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TECHNICAL REPORT

Species Identification, Molecular Sexing and Genotyping Using Non-invasive Approaches in Two Wild Bovids Species: *Bos gaurus* and *Bos javanicus*

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Since the second Indochina war, habitat destruction and overhunting has resulted in fragmentation of the remaining populations of *Bos javanicus* and *B. gaurus*. Nowadays, both species are in serious danger, especially the gaur. In Vietnam, where these species have become almost impossible to capture in the wild, non-invasive investigations are the only feasible approach to obtain data on populations. However, non-invasive derived DNA, especially in tropical areas, is usually characterized by low concentrations, poor quality and/or contamination from alien DNA. To assist in tropical conservation management, baseline information is provided here on assessing the reliability of species identification, molecular sexing and microsatellite genotyping using fecal DNA from *B. gaurus* and *B. javanicus*. For species identification using bovine fecal samples, cytochrome *b* fragment between positions 867 and 1140 was found to contain species diagnostic sites, which distinguishes the four species encountered in the region: *B. gaurus, B. indicus, B. javanicus* and *B. taurus*. For sex determination, primers were initially tested on DNA obtained from blood. Then, these primers were successfully used on DNA derived from fecal material. Finally, we also evaluate the feasibility of non-invasive microsatellite genotyping on fecal samples collected in Vietnamese nature reserves. The results presented here improve on current molecular methods based on fecal material obtained from tropical areas. Zoo Biol 0:1–10, 2008. © 2008 Wiley-Liss, Inc.

Keywords: fecal sample preservation procedures; fecal DNA; cytochrome *b*; molecular sexing

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INTRODUCTION

Within the tribe Bovini, *Bos javanicus* (banteng) and *B. gaurus* (gaur) are listed as endangered critical with less than 500 gaurs believed to be present in Vietnam: a number that is declining [Hedges, 2000a]. Current estimates for banteng are even lower, hardly reaching 200 animals [Hedges, 2000b]. These wild bovid species are very difficult to approach in nature reserves; owing to various environmental and logistical reasons making accurate estimates of species numbers and other information such as sex ratios difficult to obtain. In such a framework, the use of indirect non-invasive approaches can be of great help. In particular, the opportunity to obtain DNA from hairs and feces represents an extremely valuable and powerful tool [Higuchi et al., 1988; Taberlet and Luikart, 1999]. Nowadays, the inclusion of genetic data has become critical to any survey and conservation plan as it allows the accurate identification of species, the sex ratio in populations as well as assessments of individual membership, population size, levels of inbreeding and genetic health of populations through genotyping [Creel et al., 2003; Eggert et al., 2003; Okello et al., 2005]. Unfortunately, these kinds of data are not easily obtained as the quality and quantity of DNA extracted from non-invasive sources (such as hair or dung) are generally poor. In addition, the risk of DNA contamination, allelic drop-out or scoring of false alleles is high [Taberlet et al., 1996; Fernando et al., 2003; Bonin et al., 2004]. This risk can be minimized through considerable repetition of extractions and polymerase chain reaction amplifications, and so high number of replicates are necessary to assess genotypes with certainty [Taberlet et al., 1996, 1999; but see Miller et al., 2002 for an alternative approach using maximum likelihood].

Limitations to the use of fecal-extracted DNA for genotyping are influenced by the species and the environmental conditions in which these feces material are collected and preserved (including temperature, UV radiation and humidity). Moreover, in tropical environmental conditions the use of such methods is particularly problematic as cells are much more exposed to degenerative processes [Wehausen et al., 2004]. In the case of herbivores, the use of fecal DNA poses further problems owing to the inhibitory effects that some plant secondary compounds exert on PCR polymerases. As such, the success of PCR amplifications is highly dependent on the maximization of intact target DNA yield with the simultaneous minimization of inhibitors [Fernando et al., 2003]. In the particular case of ruminant species with compact feces pellets, the DNA extraction is easier than DNA extracted from non-pelleted feces because it can be optimized by using mucosal intestinal cells from the pellets' surface [Flagstad et al., 1999]. In summary, many factors influence the success of fecal DNA genotyping, such as the species, diet [e.g. Murphy et al., 2003], environmental processes, sample storage methodology [e.g. Nsubuga et al., 2004], as well as DNA extraction and PCR amplification methods per se [e.g. Piggott et al., 2004].

In an attempt to ensure the successful outcome of a conservation project involving wild Vietnamese bovids, we especially address the constraints of obtaining DNA data derived from non-invasive sources (fecal DNA) under tropical environmental conditions. In addition, we tested bovine microsatellites for cross species amplification between two co-occuring wild species (*B. gaurus* and *B. javanicus*) and domestic cattle (*B. taurus*) whose feces are easily confused in the field. We first applied our protocols to fecal samples of known captive animals, and

then applied them to gaur and banteng fecal samples collected in Vietnam. Our results indicate the suitability of our protocols for species identification as well as simple and rapid individual sexing.

MATERIALS AND METHODS

Sample Collection

Biological samples from captive animals

Blood, hair and fresh fecal samples were obtained from 12 specimens: *B. gaurus* (2), *B. taurus* (1) and *B. javanicus* (9). Materials were kindly provided by the Natural History Museum (MNHN Paris, France), the CIRAD (Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Montpellier, France) and the Lizieux CERZA zoo (France). In addition, feces and hair samples of the only *B. gaurus* specimen housed in captivity in Vietnam were kindly provided by the Saigon Zoo.

Biological samples from wild animals

Thirty-five fecal samples were collected in Vietnam from the Phu Dong and Bu Gia Map Parks, Yok Don National Park, Nam Nung Nature Reserve, Easo Nature Reserve and the Phong Nha-Ke Bang National Park in 2006. Fecal samples were collected from the wild without observing the animals that deposit them (i.e. no visual confirmation of target species); however, the only bovid species that occur in the region from which these fecal pellets could have originated is *B. taurus*, *B. indicus*, *B. gaurus* and/or *B. javanicus*. The location of each sample was geo-referenced using a Global Positioning System unit.

Fecal samples preservation procedure

As collection and storage protocols can significantly influence the yield and quality of DNA, several procedures were evaluated and compared (described below). PCR results obtained from DNA extracted from blood or hair from the same individual (all samples for this part of the study were taken from captive *B. gaurus, B. javanicus* and *B. taurus*) were used as reference. To our knowledge, a single *B. gaurus* individual is currently held in captivity in the Saigon Zoological Garden (Vietnam) and this individual was therefore our only source of confirmed biological material for this specific species from Vietnam. A fresh fecal sample was divided into six aliquots and then used for DNA extraction. The feces was preserved on ice until laboratory analyses 2 days later. We focused on three preservation factors including (i) temperature: fecal samples kept at ambient temperature (35°C from April to November) or chilled at 4°C, (ii) time storage: number of days (i.e. 2, 10, 20, 30 days) earlier to DNA extraction, and (iii) preservation solution (dried, in pure ethanol or in RNAlater,™ Qiagen, France). The six treatments evaluated here are outlined in Table 1. The first three procedures, all at ambient temperature (35°C), were (i) an aliquot (500 g stored in a 50 mL Falcon tube) of freshly collected feces without preservation solution, (ii) an aliquot was transferred to a 50 mL Falcon tube that was then completely filled with ethanol (100%), (iii) and thirdly, an aliquot was stored in a 50 mL Falcon tube that was then completely filled with RNAlater™. Procedures (iv)–(vi) were replicates of procedures (i)–(iii) except that the storage temperature

TABLE 1. PCR replicating success using fecal DNA (gaur; Saigon zoo) stored under six conditions

Fecal storage conditions	Temperature (Hanoi)	Storage solution	Number of days[a]	Positive PCR[b]
1	Ambient (35°C)	–[c]	2, 10, 20, 30	4-0-0-0
2	Ambient (35°C)	Ethanol (100%)	2, 10, 20, 30	4-3-3-2
3	Ambient (35°C)	RNAlater™	2, 10, 20, 30	3-3-2-2
4	4°C	–	2, 10, 20, 30	4-2-4-3
5	4°C	Ethanol (100%)	2, 10, 20, 30	4-3-3-4
6	4°C	RNAlater™	2, 10, 20, 30	3-4-3-3

[a]Number of storage days before DNA extraction.
[b]PCR was considered positive if the expected cyt *b* amplicon was detected on an agarose gel. Numbers of positive PCR obtain for a total of 96 PCR reactions as each condition (2, 10, 20 and 30 days) was tested twice by two independent persons.
[c]Aliquot of feces without preservation solution.

was 4°C. These six aliquots were then used for DNA extractions and genotyping as described below.

DNA Extraction From Blood, Hair and Feces

DNA was extracted from blood samples using the Qiagen Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. DNA was extracted from hair using a phenol/chloroform extraction protocol [Foran et al., 1997]. Fecal DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) with some modifications outlined below. DNA extractions on the six fecal aliquots were carried out in duplicates and were performed 2 days, 10 days, 20 days and 30 days collection. Two replicate extractions for each sample and method were done. While using 200 mg of fecal material for the QIAamp DNA Stool Mini Kit, we used approximately 500 mg of fecal material in 1.5 mL of ASL buffer (QIAamp DNA Stool Mini Kit). If samples were preserved in solutions (ethanol or RNAlater™), several centrifugation steps had to be performed (7,650 rcf). The supernatant was discarded until the final maximum weight reached 500 mg. The Qiagen kit manual was then followed with three alterations: (1) after addition of cold ethanol, samples were left on ice for 15 min to maximize DNA precipitation; (2) each sample was extracted twice, then eluted from a single column (approximately a total amount of 1,000 mg of fecal material per DNA extraction); (3) to elute DNA, the buffer AE (QIAamp DNA Stool Mini Kit) was preheated to 70°C and incubated (150 μL) in the spin column for 3 min (QIAamp DNA Stool Mini Kit). Ten microliter of each DNA extract was electrophoresed 3% (wt/vol) agarose gel (1 × TAE with ethidium–bromide at 10 mg/mL in the running buffer). The ethidium–bromide-stained DNA band was visualized under UV light and the DNA extraction success was evaluated. However, the success of DNA extraction from herbivores feces cannot be evaluated by direct DNA measurements (because alien DNA is frequently present). Rather, extraction success is indicated by PCR success, as alien DNA (such as protozoan or plant DNA) will not amplify with bovid

specific primers. As a consequence, 2.5–5 μL of each DNA extract (20–50 ng of DNA) was used in PCR reactions. PCRs were considered positive if the expected amplicon was detected on the agarose gel. Each PCR was run twice by two independent people, leading to four PCRs for each experimental listed in see Table 1.

Species Assessment of the Samples Collected in the Field

To select a sequence of the cytochrome *b* (cyt *b*) that (1) was variable enough between the four species *Bos gaurus*, *B. indicus*, *B. javanicus* and *B. taurus* to allow species identification, (2) that is flanked by conserved zone, and (3) a fragment short enough to be amplify using fecal-degraded DNA, we aligned Genbank sequences from the four species. Coupled with phylogenetic analyses, we observed that the fragment between positions 867 and 1,140 of the cyt *b* gene contains species-specific diagnostic sites, which allowed us to distinguish the four species unambiguously. DNA amplification of this 274 base pair (bp) sequence was performed using the following primers: L15612: 5′ -CGATCAATYCCYAAYAAACTAGG-3′ and H15915 (reverse): -TCTCCATTTCTGGTTTACAAGAC-3′ [Hassanin et al., 2006]. The cyt *b* PCRs on fecal DNA were run under the following conditions: 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl2), 0.2 mM of each dNTP, 1 μM of each primer, 0.5 units of AmpliTaq Gold™ (Applied Biosystems, Warrington, UK) and 5 μL of fecal DNA in a final reaction volume of 25 μL. PCR cycling parameters were as follows: 4 min at 95°C; 35 × 45 sec at 95°C, 90 sec at 52°C, 90 sec at 72°C with a final extension step of 10 min at 72°C. Sequencing of PCR products was carried out at Macrogen Inc. (Korea). The sequences obtained from blood and fecal DNA of the same individual of the zoo animals were aligned and the alignment optimized using *BioEdit* version 7.09 [Hall, 1999].

Molecular Sexing

To sex specimens using fecal DNA, we used the primers S4BF (5′-CAAGTGCTGCAGAGGATGTGGAG-3′) and S4BR (5′-GAGTGA-GATTTCTGGATCATATGGCTACT-3′) located in the bovine Y chromosome. Primers were defined by Kageyama et al. (2004) to sex *B. taurus* embryos and amplify two PCR products: a 178 bp long male-specific amplicon and another of 145 bp in length found in both sexes. To verify primers, we initially applied these primers to DNA extracted from blood of known individuals. As the PCR amplifications using blood-derived DNA and then from fecal-derived DNA of known individuals unambiguously confirmed the sex of each individual of the four species under study, we then applied the protocol to field collected feces. PCR reactions were similar to those described above except for the annealing step (45 sec at 52°C). Successful amplification was verified through electrophoresis in 3% agarose gels.

Genotyping

Suitability of *B. taurus* autosomal microsatellite markers for *B. gaurus* and *B. javanicus* was evaluated by 11 primers sets for the following *B. taurus* loci: TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225 and BM1824 [Bradley et al., 2004]. To reliably genotype each individual and avoid allelic dropout and false alleles, we typed all heterozygote

individuals twice and each homozygote at least three times for all loci. Heterozygote genotype had to appear in two of two replicates to be accepted and three of three replicates for homozygotes. The loci were selected from the StockMarks™ (Applied Biosystems, Warrington, UK) for cattle paternity Bovine II v. 2 PCR typing kit (PE Applied Biosystems). PCRs on blood DNA were run in duplicates whereas PCRs on fecal DNA were run in triplicates per locus and animal. When PCRs failed or yielded inconsistent results, four additional PCRs were run. Forward primers were 5′ labelled with D2, D3 or D4 fluorescent dye (Applied Biosystems). The PCR mixture (final volume of 25 μL) contained 1 × PCR buffer (see above), 0.2 mM of each dNTP, 1 μM of each primer and 0.5 units of Hot Start Taq polymerase (Fermentas) and 20–50 ng of DNA. After a first denaturation step at 95°C for 4 min, samples were subjected to 35 cycles of 45 sec at 95°C, 90 sec at 55°C and 90 sec at 72°C. PCR was terminated at 10 min at 72°C. Fragment length was assessed by the CEQ 8000 sequencer (Beckman-Coulter, Amersham) with the CEQ 400 bp DNA size standards (Beckman Coulter Inc.), including the Seq™ 8000 genetic analysis system software (Beckman Coulter).

RESULTS

DNA Extraction Procedure

Concerning fecal samples that had been kept at ambient temperature (25–35°C) and from which DNA was extracted 2 days later, the highest proportion of positive PCRs for cyt *b* was obtained using feces preserved either in ethanol or RNAlater™ (Table 1). At ambient temperature, after 10 days, we were unable to successfully amplify the cyt *b* gene from unpreserved fecal samples (Table 1). The proportion of scored amplifications was significantly higher for DNA extracted from fresh fecal samples stored immediately at 4°C. It reached 100%, even after 30 days of storage. No difference was noted between ethanol and RNAlater™ preservation procedures. Therefore, we recommend the use of ethanol preservation procedure because it is widely accessible and far more economical.

Species Identification and Molecular Sexing

Another aim of our study was to develop a simple species identification protocol based on noninvasive DNA. DNA was extracted from blood (as reference) and feces from the same individual. A 274 bp long DNA fragment was amplified, sequenced and subsequently aligned with known sequences. A multiple nucleotide sequences alignment of the three species under study included seven references from the Genbank database (*B. taurus AY676873 and AY676872; B. indicus AF419237 and AF531473*; *B. gaurus DQ319905; B. javanicus AY689188 and BOVMTCBB*) (Fig. 1) shows that species could be identified without ambiguity, as paired blood and fecal fragments yielded identical and species-specific sequences. All new sequences have been submitted to Genbank under the accession numbers EU255781 (*B. taurus*), EU255782 (*B. gaurus*) and EU255783 (*B. javanicus*).

The primers defined by Kageyama et al. (2004) were applied to 12 known individuals representing the four species and consistently yielded accurate results in the molecular sexing of fresh fecal samples. However, Kageyama et al. (2004) pointed out that the 145 bp fragment was derivation-unknown. It seems to be located

Species	Accession number*	Sequences
Bos taurus	*AY676873*	CCCAACAAACTAGGAGGAGTACTAGCCCTAGCCTTCTCTATCCTAATTCTTGCTCTAATCCCCCTACTACACACCTCCAAACAACGAAGCATAATATTCC
	B1 *EU255781*	..T..T.....
	F1 *EU255781*	..T..T.....
Bos indicus	*AF419237*	.....
Bos gaurus	*DQ319905*	.....G.....T.....C..CAT..T.....T.....
	B2/F2 *EU255782*	..T..T.....G.....T.....C..CAT..T.....T.....
Bos javanicus	*AY689188*	.....T.....C..C..C..C..T.....
	B3/F3 *EU255783*	..T..T.....T.....C..C..C..C..T.....
	B4/F4 *EU255783*	..T..T.....T.....C..C..C..C..T.....
(continued)		(continued)
Bos taurus	*AY676873*	GACCACTCAGCCAATGCCTATTCTGAGCCCTAGTAGCAGACCTACTGACACTCACATGAATTGGAGGACAACCAGTCGAACACCCATATATCACCATCGG
	B1 *EU255781*	.....
	F1 *EU255781*	.....
Bos indicus	*AF419237*	.....T..
Bos gaurus	*DQ319905*	.....T.....T...A.T.....A.....T.....T.....T..
	B2/F2 *EU255782*	.....T.....T...A.T.....A.....T.....T.....T..
Bos javanicus	*AY689188*	.....A.....T...ATTT..A.....T.....A.....C.....G.....T.....T..
	B3/F3 *EU255783*	.....A.....T...ATTT..A.....T.....A.....C.....G.....T.....T..
	B4/F4 *EU255783*	.....A.....T...ATTT..A.....T.....A.....C.....G.....T.....T..
(continued)		(continued)
Bos taurus	*AY676873*	ACAACTAGCATCTGTCCTATACTTTCTCCTCATCCTAGTGCTAATACCAACGGCCGGCACAATCGAAAACAAAT(-)TAATAT
	B1 *EU255781*	.....(-).....
	F1 *EU255781*	.....(-).....
Bos indicus	*AF419237*	.....A.....T.....A.....A.....G.T.....(-).....
Bos gaurus	*DQ319905*	.....CA.TA.....T.....A.....A.....G.....C(-)C....C
	B2/F2 *EU255782*	.....CA.TA.....T.....A.....A.....G.....C(-)C....C
Bos javanicus	*AY689188*	.....CA.TA.....T.....A.....A.....G.....T...C(-).....C
	B3/F3 *EU255783*	.....CA.TA.....T.....A.....A.....G.....T...C(-).....C
	B4/F4 *EU255783*	.....CA.TA.....T.....A.....A.....G.....T...C(-).....C

Fig. 1. Alignment of partial cytochrome *b* sequences for individuals and species of the genus *Bos* indicating nucleotide variability. *The sequence of *B. gaurus*, *B. javanicus, B. taurus* obtained in this study are available on GenBank under the accession numbers listed below. Letters indicate source of DNA: B, blood; F, feces. Numbers indicate individual; (-) indicate long stretches of conserve sequences and letters indicate nucleotide variation in the alignment.

on the X chromosome or duplicates, which might be dispersed throughout the bovine genome. For this reason we first tested these primers on DNA extracted from blood for the four species because duplicates or pseudogenes may perform differently in different species (an electrophoresis picture is available on request). Importantly, no multiple bands on the PCR electrophoresis gel were observed in the four species, thus strongly suggesting that no duplicated copies of the Y-linked S4BF loci was amplified [Kageyama et al. 2004], although sometimes up to six replicates were necessary for the driest samples.

Microsatellites Genotyping

Our experiments were mainly focussed on the amplification success of microsatellites on fecal DNA collected in tropical areas rather than on genetic diversity per se. To establish the true genotype, all loci were typed twice for each individual using DNA from blood. Alleles were subsequently compared with fecal DNA amplification. As expected, the proportion of amplifications was higher for blood and hair-derived DNA, compared with that scored from fecal DNA especially for material collected in the wild, which was generally old and dry feces. It is important to highlight the fact that up to five PCR reactions were sometimes needed to obtain a product from fecal samples. PCR amplifications were much more successful using the AmpliTaq Gold™ (Applied Biosystems)

compared with the standard Taq (Fermentas) polymerase. The 11 bovine primers yielded alleles for both *B. javanicus* and *B. gaurus* species (available on request).

DISCUSSION

DNA Extraction and Amplification Procedure

Using DNA extracted from non-pellet herbivore feces, the main difficulties included low-quantity DNA template or/and DNA degradation owing to degenerative environmental processes [Wehausen et al., 2004]. Tropical climatic conditions encountered in Vietnam such as high temperature, humidity and a high UV radiation must contribute strongly to the degradation of cellular DNA. The aim of this study was to evaluate the use of non-invasive DNA sources as DNA derived from feces or hairs that can be collected at feeding points where hair catchers could be installed. Under some field conditions, as those encountered in Vietnam, non-invasive approaches are the only way to perform molecular analyses on wild bovid species if the study is to be completed in a reasonable timeframe. We found that storage temperature and preservation methods had a strong effect on DNA extraction success and DNA amplification rate as demonstrated by previous studies [Goossens et al., 2000; Huber et al., 2003]. Clearly, our results show that optimal PCR amplification rates are obtained with fresh feces immediately preserved in pure ethanol. RNAlater™ does not seem to improve the outcome compared with the results obtained with ethanol. In addition, as expected, it is preferable to store these fecal samples at 4°C as soon as possible. This confirms that quantity and quality of DNA extracted from feces are greatly influenced by storage temperature especially when samples are collected in tropical areas. As an example, Maudet et al. [2004] showed that 99% of the genotyping repetitions provided the expected genotype using winter feces of *Ibex capra* species whereas spring samples provided only 52% of correct genotypes. However, in their study, even in spring, average temperatures were always much lower than the ones encountered in Vietnam.

Species Identification, Molecular Sexing and Microsatellites Genotyping

It has become almost impossible to observe and capture gaur or banteng in the wild in Vietnam. Species identification based solely on feces appearance is not feasible. Here, we consistently succeeded in sequencing a diagnostic DNA fragment even from degraded DNA, which allowed us to unambiguously identify *B. javanicus*, *B. gaurus* and *B. taurus*. This method is of great assistance for ecological surveys such as those conducted in Vietnam. Another important aspect of our study was to perform molecular sexing from DNA extracted from old feces. Because the fragments amplified are short enough to be obtained even on degraded fecal DNA, this simple protocol will provide an economic, quick and reliable method to determine the sex of the feces shedding animal. However, non-invasive approaches, as previously demonstrated by Taberlet and Luikart [1999], require repeated PCR amplification rounds, which appear to be the only approach to investigate wild bovid populations whose individuals are so difficult to locate. Finally, the data presented here (such as optimal sampling techniques, polymorphic

microsatellite loci and amplification conditions) provide the groundwork for further studies to investigate specific, and much needed population parameters for endangered Asian species.

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