig. 3). These patterns suggest that three of the samples collected between 2003–2006 are from the animal that was still alive in 2009–2010.

4. Discussion and conclusions

4.1. Extinction of the Javan rhinoceros from Vietnam

Although the 2009–2010 field survey, genotyping and genetic sexing of 2009–2010 javan rhinoceros faecal samples, and the bacterial diversity assay of 110 faecal samples from 2003 to 2006 and 2009 to 2010 alone may not conclusively point to the extirpation of the javan rhinoceros from Vietnam, we believe that the results of the three in combination strongly indicate the extinction of this taxa. We discuss each of these lines of evidence below.

This survey achieved good coverage of the known (approx. 6500 ha) and previous (approx. 5500 ha) range of javan rhinoceros in Cat Loc (approximately 12,000 ha in total). We ensured that the survey effort was sufficient to collect dung samples from throughout the rhinoceros range; dung was collected from the northern and southern extremities of the rhinoceros core area 5 km distant from each other, and 3 km apart from east to west. The whole of the wider area was also surveyed once, with parts of the area surveyed twice, despite there being no confirmed rhinoceros records in this area since the early 1990s, to ensure that no individual was missed by the survey team. No signs of rhinoceros were found outside of the rhinoceros core area at any point during the survey, despite targeting potential hotspots. By that time of year, most of the swamps and streams in the wider area were almost or completely dry. Since javan rhinoceros require wallows and swampy areas on a regular basis for several purposes, including thermoregulation, removal of parasites and skin conditioning, (Groves and Leslie, 2011), it is very unlikely that the rhinoceros would be able to inhabit the wider area in the dry season where such wallows and swampy areas are scarce. The javan rhinoceros has not been recorded outside of the protected area for more than 20 years (last confirmed record was Schaller's survey in 1989) and with much of the surrounding habitat to the north, south and west of the protected area converted to agricultural land and urban areas and where suitable habitat does exist now disconnected from the rhinoceros core area, it is highly unlikely that rhinoceros survive in the wider landscape. These results are consistent with past survey findings and recent anecdotal reports from local villagers.

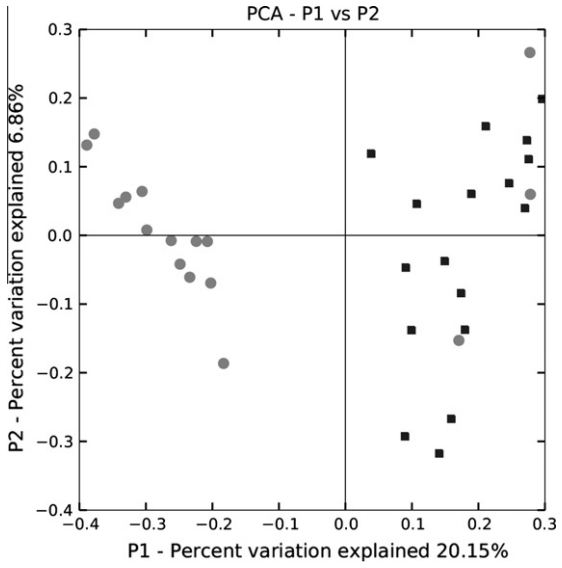

Fig. 3. Bacterial diversity comparisons for faecal samples in 2003–2006 (circles) and 2009–2010 (squares). Plotted are principal coordinates from a principal coordinate analysis of the unweighted UniFrac distances between samples, the first two PC's (explaining the majority of the variance) are plotted (variance explained by the PC's is indicated in parentheses on the axes). Samples that cluster close together have similar bacterial community composition (unaffected by abundances of specific sequence types).

The field survey data provides evidence that all of the samples belonged to only one individual, the animal that was found dead in CTNP in April 2010. All 22 dung samples were collected in the first 4 months of the 6-month survey and no new dung samples or footprints were found after 4th February 2010, even though prior to this fresh dung were recorded on a regular basis. Although a pathological examination could not determine precisely when the animal died (Streicher et al., 2010) due to the absence of most of the skin and soft tissue which had already decomposed, it is therefore suspected from the field data that the last rhinoceros died in late January/early February 2010.

Genotype and genetic sex data was extracted from 17 of the 22 faecal samples (77%) collected by the survey teams, the remainder were likely too old and decomposed to extract host DNA. The identical genotype of all the faeces point to a single rhinoceros being present at the start of the survey in 2009 and that this was the same individual that was found dead in 2010 – its genotype was identical to that of the faeces. The genetic sex of faeces that amplified were all female – the same as the deceased animal. While the variability of the microsatellite markers optimised to date suggest that the number of animals in larger populations may be underestimated (see below) the absence of tracks in the third phase of the field survey and bacterial diversity data support the recent existence of a single female that is now dead.

We used bacterial 16S rRNA gene sequence analysis from the faecal samples to provide an independent inquiry into the rhinoceros count. This approach is based on the observation that when using the unweighted UniFrac analysis, repeat samples from the same individual host often cluster more closely together than samples from unrelated individuals. This pattern has been observed for humans (Ley et al., 2006; Turnbaugh et al., 2009), and limited data are available for other species of mammal (Ley et al., 2008). However, a few caveats need to be acknowledged: we have no baseline data to know if this is true for wild rhinoceros, and other factors may also drive this type of pattern, especially diet. The extent to which diet drives faecal bacterial diversity in wild rhinoceros is not known. The clustering pattern observed for these data is consistent with the interpretation that only one individual rhinoceros was present in 2009–2010. However it is possible that, other factors may have caused this pattern of clustering, such as differences in diet items that formed the faeces. Interestingly, from this work it may also be inferred that this and at least one other javan rhinoceros was alive when the other faecal samples were collected in CTNP from 2003–2006. This finding suggests that another individual has been lost from CTNP between that time and prior to the beginning of the survey in 2009.

Consequently, we are confident in reporting that the death of the individual javan rhinoceros in 2010 represents the extinction of javan rhinoceros in Vietnam and of the *annamiticus* subspecies. Although this population estimate is lower than past surveys, we suggest that the inaccuracies of previous surveys and analytical methods led to over-estimates of the population in the past, particularly in earlier surveys. Furthermore, the bacterial diversity analysis indicates that at least one individual has died between 2003 and 2004 and the start of the 2009 survey, which has never been found. The only remaining population of javan rhinoceros therefore survives in Java, Indonesia. The protection and expansion of this population is the utmost priority for conservation of this critically endangered species. A recent paper using video-trapping mark-recapture methods showed a minimum of 29 animals were identified in Ujung Kulon National Park over an 18 month period between 2008 and 2009 (Hariyadi et al., 2011), and recent unpublished data by Ujung Kulon National Park suggest a minimum of 35 individuals have now been identified using camera and video traps; both being considerably lower than past estimates.

4.2. Progress on non-invasive survey techniques of rhinoceros species

The above findings are disheartening and point to ongoing conservation failures in the region (Bennett, 2011; Van Song, 2008; TRAFFIC, 2008). Against this failure we evaluate the potential of the three non-invasive components detailed above to assist in accurate census and monitoring of the remaining Asian and African rhinoceros populations.

4.2.1. Scat detection dogs

Scat detection dogs are widely acknowledged as a successful wildlife survey method (Browne et al., 2006). Detection rates of faecals with scat detection dogs are higher than more opportunistic methods (Rolland et al., 2006) and are equal to or higher than detection rates of camera trapping, track plates and hair snare studies (Long et al., 2007; Vynne et al., 2010; Wasser et al., 2004). Dogs can even identify between different individuals from their dung with accuracy rates of greater than 80% (Kerley, 2004).

However, the relative merit of detection dogs to any survey is likely to depend on a few different factors, namely attributes of the species (or its faeces) that the dogs have been trained to detect, and secondly, environmental features of the habitats in which they work. Although this survey successfully detected 22 javan rhinoceros faecal samples, the added value of scat detection dogs to this particular survey is unclear, but for reasons we discuss below, we think they are unlikely to be as significant for rhinoceros surveys as for other species, or for surveys in less complex environments.

The vast majority of the studies listed above have been carried out in structurally, and in some instances also topographically, less complex environments than the dense tropical forest environment of the study area. With large areas of the study area covered in impenetrable rattan and bamboo thickets, the survey teams and detection dogs were consequently restricted to searching existing trail systems much more than would have been preferable. Although the dogs did range off the trails, their ranging ability was certainly restricted by the habitat which at times may have resulted in more distant dung piles being missed by the dogs and human observers, although if dogs could not access areas the likelihood of rhinoceros accessing these areas is also low. The majority of faecal samples that were detected were on or very close to existing trails, which were also easily located by the human observers. Based on tracks and signs from the field survey, the javan rhinoceros seemed to use existing trails quite heavily, certainly in areas with very dense vegetation. With complexities of scent dispersion (e.g. environmental factors, vegetation, weather) affecting how quickly the dogs could home in on the exact location of the faeces (Reed et al., 2011; Wasser et al., 2004), in many instances the dung pile itself was located by the human observers before the dogs, despite the dogs picking up on the scent a significant distance before the dung was visible. This may not be a factor for other species with smaller dung, but rhinoceros dung piles are hard to miss by human observers when located on or within a few metres of the trail. Several other sites for javan and sumatran rhinoceros are structurally similar to Cat Loc (e.g. Tabin Wildlife Reserve). Consequently, future rhinoceros surveys should carefully consider whether scat detection dogs will contribute significantly to increasing detection probabilities for rhinoceros faeces at a particular site, to ensure the additional expense and logistical constraints incurred with scat detection dog surveys are justified and necessary.

4.2.2. Genotyping and genetic sexing of faecal samples

Our findings clearly demonstrate that the genotyping of faecal samples is a valid census method for javan rhinoceros populations. Of the three storage methods of fresh faecal samples tested – EtOH, EDTA Buffer and Dried – EtOH storage worked the best with 17/22 EtOH faecals being genotyped. Of those EtOH extracts for which microsatellite genotypes and genetic sex were obtained, there was substantial variation in amplification success of faecal extracts across loci with a 31–85% chance of amplifying the correct amplicon in a given faeces. Of 40 non-javan rhinoceros loci evaluated, we have optimised 12 non-javan rhinoceros loci to reliably and accurately amplify javan rhinoceros microsatellites from javan rhinoceros faeces (Van Coeverden de Groot et al., 2011). By this it is meant the same sized DNA amplicon is produced in replicate faecal extracts and in non-faecal javan rhinoceros tissue extracts. The latter step was not included in previous faecal DNA studies (Fernando and Melnick, 2006).

Despite this progress we were unable to reliably optimise our nine javan rhinoceros primers (Van Coeverden de Groot et al., 2011) on the non-radioisotopic Licor platform despite redesigning primers for four of these loci. Our GCR-PCR library was constructed from DNA combined from all the control set samples and only yielded 38 clones from which to design primers (Van Coeverden de Groot et al., 2011). Many of these had insufficient flanking sequence from which to design good primers. Our next step is to generate a large number of javan rhinoceros microsatellites containing clones using 454 pyrosequencing. With the large number clones we are confident we will optimise variable javan rhinoceros microsatellites that amplify javan rhinoceros DNA from faecal material.

A better set of control samples is required for additional javan rhinoceros microsatellite optimisation; the samples in our control set are predominantly very old museum samples. This DNA was generally of low molecular weight and relatively degraded. As a result the polymorphic/non-polymorphic characterisation of microsatellites in javan rhinoceros determined from assaying this set should be regarded as preliminary. This control set could be augmented with hair-snag or biopsy samples from living rhinoceros and by the large museum collections in Holland, Britain and Indonesia. Finally, we have extended universality of current sexing primers to all rhinos (Peppin et al., 2010; Van Coeverden de Groot et al., 2011). However, good tissue and faecal samples from known males is required before genetic sexing of males from their faecal samples can be confidently determined.

4.2.3. Bacterial survey

In contrast to the varied genotyping success for the extracted javan rhinoceros faecal samples the characterisation of their faecal bacterial diversity was more successful and from a technical point of view holds great promise. However, before these data can be considered useful for census objectives the cautions above need to be considered. With this in mind, a study of the variation in

bacterial diversity within and among captive white and Indian rhinoceros over time is underway. A similar collection for the black rhinoceros may be easily obtained while the one for the sumatran rhinoceros may be obtained with more difficulty. No such samples for javan rhinoceros can be easily obtained and the results from the other species will used to evaluate the census value of these data in the javan rhinoceros.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2012.06.008.

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