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CHAPTER 8

**On the identity of the first rhinoceros
owned by 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 fifty 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 among others by 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 bears 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 the 3rd of August 1864 (Rookmaaker, 1994). Unfortunately the young rhinoceros never settled in Dublin; zoological records show that it was frequently 'crabby' and did not eat very 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 today.

The rhinoceros had been in captivity since at least October 1863 and Samuel Haughton identified it as a three year old in the autopsy report (Haughton, 1867). 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, *Rhinoceros sondaicus annamiticus* [Heude, 1892] in Cat Tien (Vietnam; Polet *et al.*, 1999) were officially considered extinct in a recent WNF report (Brook *et al.*, 1011) 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 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 and 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 already 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 the *R. sondaicus* 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 2 feet (61 cm), whereas only the male *R. sondaicus* possesses a horn, which regularly does not exceed 10 inches (25 cm). 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 groove, 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 for *R. unicornis*, but has a cracked scaly (reticulated) appearance. Guggisberg (1966) describes it as a "mosaic-like pattern", which indeed can be seen



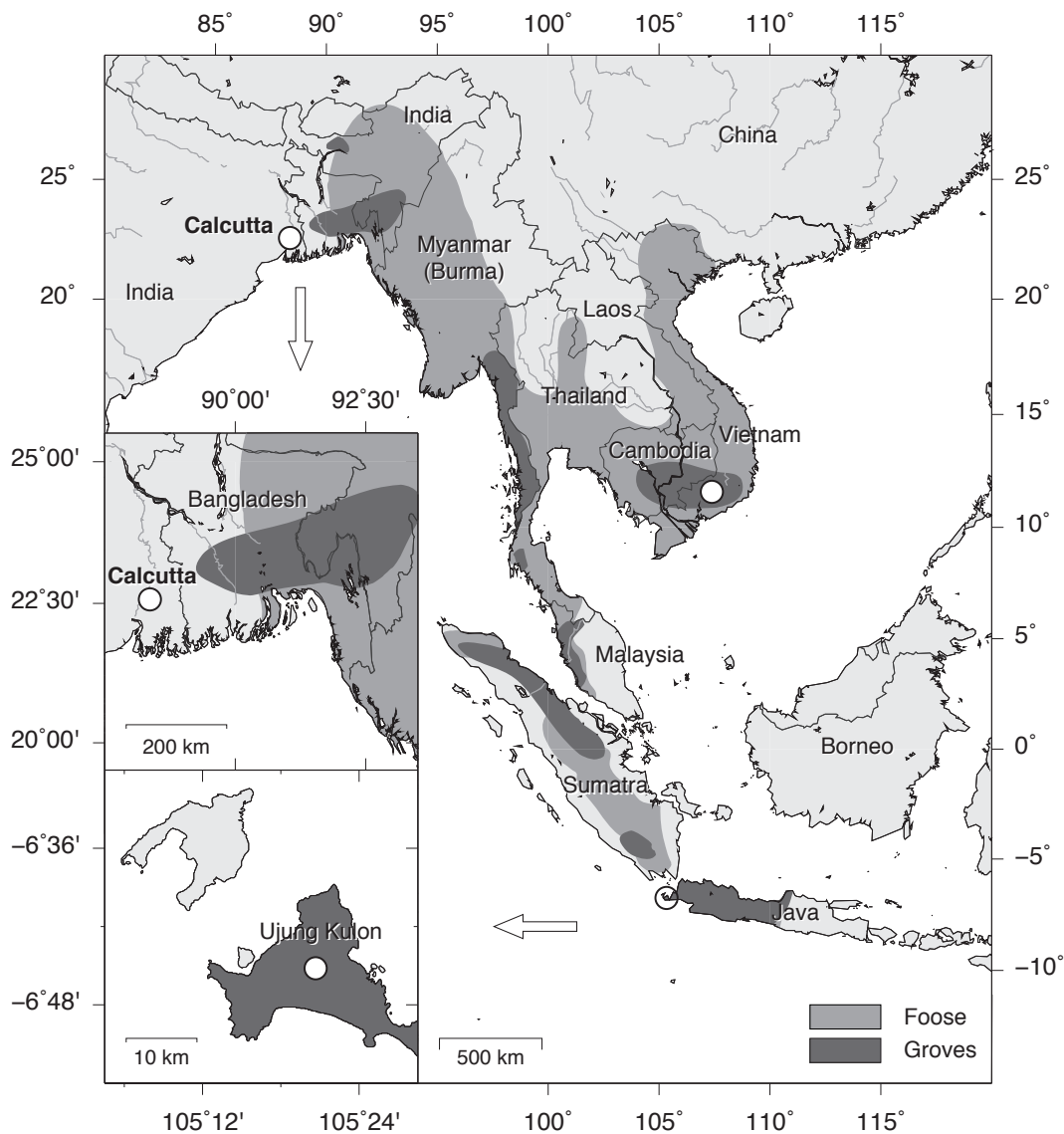


Figure 1. Historic distribution of *Rhinoceros sondaicus* (after Foose and van Strien, 1997 and Groves and Leslie, 2011).

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 Ujung Kulon National Park, where *R. sondaicus sondaicus* still survives.



in the photographs of several specimens killed in Sumatra that illustrate an article published by JC Hazewinkel in 1933. Finally, the upper lip of *R. sondaicus* might be longer and more prehensile than that of *R. unicornis* (Sclater, 1874 and Groves and Leslie, 2011).

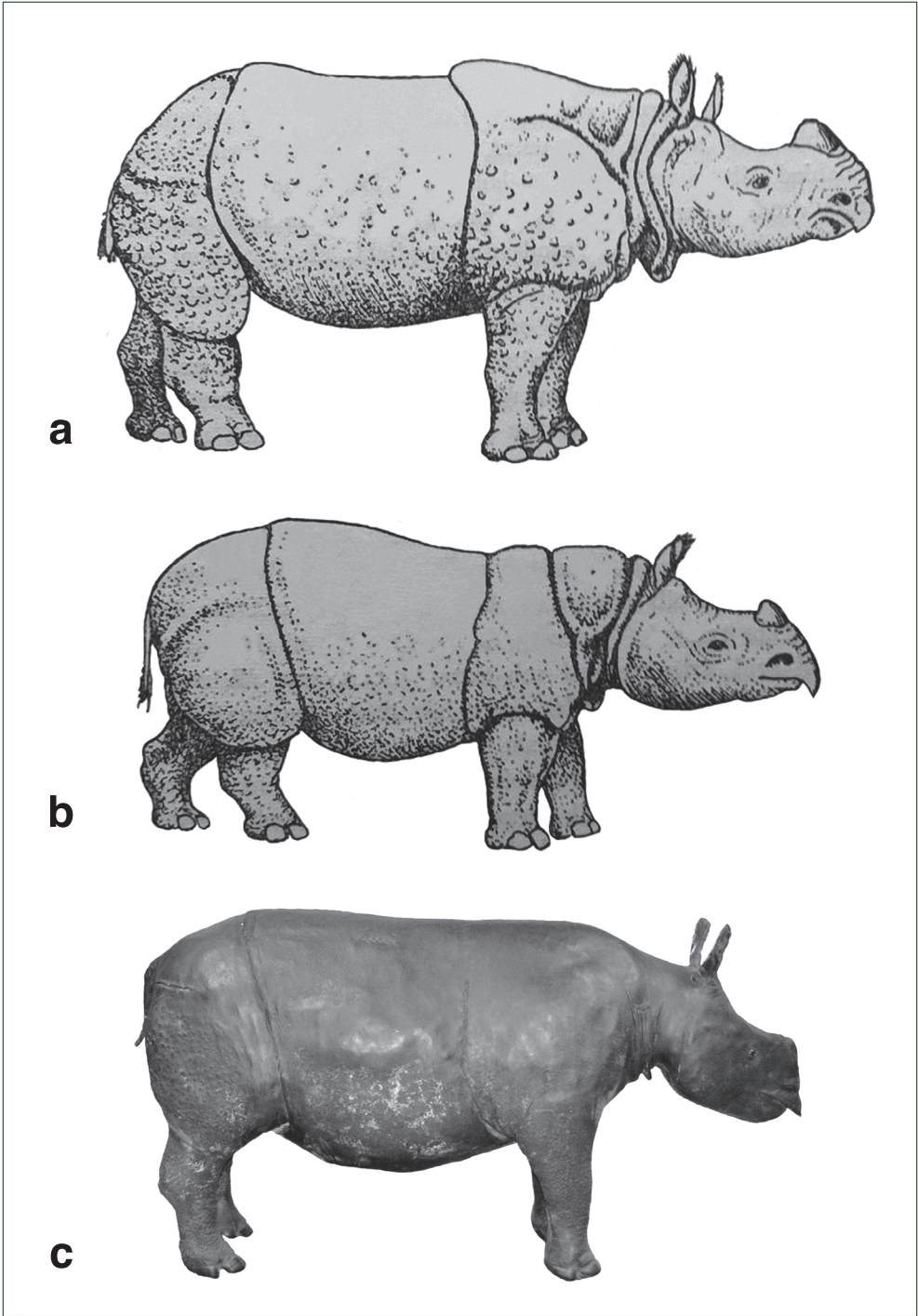
Despite these differences, confidently identifying the 1865 Dublin rhinoceros (Fig. 2) on sheer morphology turned out to be difficult for two main reasons. Firstly, the specimen is small for a three year old *R. unicornis*. Secondly, some of the before mentioned characters are absent or are not discernable. Thirdly, because of the poor way in which the skin was preserved, a number of 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 as well, 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

Prior to sending samples to Leiden (the Netherlands) for molecular analysis permission was granted by the Irish CITES Management Authority (Fig. 4). 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 of which drilling chips and dust were collected. DNA extractions were performed in a dedicated ancient DNA facility (LAF, Leiden), where no work on Rhinocerotidae was previously performed and 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 GuanidineThioCyanate (GuSCN) protocol described by Rohland and Hofreiter (2007). Final extraction volume was 40 μ l and DNA extraction and PCR blanks were included to monitor for contamination.

Based on 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 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 (396 bp: 14613-F and 15008-R) followed by successively smaller fragments (186 bp: 14823-F and 15008-R; 74 bp: 14719-F and 14792-R), if PCRs would not succeed. PCR reactions were carried out on a PTC-200 thermocycler (MJ research) in 25 μ l volumes, using 2.5 μ l genomic DNA extract, 0.4 μ M of each primer, 1.5 mM MgCl₂ (in buffer), 0.2 mM dNTPs and 0.5 μ l Phire™ Hot Start DNA Polymerase (Finnzymes). The PCR thermal cycling profile started with 5 min. initial denaturation at 98°C, followed by 40 cycles of 5 sec. denaturation at 98°C, 20 sec. annealing at 60°C, 30 sec. extension at 72°C and a final extension of 1 min. 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 A. and Poinair H.N., 2000) and genuine sequence data. Because the main objective of this study was identification of the 1865 specimen and *Cyt B* sequences of the Javan and Indian rhinoceros are sufficiently divergent (genetic distances are 8.6%, 10.9% and 15.4% for the 396, 186 and 74 bp fragments respectively; also see Tougard *et al.*, 2001 and 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 only the smallest PCR product (74 bp) was cloned using a TOPO TA Clonig kit (Invitrogen) following the manufacturers instructions, albeit using only half of the prescribed reaction volumes. Colony PCRs were performed with the same primers (14719-F and 14792-R) and with 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 PCR primers) on an ABI3730XL. The obtained forward and reverse sequences were assembled using Sequencher v.4.10.1 (GeneCodes Cooperation), checked for indels and stopcodons, and subsequently submitted to GenBank.

Figure 2. Schematic overview of a) *Rhinoceros unicornis*, b) *Rhinoceros sondaicus* (both from Sody, 1959; used with permission) and a photo of c) the Dublin rhinoceros (†1865) as currently housed in the Trinity museum.

To display the juvenile Dublin rhinoceros as detailed as possible it is depicted roughly the same size as the Javan rhino. The relative size of *R. unicornis* compared to *R. sondaicus* is after Sody (1959).



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

Primer name	Sequence (5'→ 3')
14613-F	ATTACAAATCTCCTCTCAGCCATC
14719-F	TCCACTTCATCCTTCCCTTTATTA
14792-R	GGATCCTGTTTCGTGTAGGAATAG
14823-F	GACAAAATTCCATTTACACCCTTAC
15008-R	AGCAAATAGGAAATACCATTCTGG
M13/pUC-F	GCCAGGGTTTTCCCAGTCACGA
M13/pUC-R	GAGCGGATAACAATTTACACAGG

RESULTS

The initial PCRs for the 396 bp fragment only worked for the right front toe sample. The amplification of the 187 bp 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 (74 bp) 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 same *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 very 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 for the Great Indian. On the other hand the skin does not have the reticulate appearance typical for *R. sondaicus* either. 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 since this specimen appears to be a juvenile. Compared to the rest of the body the head seems relatively small, also rather untypical for *R. unicornis*. The



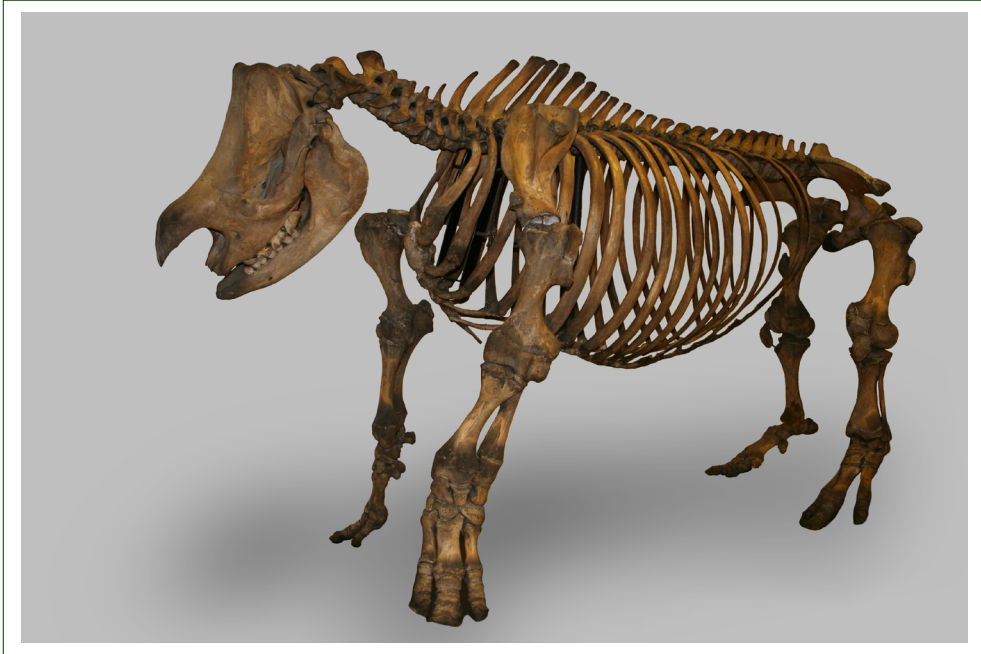



Figure 3. Mounted skeleton most likely belonging to the Dublin rhinoceros (+1865).

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 originate 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; Lulueza-Fox *et al.*, 2009), 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.



EUROPEAN COMMUNITY

ORIGINAL	1. Holder		CERTIFICATE		No			
	Name		Trinity College Dublin		ECR 022/10			
	Address		Zoology Department		Not for use outside the European Community			
			Trinity College Dublin		Council Regulation (EC) No 338/97 and Commission Regulation (EC) No 865/2006 on the protection of species of wild fauna and flora by regulating trade therein			
			Dublin 2					
			Ireland					
	2. Authorised location for live specimens of Annex A species		N/A		3. Issuing Management Authority Irish CITES Management Authority National Parks & Wildlife Service Department of Environment, Heritage & Local Government 7 Ely Place Dublin 2 Ireland e-mail: CITES@environ.ie			
	4. Description of specimens (incl. marks, sex/date of birth for live animals)		SKP Small sample piece of Rhinoceros skin taken from circa 1860's taxidermy specimen, being sent to the Netherlands for DNA analysis to confirm species type.		5. Net mass (kg) N/A		6. Quantity 1	
					7. CITES Appendix I		8. EC Annex A	
					9. Source W		10. Country Of Origin Unknown	
				11. Permit No N/A		12. Date of issue N/A		
				13. Member State of import N/A		14. Document No N/A		
				15. Date of issue N/A				
16. Scientific name of species		<i>Rhinoceros unicornis</i>		17. Common name of species		Great Indian Rhinoceros		
18. It is hereby certified that the specimens described above:								
<input type="checkbox"/> were taken from the wild in accordance with the legislation in force in the issuing Member State <input type="checkbox"/> are abandoned or escaped specimens that were recovered in accordance with the legislation in force in the issuing Member State <input type="checkbox"/> are captive born-and-bred or artificially propagated specimens <input type="checkbox"/> were acquired in or introduced into the Community in compliance with the provisions of Council Regulation (EC) No 338/97 <input type="checkbox"/> were acquired in or introduced into the Community before 1 June 1997 in accordance with Council Regulation (EEC) No 3626/82 <input type="checkbox"/> were acquired in or introduced into the Community before 1 January 1984 in compliance with the provisions of CITES <input checked="" type="checkbox"/> were acquired in or introduced into the issuing Member State before the provisions of Regulations (EC) No 338/97 or (EEC) No 3626/82 or of CITES became applicable in this territory <input type="checkbox"/> are to be used for the advancement of science/breeding or propagation/research or education or other non-detrimental purposes								
19. This document is issued for the purpose of:								
<input type="checkbox"/> confirming that a specimen to be (re-) exported has been acquired in accordance with the legislation in force on the protection of the species in question <input checked="" type="checkbox"/> exempting Annex A specimens from the prohibitions relating to commercial activities listed in Article 8.1 of Regulation (EC) No 338/97 <input type="checkbox"/> authorising the movement within the Community of a live Annex A specimen from the location indicated in the import permit or in any certificate								
20. Special Conditions <u>Transaction Specific Certificate</u>								
This Certificate is valid solely for the purposes of one transaction. After this sample has been analysed in the Netherlands this Certificate will no longer be valid. (See section 4 above).								
<input type="checkbox"/> certificate valid only for holder named in box 1 (issued under Article 48(1) (d) of Regulation (EC) No 865/2006)								
Kevin Cahillane		Dublin, Ireland.						
Name of issuing official		Place and date 10 March 2010				Signature and stamp		

Ataset Ltd. - 38770-002



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, 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.

ACKNOWLEDGEMENTS

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Figure 4. CITES certificate which allowed the exportation of samples from the Dublin Zoo rhinoceros (+1865) from Ireland to the Netherlands for genetic analysis.



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