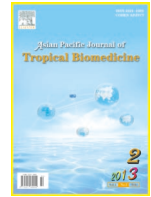




Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb



Document heading

doi:10.1016/S2221-1691(13)60031-3

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Blood meal analysis of tabanid fly after it biting the rare Sumatran rhinoceros

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PEER REVIEW

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Comments

I strongly support this paper to be published as it has improved the field method by cleverly using molecular genetics via tabanids feeding on host species of rhinos. It has greater use to other species of wildlife in the thick rain forest.

(Details on Page 99)

ABSTRACT

Objective: To demonstrate a noninvasive large mammalian genetic sampling method using blood meal obtained from a tabanid fly. **Methods:** Blood meal was recovered from the abdomen of an engorged tabanid fly (*Haematopota* sp.) which was captured immediately after biting a Sumatran rhino in captivity. The blood was applied on to a Whatman FTA[®] blood card. Subsequent laboratory work was conducted to extract, amplify and sequence the DNA from the sample. Validation was done by sampling the hair follicles and blood samples from the rhinoceros and subjecting it to the same laboratory process. **Results:** BLAST search and constructed phylogenetic trees confirmed the blood meal samples were indeed from the rhino. **Conclusions:** This method could be used in the field application to noninvasively collect genetic samples. Collection of tabanids and other haematophagous arthropods (*e.g.* mosquitoes and ticks) and other blood-sucking parasites (*e.g.* leeches and worms) could also provide information on vector-borne diseases.

KEYWORDS

Noninvasive DNA sampling, Blood meal, Tabanid fly, Sumatran rhino

1. Introduction

Collecting genetic material from free-ranging, elusive and rare animal species that live in remote tropical rainforests is difficult or even impossible. Coupled with the dense nature of the rainforest, even spotting an individual in the wild is almost impossible, making the acquisition of DNA samples almost extremely difficult and in practice

prohibitively expensive in terms of manpower, money and time mosquitoes and ticks. Even collection of faecal samples has proven almost impossible, under conditions of frequent rain on remote, extensive forested hills. However, plenty of studies have demonstrated the usefulness of the noninvasive genetic sampling in the research on free-ranging and elusive animals through the ability to use low quantity (and sometimes quality) of shed genetic materials

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Foundation Project: This work was financially supported by the Sime Darby

Foundation (Grant code: P23 071000490001).

Article history:

Received 8 Dec 2012

Received in revised form 18 Dec, 2nd revised form 25 Dec 2012, 3rd revised form 3 Jan 2013

Accepted 20 Jan 2013

Available online 28 Feb 2013

with the advent of polymerase chain reaction (PCR)–based technology^[1–5]. This advancement had truly escalated the field of conservation biology particularly when dealing with rare and elusive, endangered, or cryptic fauna^[1–3,5–8].

Noninvasive sampling is described as a method by which scientists gather the genetic materials shed by animals through sources that can be collected without having to catch or disturb the animal^[9]. Among the types of samples that may contain genetic materials are dung, shed hairs and feathers, urine, snake skins, sloughed skin, eggshells and even skulls in owl pellets^[9,10]. However, blood meal collected from haematophagous insects have not yet been listed in public domain as a potential practical source for noninvasive samplings.

Analysis of blood meals of haematophagous insects (*e.g.* mosquitoes, tsetse flies, and sand flies) has been widely used to determine their main hosts, host preferences and feeding patterns, but very little research has been conducted on blood meal from horseflies of the Family Tabanidae^[11]. In addition, limited or no studies have been done to look at the effects of tabanids bites on wildlife species.

The genus *Haematopota* (Insecta: Tabanidae) consists of a growing number of species, females of which suck bloods from mammalian hosts. Although it is a common froup livestock attacking flies, members of this genus still poorly understood. A large number of specimens are available in various Southeast Asian museums but lacking reliable taxonomic work that allows specific identification.

In this study, we demonstrate the use of a noninvasive technique to sample genetic material from the critically endangered Sumatran rhinoceros (*Dicerorhinus sumatrensis*) (*D. sumatrensis*) by collecting blood meals from a tabanid horsefly (*Haematopota* sp.) which was captured immediately after biting and feeding on a captive individual Sumatran rhino.

2. Materials and methods

2.1. Study site and sample collection

This work was conducted at the rhino captive facility managed by the Borneo Rhino Alliance (BORA) in Tabin Wildlife Reserve, Lahad Datu District, Sabah. Two captive Sumatran rhinos were housed at the facility (Table 1). Flies were collected from around the vicinity and identified to the genus level (Figure 1). Blood samples were withdrawn from the abdomen of blood-engorged flies and applied on to a FTA[®] Blood Card (Whatman) (Figure 2). For the purpose of validation, hair follicles and blood samples were taken from both rhinos and similar laboratory processes were conducted for all sample types. The hair follicles were preserved in 95% ethanol while the blood spots were left to air-dry and later stored at room temperature.

2.2. Laboratory analysis

The DNA extraction, PCR amplifications and sequencing reaction were conducted on the blood meals from the fly, the hair follicles, and blood samples from both rhinos. DNA extraction from the blood cards was carried out by incorporating the protocol for dried blood spots (for both blood meals from the flies and blood samples from the rhinos) while the hair follicles were extracted using the forensic case work samples (hair roots) protocol implemented in the QIAamp[®] DNA Micro Kit (Qiagen).

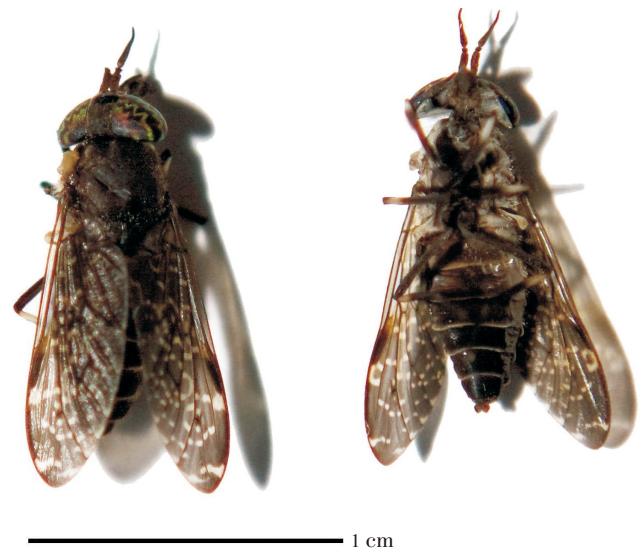


Figure 1. The tabanid flies collected (*Haematopota* sp.) (Photo by Zainal Zahari Zainuddin).

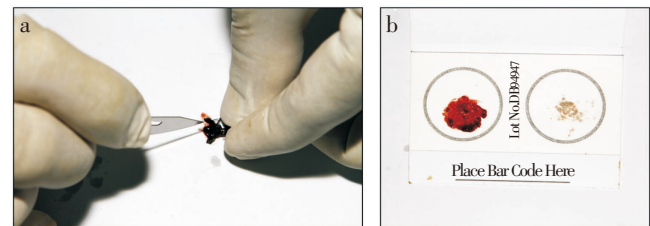


Figure 2. Collecting the blood meal from the *Haematopota* sp. (a) Extracting the blood meal from the fly's abdomen. (b) Spreading of the blood meal onto the blood card (Photos by Zainal Zahari Zainuddin).

Two primer sets were used to amplify the cytochrome b (*cytb*) and the control region (CR) of the mitochondrial DNA (mtDNA) gene. The *cytb* primer set are known as, GluDG–L, 5'–TGA CTT GAA RAA CCA YCG TT–3' and CB2–H, 5'–CCC TCA GAA TGA TAT TTG TCC TCA–3'^[12], while the CR primer set are known as L15 926, 5'–TAC ACT GGT CTT GTA–3', and H00 651, 5'–AAG GCT AGG ACC AAA CCT–3'^[13]. The PCR amplifications (reaction mixtures and PCR profile) and sequencing reaction are described elsewhere^[14].

2.3. DNA characterization and phylogenetic analyses

Alignments of the sequences were done by using the program Geneious v5.5^[15]. Pair–wise distance analysis using the Kimura two–parameter model was done to estimate the genetic distances among the sequences as performed using MEGA version 5^[16,17]. Sequence characterization (variable sites, conserved sites and parsimony–informative sites) were also done using MEGA.

The sequences were later aligned with the Basic Local Alignment Search Tool (BLAST) program^[18] to match the query sequence with the available sequence database in GenBank which is produced and maintained by the National Center for Biotechnology Information (NCBI) (available at <http://www.ncbi.nlm.nih.gov/>). Depending on the availability of the sequences at the database, BLAST will provide the closest matching sequence to the sequence under query, hence, suggesting the species identification.

Phylogenetic tree was later constructed for both the *cytb* and CR segment by using the neighbour–joining (NJ) method implemented in MEGA. The NJ clustering was performed by using the Kimura 2–parameter distance model^[16] with

Table 1

Information of the Sumatran rhinoceros housed at the rhino captive centre and details of the other rhinoceros species and representatives of the Order Perissodactyla obtained from the GenBank.

No	Species	Sample abbr.	Common name	House name	Population	Studbook No./NCBI Acc. No.
1	<i>D. sumatrensis</i>	SR04	Sumatran rhino	Kretam	Sabah	N/A/JF290494, JQ281903
2	<i>D. sumatrensis</i>	SR05	Sumatran rhino	Gologob	Sabah	#40/JF290495, JQ281904
3	<i>D. sumatrensis</i>	NC012684	Sumatran rhino	Suci	Sumatra	#43/NC012684
4	<i>D. sumatrensis</i>	AJ245723	Sumatran rhino	N/A	Sumatra	AJ245723
5	<i>D. sumatrensis</i>	JF718875	Sumatran rhino	N/A	Sumatra	N/A/JF718875
6	<i>Coelodonta antiquitatis</i>	FJ905813	Woolly rhino			FJ905813
7	<i>Coelodonta antiquitatis</i>	NC012681	Woolly rhino			NC012681
8	<i>Ceratotherium simum</i>	NC001808	White rhino			NC001808
9	<i>Ceratotherium simum</i>	Y07726	White rhino			Y07726
10	<i>Diceros bicornis</i>	NC012682	Black rhino			NC012682
11	<i>Diceros bicornis</i>	FJ905814	Black rhino			FJ905814
12	<i>Rhinoceros sondaicus</i>	NC012683	Javan rhino			NC012683
13	<i>Rhinoceros sondaicus</i>	FJ905815	Javan rhino			FJ905815
14	<i>Rhinoceros unicornis</i>	X97336	Indian rhino			X97336
15	<i>Rhinoceros unicornis</i>	NC001779	Indian rhino			NC001779
16	<i>Tapirus indicus</i>	AF145734	Malayan tapir			AF145734
17	<i>Tapirus terrestris</i>	AJ428947	Brazilian tapir			AF056030
18	<i>Tapirus pinchaque</i>	GQ259955	Mountain tapir			GQ259957
19	<i>Equus asinus</i>	NC001788	Donkey			NC001788
20	<i>Equus caballus</i>	NC001640	Horse			DQ297663
21	<i>Equus przewalskii</i>	HQ439484	Przewalskii horse			DQ223534
22	<i>Equus hemionus</i>	NC016061	Plains zebra			DQ470805

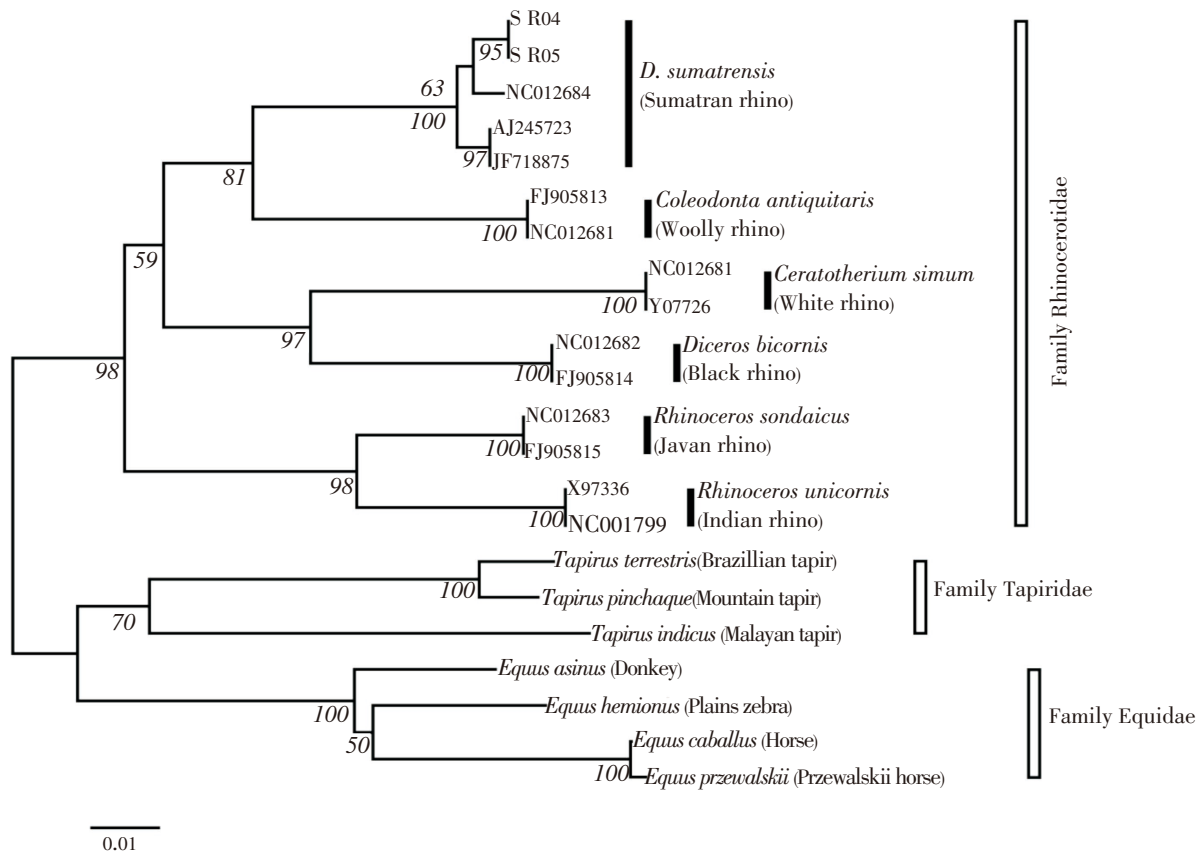


Figure 3. Phylogenetic tree (NJ method) constructed using the *cytb* gene segment.

pair-wise deletion option. Sequences of the *D. sumatrensis* and the representatives of the other species from the Order Perissodactyla were also obtained from the GenBank (Table 1).

Phylogenetic confidence was estimated by bootstrapping^[19] with 1000 replicate data sets.

3. Results

DNA fragments with the sizes of 446 bp and 500 bp from the *cytb* and CR respectively were successfully amplified and sequenced from all sample types. All sequences generated from this study were submitted to the GenBank and given the accession number JQ281903–JQ281904, JF290494–JF290495. Sequences from the different types of samples (blood meal, hair follicles, and host blood) from both rhinos produced identical DNA sequences.

In total, 19 variable sites with no parsimony-informative sites (Table 2) were observed when the rhino sequences

from both gene segments were aligned against the species *D. sumatrensis* from the NCBI database (Accession No.: NC012684). Genetic distances calculated between the rhinos from Sabah and Sumatra showed the value of 1.0–1.2% for the *cytb* gene segment while in the CR segment the value ranges from 2.7–3.1%. BLAST search matched the *cytb* segment with a 99.1% of similarity (both SR04 and SR05) while the sequences of the CR segments matched 97.2% (SR04) and 96.8% (SR05). Phylogenetic tree constructed using the NJ method (Figure 3) further confirmed that the blood meal samples were indeed from the rhino.

Table 2

Variable sites observed in the *cytb* and CR gene segment of the rhinos in the present study as compared to the *D. sumatrensis* from the GenBank. Bold nucleotides show the variable sites between the rhinos in the present study.

Gene	CYTB										CR									
Position	14220	14350	14530	14584	15475	15577	15582	15614	15617	15632	15658	15672	15685	15695	15718	15733	15774	15775	15841	15897
NC012684	G	C	C	T	–	A	A	A	A	A	G	A	T	T	T	A	A	T	C	C
SR04	A	T	T	A	A	G	G	G	G	G	A	G	C	C	C	G	A	C	T	C
SR05	A	T	T	A	A	G	G	G	G	G	A	G	C	C	C	G	C	C	T	T

4. Discussion

Our original interest in sampling Sumatran rhino blood was linked to our concern over the implications of a rigid belief in a perceived need to keep separate the different Sumatran rhino populations (on Borneo and Sumatra islands) based on classical divisions into sub-species. Phylogenetic tree constructed supports previous work where similarly, three sister-pairings were observed: (i) the black/white, (ii) the woolly/Sumatran, and (iii) the Javan/Indian. Furthermore, the tree showed a weak support (63%) for the structuring between the Sabah and Sumatra rhino populations suggesting the close relationship between them^[20]. Three subspecies of Sumatran rhinos are currently recognized based on morphology: (i) *D. sumatrensis* (Peninsular Malaysia and Sumatran population), *D. harrissoni* (Sabah population) and, *D. s. lasiotis* (unconfirmed reports from Burma, extinct in India and Bangladesh). With the current population number both in the wild and captivity showing continuous and alarming decline over the past few decades^[21,22], there is a dire need to boost the number of rhinos through population inter-mixing regardless of their subspecies designation.

Our interest and approach led to the findings reported here, which demonstrate the usefulness of using blood meals collected from tabanid flies in determining the identity of the host species, using PCR. A recent study conducted demonstrates the use of blood meals to identify host species through PCR^[23]. This approach is potentially important when working with rare, elusive and endangered species like the Sumatran rhino. A recent work describing a new screening tool using haematophagous leeches (*Haemadipsa* spp.) also demonstrated the effectiveness of using micropredators to assess mammalian biodiversity by DNA profiling^[5]. Due to the non-destructive nature of this method, we suggest that the sampling of blood meals from haematophagous micropredators should be considered as a targeted noninvasive sampling technique for Sumatran rhinos and other rare rainforest mammals. In our experience, tabanid

flies tend to be associated closely with their normal hosts (large wild mammals) in rainforests, but these flies do sometimes locate and bite humans who are seeking the animals in the forest. Thus, researchers and wildlife patrol and monitoring teams do have the opportunity to collect horsefly blood meals, if they are often out in the forest seeking signs of mammals such as Sumatran rhinos.

We conclude that this method could be used in the field application to noninvasively collect genetic samples especially on the rare, elusive and endangered species. Collection of haematophagous mosquitoes, ticks, leeches and worms from the wild could be conducted at sites frequented by animals including salt-licks, wallows, wildlife main trails or “highways” and river banks. Additionally, information on vector-borne diseases could also be obtained through analyses of the blood meals. Further research needs to be conducted, however, to evaluate and measure the longevity of a particular blood meal in order for positive detection to occur.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are grateful to the Department of Wildlife and National Parks (DWNP) of Peninsular Malaysia and the Sabah Wildlife Department for their support and for providing the research facilities and permits for this study. This work was financially supported by the Sime Darby Foundation (Grant code: P23 071000490001); years 2009–2015 grant to Borneo Rhino Sanctuary programme. We also thank MT Abdullah from the Universiti Malaysia Sarawak (UNIMAS) for critical comments, the Universiti Malaysia Sabah (UMS) and the Borneo Rhino Alliance (BORA) for providing travel assistance and accommodation for Rovie-Ryan JJ during the sampling work. Also many thanks to the staff of BORA for their assistance in sample acquisition.

Comments

Background

Noninvasive Genetic Sampling on the Rare Sumatran Rhinoceros (*Dicerorhinus sumatrensis*): Identification of the Host Species from the Blood Meal Collected from the tabanid fly (Tabanidae: *Haematopota* sp.).

Sampling of the rare and endangered Sumatran rhinoceros is a very costly and difficult task in the thick tropical rain forest in Borneo, and the normal method of indirect observation methods can only provide rough estimate of the population size.

Research frontiers

This noninvasive genetic sampling using specific molecular marker method is novel and innovative and could solve the problem of sampling of the illusive rhinos and other wildlife in the thick Bornean rain forest.

Related reports

Molecular methods had been used to identify species and species boundary. But the use of tabanids as a source of DNA for wildlife species has never been used in Malaysia. There was a similar attempt to use leeches to sample mammals in Vietnam.

Innovations and breakthroughs

The noninvasive genetic sampling using specific molecular marker method is novel and innovative.

Applications

Having high potentials for noninvasive genetic sampling of other animals such as tigers, elephants, wild gaur, wild pigs, serow and tapirs.

Peer review

I strongly support this paper to be published as it has improved the field method by cleverly using molecular genetics via tabanids feeding on host species of rhinos. It has greater use to other species of wildlife in the thick rain forest.

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