ON THE IDENTITY OF THE FIRST RHINOCEROS OWNED BY OF THE DUBLIN ZOO (†1865); GENETIC CHARACTERISATION OF A POORLY PRESERVED MUSEUM SPECIMEN

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ABSTRACT

Understanding past and present genetic diversity within endangered species is crucial for the identification of evolutionary significant units (ESUs) and subsequent conservational decisions. Direct access to genetic diversity of extinct populations can only be gained from (sub)fossils or specimens housed in natural history collections. With probably less than 50 extant specimens, the Javan rhinoceros, *Rhinoceros sondaicus*, is a critically endangered species. It is rare even in museum collections, thus each newly discovered specimen is of conservation importance. Although the Indian (*Rhinoceros unicornis*) and Javan rhinoceros differ in size, skinfolds and skin texture, the two have been confused on several occasions in the recorded history of both species. Examples can be found in textbooks, zoological gardens and museums. As for the latter, identification of mounted specimens can be compromised by among other factors poor preservation of the skin. An example of such an ambiguous specimen is the Dublin Zoo rhinoceros (†1865) housed in the Zoological Museum at Trinity College Dublin. Ever since it was mounted, it has borne a name plate that claims it represents a specimen of *R. unicornis*, but this is not necessarily supported by a number of morphological characters. With this study, we determine the identity of this one-and-a-half century old specimen by DNA sequencing a fragment of mitochondrial *Cytochrome B*.

INTRODUCTION

Throughout the nineteenth century, exotic animals were scarce and expensive. In the early years of the Dublin Zoo (opened 1831), animals such as elephants and rhinoceroses were hired from travelling menageries, acquired by gift or exchange from other zoos and, on rare occasions, purchased. In 1864, Sir Charles Trevelyan (1807–1886), finance minister in India and a friend of Dublin Zoo, donated a young, male rhinoceros. Together with a consignment of animals for the London Zoo, the rhinoceros was shipped from Barrackpore Park, Calcutta, to London. From there on it was shipped to the Dublin Zoo, where it arrived on 3 August 1864 (Rookmaaker et al. 1994). Unfortunately the young rhinoceros never settled in Dublin; zoological records show that it was frequently 'crabby' and did not eat well (de Courcy 2009, and references therein); it died in horrific circumstances in April 1865. Generally, deceased animals were sold by the Zoo to generate some additional income. In this case, the carcass was bought by the Zoological Department of Trinity College Dublin for a post-mortem and anatomical study, after which the skin and skeleton were donated to the Zoological Museum of Trinity College, where they are still housed.

The rhinoceros had been in captivity since, at least, October 1863, and Haughton (1867) identified it as a three year old in the autopsy report. And, this is where the questions arise. The mounted specimen in the Zoological Museum in Trinity College, which has to be the Dublin Zoo rhinoceros, seems remarkably small for an Indian rhinoceros, *Rhinoceros unicornis* [Lin. 1758], of this age. Given the rarity of the Javan rhinoceros in museum collections, it was felt worthwhile to investigate this specimen further.

Nowadays only a small group (probably less than 50 specimens) of Javan rhinoceros, *Rhinoceros sondaicus sondaicus* [Desmarest 1822], survive in Ujung Kulon (Java). The few 'rediscovered' Javan rhinoceroses, *R. sondaicus annamiticus* [Heude, 1892], in Cat Tien (Vietnam; Polet *et al.* 1999) were officially considered extinct in a recent Working Neighbourhoods Fund (WNF) report (Brook *et al.* 2011) after the last one was found

dead in April 2010 as a result of poaching. When the Dublin rhinoceros was shipped in 1864, however, the Javan rhinoceros (*R. sondaicus inermis*) still inhabited the Sunderbunds of Bangladesh (Guggisberg 1966) and *R. sondaicus* was occasionally kept in Barrackpore Park, Calcutta. Fig. 1 shows the historic distribution of *R. sondaicus* (after Foose and van Strien 1997; Groves and Leslie 2011). Note that Groves and Leslie (2011) consider the historical distribution of *R. sondaicus* in Foose and van Strien (1997) to be overstated.

Unfortunately, *R. unicornis* and *R. sondaicus* share a history of confusion; in 1959, Sody listed four books that showed illustrations of *R. unicornis* accompanying descriptions of *R. sondaicus*, and in 1966, Guggisberg wrote, 'A Javan rhinoceros shown in the Berlin Zoo sometimes in the last century was in fact an Indian one, while an Indian rhino which died in the Zoo of Adelaide, Australia, was found to belong to the Javan species'. More recently, Rookmaaker concluded that the so-called Javan rhinoceros that once lived in the Zoological Garden of Liverpool likely to have been an Indian rhinoceros (Rookmaaker 1993).

There are a number of clear morphological differences between adult *R. unicornis* and *R. sondaicus*, however. The Javan rhinoceros has a skin fold protruding around the shoulder that results in an additional skin shield (also referred to as nape shield or saddle), which lacks in *R. unicornis*. The skin folds on *R. sondaicus* are also shallower than that on *R. unicornis*, and *R. sondaicus* does not develop a 'bib' (pronounced in the adult Indian rhinoceros); nor does it develop the deep cheek and neck folds that are seen in *R. unicornis* (Groves and Leslie 2011). Moreover there is a difference in the form of the head (which is more slender in the Javan rhinoceros) and the overall size of *R. sondaicus* is less than that of the Great Indian. Another obvious distinction is the presence of a prominent horn in both sexes of *R. unicornis*, which can reach up to 61cm (2 feet; Tun Yin 1967), whereas, in general, only male *R. sondaicus* possesses a horn, which normally does not exceed 25cm (10 inches; Guggisberg 1966). According to Lydekker (1907), 'the tail of *R. sondaicus* stands out quite distinct from the hind-quarters so that its whole extent is exposed in a side view', whereas in *R. unicornis,* 'the tail is enclosed in a deep grove, in such a manner that only its terminal portion is visible in a side view' (Fig. 2). The skin of the Javan rhinoceros does not show the tubercles characteristic of *R. unicornis*, but has a cracked scaly (reticulated) appearance. Guggisberg (1966) describes it as a 'mosaic-like pattern', which indeed can be seen in the photographs of several specimens killed in Sumatra that illustrate an article published by Hazewinkel in 1933. Finally, the upper lip of *R. sondaicus* might be longer and more prehensile than that of *R. unicornis* (Sclater 1874; Groves and Leslie 2011).

Despite these differences, confidently identifying the 1865 Dublin rhinoceros (Fig. 2) on sheer morphology turned out to be difficult for the following reasons. First, the specimen is small for a three-year-old *R. unicornis*. Second, some of the before mentioned characters are absent or are not discernable. Third, because of the poor way in which the skin was preserved, morphological details were either missing or ambiguous. Consequently, only genetic analysis could result in a reliable identification of this specimen. Next to extracting DNA from the skin, we tried to extract DNA from the skeleton, because *a priori* it was unclear which body part (and hence preservation method) provided the best conditions for DNA conservation. Moreover, if the analysis proved successful for both objects, it would allow us to see if the skin and skeleton indeed belonged to the same species. At first sight it seems hardly imaginable how the skin (Fig. 2) and skeleton (Fig. 3) could not belong to the same individual, given the age, size and origin of these body parts, but once separated, different parts can end up in different institutions (Rookmaaker 1993) and objects displayed together today, do not necessarily share the same history.

MATERIAL AND METHODS

Before sending samples to Leiden (the Netherlands) for molecular analysis, permission was granted by the Irish CITES Management Authority. In total, eight samples were taken, four from the skin (under the head region, tail region, left rear toe and right front toe) and four from the skeleton (right shoulder blade, pelvic region, underside of the jaw and left rear heel). For the skin samples, small pieces of tissue were cut from the mounted specimen using gloves and sterile blades; for the bone samples, tiny holes were drilled in the skeleton and drilling chips and dust were collected. DNA extractions were performed in a dedicated ancient DNA facility (LAF, Leiden). No work on Rhinocerotidae was previously performed in this facility, which is physically isolated from the main laboratory (where post-PCR work was carried out). All samples were pulverized with an MM200 mixer mill (Retsch) using steel balls and grinding jars. DNA was extracted using the guanidine thiocyanate (GuSCN) protocol described by Rohland and Hofreiter (2007). Final extraction volume was 40µl, and DNA extraction and PCR blanks were included to monitor for contamination.

On the basis of available sequence data (GenBank) for both the Indian (Xu *et al.* 1996) and Javan rhinoceroses (Tougard *et al.* 2001), internal primer sets were designed using Primer 3 (Rozen and

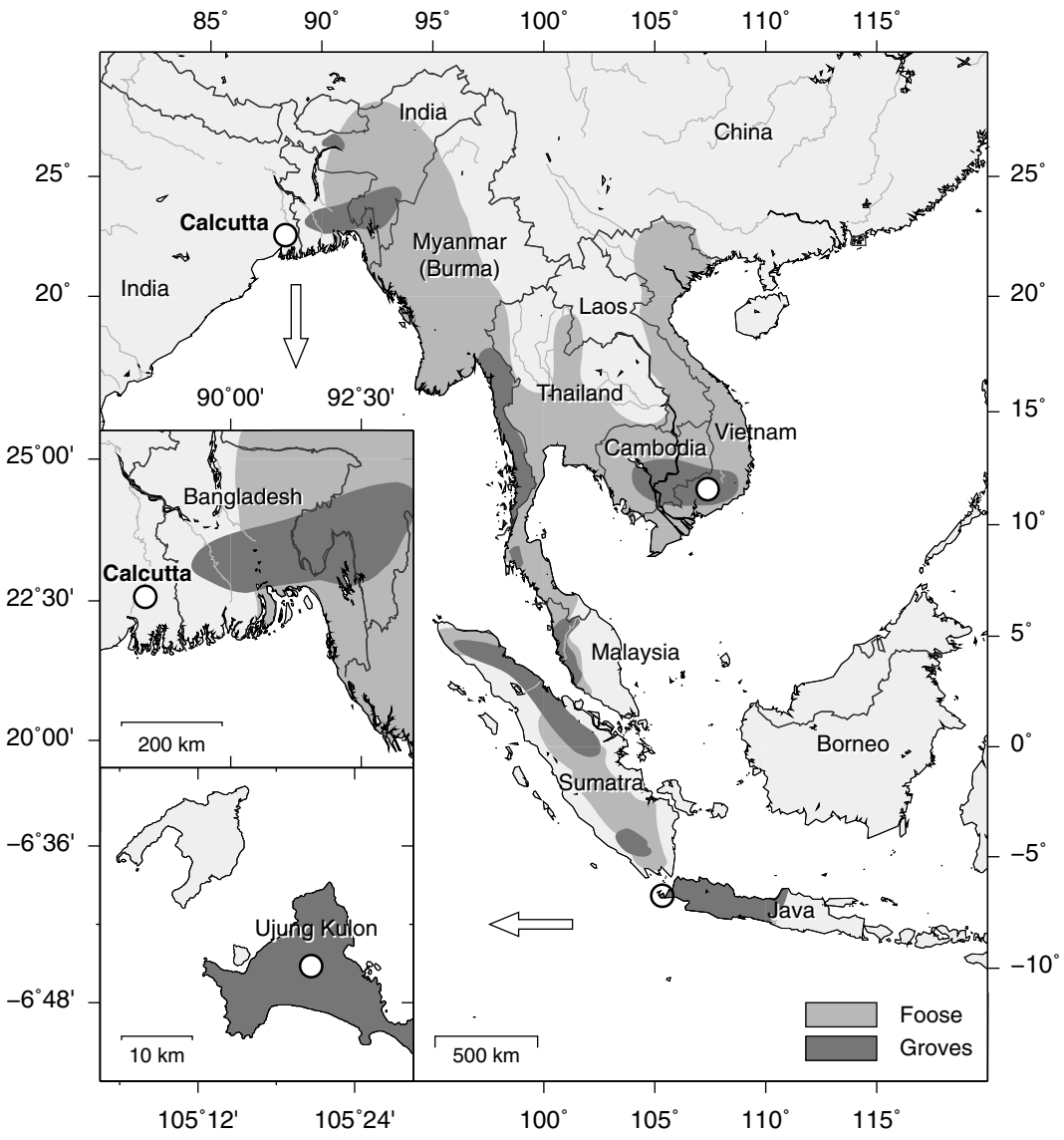

Fig. 1—Historic distribution of *R. sondaicus* (after Foose and van Strien 1997; Groves and Leslie 2011). The three circles in the main picture represent Calcutta, the origin of the Dublin rhinoceros (†1865), Cat Loc (within Cat Tien National Park, Vietnam), where *R. sondaicus annamiticus* recently died out and Ujong Kulon National Park, where *R. sondaicus sondaicus* still survives.

Skaletsky 2000) to amplify small fragments (suitable for ancient DNA studies) of *Cytochrome B* (*Cyt B*). Primer names and sequences can be found in Table 1; numbers relate to the position of these primers relative to GenBank reference sequence X97336 (Xu *et al.* 1996). Although the primers were designed to work on both *R. unicornis* and *R. sondaicus*, we selected the nucleotide found in *R. sondaicus* (FJ905815; Tougard *et al.* 2001) for the minority of positions that were not identical. Initially, we attempted to amplify the largest fragment (396bp: 14613-F and 15008-R) followed by successively smaller fragments (186bp: 14823-F and 15008-R; 74bp: 14719-F and 14792-R), if PCRs would not succeed. PCR reactions were carried out on a PTC-200 thermocycler (MJ research) in 25µl volumes, using 2.5µl genomic DNA extract, 0.4µM of each primer, 1.5mM $MgCl_2$ (in buffer), 0.2mM dNTPs and 0.5µl Phire™ Hot Start DNA Polymerase (Finnzymes). The PCR thermal cycling profile started with 5min initial denaturation at 98°C, followed by 40 cycles of 5sec denaturation at 98°C, 20sec annealing at 60°C, 30sec extension at 72°C and a final extension of 1min at 72°C.

To establish the authenticity of 'ancient' DNA sequences, PCR products are generally cloned to distinguish between post-mortem changes (damage induced errors; Cooper and Poinair 2000) and genuine sequence data. Because the main objective

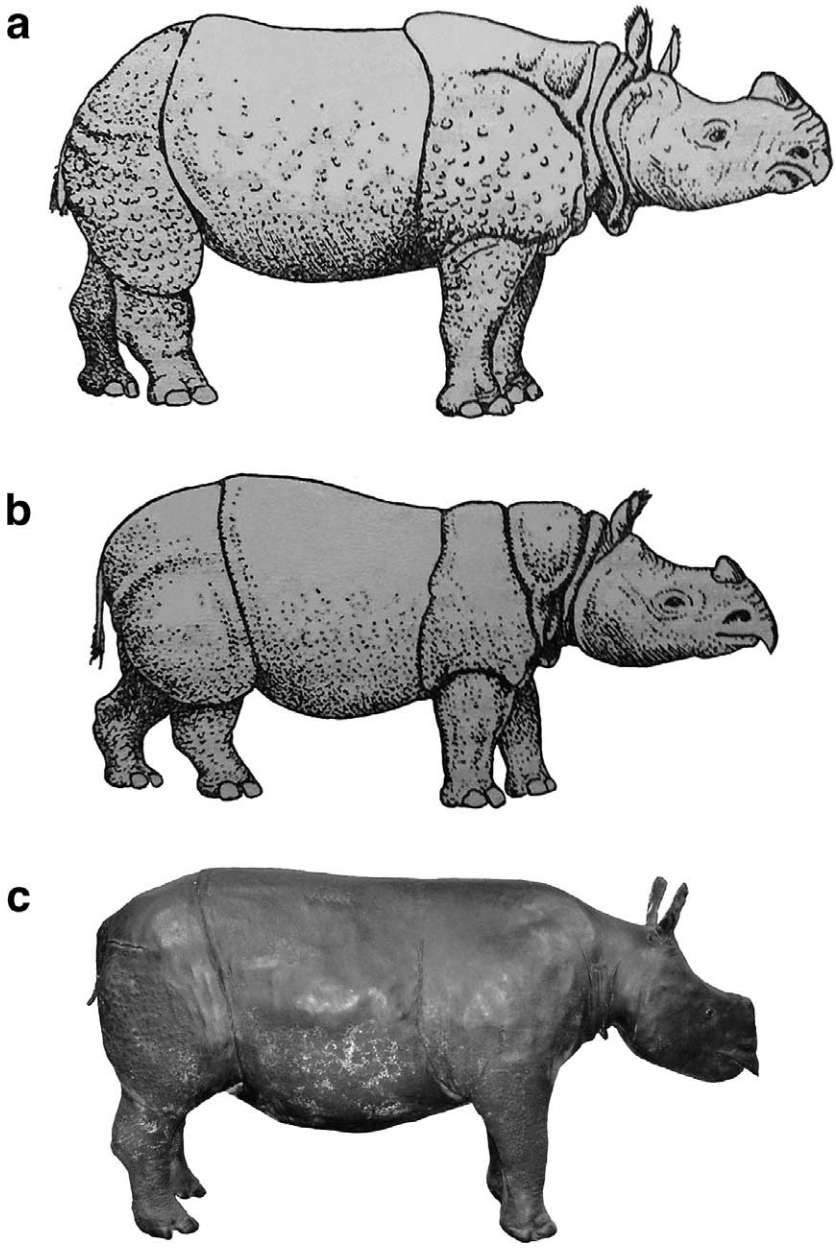

Fig. 2—Illustrations of (a) *R. unicornis*, (b) *R. sondaicus* (both from Sody 1959; used with permission) and a picture of (c) the Dublin rhinoceros (†1865) as currently housed in the Trinity museum. To display the juvenile Dublin rhinoceros in as much detail as possible, it is depicted roughly the same size as the Javan rhino. The relative size of *R. unicornis* compared with *R. sondaicus* is after Sody (1959).

of this study was identification of the 1865 specimen and as *Cyt B* sequences are divergent enough to distinguish between the Javan and Indian rhinoceros (uncorrected pairwise genetic distances are 8.6%, 10.9% and 15.4% for the 396bp, 186bp and 74bp fragments, respectively; also see Tougard *et al*. 2001; Hsieh *et al*. 2003), authenticity was not a significant issue here (as long as the recovered sequences would represent a species of *Rhinoceros*). To facilitate DNA sequencing, the smallest PCR product (bone sample) was cloned; other PCR products were sequenced directly. Cloning was done using a TOPO TA Cloning kit (Invitrogen) following the manufacturers instructions, *albeit* using only half of the prescribed reaction volumes. Colony PCRs were done with the same primers (14719-F and 14792-R) and with the M13 primers (M13/pUC-F and M13/pUC-R, see Table 1). All PCR products were sent to Macrogen Europe (Amsterdam) where they were purified with a Montage purification kit (Millipore) and sequenced in both directions (with the same primers used to

Fig. 3—Mounted skeleton most likely belonging to the Dublin rhinoceros (†1865).

obtain the PCR products) on an ABI3730XL. The obtained forward and reverse sequences were assembled using Sequencher v.4.10.1 (Gene Codes Corp.), checked for indels and stop codons and subsequently submitted to GenBank.

RESULTS

The initial PCRs for the 396bp fragment only worked for the right front toe sample. The amplification of the 187bp fragment worked for the same sample as well as for the left rear toe sample (thus only skin samples). Of the skeleton samples, only the sample from the pelvic region yielded an amplicon for the smallest (74bp) fragment. The sequences have been deposited in GenBank under accession numbers JN935370–JN935374. Except for sequence JN935373 (colony PCR from the pelvic bone sample using the M13 primers; Table 1) which had one A to G substitution (corresponding to position 14753 in the reference sequence) the five obtained sequences were identical to each other as well as to the *R. unicornis* reference sequence (X97336; Xu *et al.* 1996).

DISCUSSION

Despite the fact that the DNA sequences unambiguously show that the 1865 Dublin Zoo rhinoceros represents *R. unicornis*, it is remarkable to see how shallow (if visible at all) its skin folds are (especially in the neck area); this contrasts sharply with the prominent folds that are normally seen in this species. Also, the skin is rather smooth hardly showing the large tubercles characteristic of the Great Indian.However, the skin does not have the reticulate appearance typical for *R. sondaicus*. From side view, the tail does not stand out distinctly and, indeed, only the terminal portion was visible. The shape of the head is more difficult to appraise, especially because this specimen appears to be a juvenile. Compared to the rest of the body the head seems relatively small, also rather untypical for

Table 1—Internal *Cytochrome B* primers designed for the genus *Rhinoceros* and M13 primers used for colony PCR.

Primer name	*Sequence (5′ → 3′)*
14613-F	ATTACAAATCTCCTCTCAGCCATC
14719-F	TCCACTTCATCCTTCCCTTTATTA
14792-R	GGATCCTGTTTCGTGTAGGAATAG
14823-F	GACAAAATTCCATTTCACCCTTAC
15008-R	AGCAAATAGGAAATACCATTCTGG
M13/pUC-F	GCCAGGGTTTTCCCAGTCACGA
M13/pUC-R	GAGCGGATAACAATTTCACACAGG

R. unicornis. The upper lip is long and shaped into a point, seemingly more resembling *R. sondaicus* than *R. unicornis*. But if it is a juvenile male *R. unicornis*, the 1865 specimen would not have yet developed characteristics typical for adult males of this species, such as a pronounced 'bib' and large horn. The overall size of the Dublin rhinoceros is unarguably small for a three-year-old Indian rhinoceros, even one that was malnourished (de Courcy 2009, and references therein). Although the results of both skin and bone samples identify the specimen as *R. unicornis*, the *Cyt B* sequence alone does not provide enough information to genotype individuals. Together with the rather well documented history of this specimen, the results leave little doubt that the mounted skin and skeleton originated from the same specimen. Even though there is no evidence for lesions in ancient DNA that result in A to G substitutions (Stiller *et al.* 2006; Lalueza-Fox *et al.* 2007), sequence JN935373 provides yet another example that these substitutions are being observed. Nevertheless, we cannot rule out that these substitutions can be attributed to the type of polymerase (Stiller *et al.* 2006) or reaction conditions used.

CONCLUSION

Despite its small size and ambiguous morphology, the first rhinoceros owned by the Dublin Zoo was a specimen of *R. unicornis*, not *R. sondaicus*. Although this study, unfortunately, did not result in the discovery of another specimen of the increasingly threatened Javan rhinoceros (Brook *et al.* 2011), it shows the possibility of extracting genetic information from museum specimens and emphasizes the potential of these specimens as a tool in conservation of endangered species (Tracy and Jamieson 2011). In cases where morphology based identifications are inconclusive, which (as shown here) could be due to poor preservation of collections specimens, molecular identifications can be of decisive importance.

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REFERENCES

Brook, S., Van Coeverden de Groot, P., Mahood, S. and Long, B. 2011 Extinction of the Javan Rhinoceros (*Rhinoceros sondaicus*) from Vietnam. WWF report.

Cooper, A. and Poinar, H.N. 2000 Ancient DNA: do it right or not at all. *Science* **289**, 1139.

de Courcy, C. 2009 *The Dublin Zoo: an illustrated history*. Cork. Harper Collins Publishers Limited.

Foose, T.J. and van Strien, N.J. 1997 *Asian Rhinos – status survey and conservation action plan*. Gland. The World Conservation Union, Asian Rhino Specialist Group.

Groves, C.P. and Leslie, Jr., D.M. 2011 *Rhinoceros sondaicus* (Perissodactyla: Rhinocerotidae). *Mammalian species* **43**, 190–208.

Guggisberg, C.A.W. 1966 *S.O.S. rhino*. London. Andre Deutsch.

Haughton, S. 1867 On the muscular anatomy of the rhinoceros. *Proceedings of the Royal Irish Academy* **9**, 515–26.

Hazewinkel, J.C. 1933 The one-horned Javanese rhinoceros found also in Sumatra. *The illustrated London news*, 1018–19.

Hsieh, H.-M., Huang, L.-H., Tsai, L.-C., Kuo, Y.-C., Meng, H.-H., Linacre, A. and Lee, J.C. 2003 Species identification of rhinoceros horns using the *cytochrome b* gene. *Forensic Science International* **136**, 1–11.

Lalueza-Fox, C., Römpler, H., Caramelli, D., Stäubert, C., Catalano, G., Hughes, D., Rohland, N., Pilli, E., Longo, L., Condemi, S., de la Rasilla, M., Fortea, J., Rosas, A., Stoneking, M., Schöneberg, T., Bertranpetit, J. and Hofreiter, M. 2007 A melanocortin 1 receptor allele suggests varying pigmentation among Neanderthals. *Science* **318**, 1453–5.

Lydekker, R. 1907 *The game animals of India, Burma, Malaya, and Tibet, being a new and revised edition of* '*The great and small game of India, Burma, and Tibet.*' London. Rowland Ward.

Polet, G., Tran, V.M., Nguyen, X.D., Bui, H.M. and Baltzer, M. 1999 The Javan rhinoceros, *Rhinoceros sondaicus annamiticus*, of Cat Tien National Park, Vietnam: current status and management implications. *Pachyderm* **27**, 34–48.

Rohland, N. and Hofreiter, M. 2007 Ancient DNA extraction from bones and teeth. *Nature Protocols* **2**, 1756–62.

Rookmaaker, L.C. 1993 The Mysterious "Liverpool Rhinoceros". *Der Zoologische Garten Neue Folge* **63**, 246–58.

Rookmaaker, L.C., Jones, M.L., Kloes, H.G. and Reynolds, R.J. 1994 *The rhinoceros in captivity: a list of 2439 rhinoceroses kept from Roman times to 1994*. The Hague. SPB Academic Publishing.

Rozen, S. and Skaletsky, H.J. 2000 Primer3 on the WWW for general users and for biologist programmers. In S. Krawetz and S. Misener (eds), *Bioinformatics methods and protocols: methods in molecular biology*, 365–86. Totowa, NJ. Humana Press.

Sclater, P.L. 1874 Announcement of the arrival in the Society's menagerie of a Javan rhinoceros. *Proceedings of the Zoological Society of London*, 182–83.

Sody, H.J.V. 1959 Das Javanische Nashorn *Rhinoceros sondaicus* historisch und biologisch. *Zeitschrift für Säugetierkunde* **24**, 109–240.

Stiller, M., Green, R.E., Ronan, M., Simons, J.F., Du, L., He, W., Egholm, M., Rothberg, J.M., Keates, S.G., Ovodov, N.D., Antipina, E.E., Baryshnikov, G.F., Kuzmin, Y.V., Vasilevski, A.A., Wuenschell, G.E., Termini, J., Hofreiter, M., Jaenicke-Després, V. and Pääbo, S. 2006 Patterns of nucleotide misincorporations during enzymatic amplification and direct large-scale sequencing of ancient DNA. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 13578–84.

Tougard, C., Delefoss, T., Hànni, C. and Montgelard, C. 2001 Phylogenetic relationships of the five extant rhinoceros species (Rhinocerotidae, Perissodactyla) based on mitochondrial *Cytochrome b* and *12S* rRNA Genes. *Molecular Phylogenetics and Evolution* **19**, 34–44.

Tracy, L.N. and Jamieson, I.G. 2011 Historic DNA reveals contemporary population structure results from anthropogenic effects, not pre-fragmentation patterns. *Conservation Genetics* **12**, 517–26.

Tun Yin, U. 1967 *Wild animals of Burma*. Rangoon. Rangoon Gazette.

Xu, X., Janke, A. and Arnason, U. 1996 The complete mitochondrial DNA sequence of the greater Indian rhinoceros, *Rhinoceros unicornis*, and the phylogenetic relationship among Carnivora, Perissodactyla, and Artiodactyla (+ Cetacea). *Molecular Phylogenetics and Evolution* **13**, 1167–73.