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TECHNICAL NOTE

Rapid, field-based screening for chital (*Axis axis*) DNA in illegal meat markets

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Abstract The Bangladesh Sundarbans are a globally significant ecosystem for biodiversity conservation. However, illegal hunting has resulted in the extinction of a number of species and threatens the persistence of many others, including the chital deer (*Axis axis*). Wildlife officials are able to easily recognise chital hides by their distinctive markings, but the meat of this protected species is much harder to identify. Here, we describe a genetic method to detect chital DNA in meat products confiscated from market traders.

Keywords Chital · Spotted deer · *Axis axis* · Species identification · LAMP · Loop-mediated isothermal amplification · Strand displacement enzyme

Introduction

Tiger prey species such as chital are under threat from illegal hunting, primarily for their meat and pelts. Despite being protected under Bangladesh Wildlife Law, Forest Department officials commonly seize skins from poachers, and markets bordering protected areas are thought to trade chital meat. In order to assist wildlife officials in identifying these wildlife traders I have designed a screening tool to detect chital DNA in meat samples.

The test uses loop-mediated isothermal amplification (LAMP), first described by Notomi et al. (2000). LAMP uses a strand displacement enzyme and a set of 6 primers to amplify a target sequence. Using six primers to target one DNA fragment helps to increase the specificity of the reaction. Two outer primers (F3 and B3) initiate the reaction and two inner primers (FIP and BIP) facilitate the amplification (Fig. S1). Standard LAMP reactions typically take 60–90 min, but adding two loop primers (Loop F and Loop B) reduces this to 15–30 min (Nagamine et al. 2002; Chander et al. 2014). The LAMP amplicon can be detected by various methods including gel electrophoresis, turbidity, dye colour change or fluorescence (Zhou et al. 2014; Tanner et al. 2015). In this study I used a portable fluorescence detection device, Genie II (Optigene Ltd.), to monitor the LAMP reaction. The Genie II is lightweight, has a touchscreen interface, a battery life of 6–8 h, and displays fluorescence in real time. LAMP reactions can also be run with crude DNA extracts obtained by heating a tissue sample in lysis buffer to 95–100 °C for 10 min. An aliquot of the resultant supernatant can then be used directly in a LAMP reaction (Centeno-Cuadros et al. 2017).

Methods

The complete mitogenomes of chital and other mammal species were aligned using the MAUVE plugin (Darling et al. 2010) in Geneious vR9 (Biomatter Ltd.) to identify the most polymorphic regions for primer design (Table S1). Mitochondrial genes with a homology of <90% compared to the chital reference sequence were used as targets for primer design. Candidate primer sets were designed using LAMP Designer v1.14 (Premier Biosoft) and evaluated for specificity based on an NCBI BLAST search to shortlist

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primer sequences specific for *Axis axis*. Some primer sequences were then modified to include 3′ SNPs specific to chital to reduce the likelihood of non-specific binding (Wetton et al. 2004; Newton et al. 1989).

Table 1 The LAMP primers used to amplify a 292 bp Cytochrome b fragment in chital deer

Primer	Sequence
F3	CCC TTA CTA TAC CAT TAA AGA TAC
B3	TCG GAA TAT TAT GCT GCG TT
FIP (F1c + F2)	ATT GAG TGG GTT TGC TGG GGT AGC TAT TTG CAC CAG ATA
BIP (B1c + B2)	TGC ATA CGC AAT CCT ACG ATC AGG ATC AGG ATG GAT GAA GC
Loop F	TTG TCT GGG TCT CCA AGC ATA
Loop B	CTA GGA GGA GTC TTA GCC CTA

Table 2 Time to peak amplification and anneal temperatures for 11 chital reference samples

Sample	Time to peak amplification (min:s)	Anneal temperature (°C)
Axis1	19:15	82.6
SP1	19:45	82.4
SP2	18:15	82.3
SP3	18:15	82.2
SP4	17:45	82.4
SP5	17:45	82.4
SP6	18:45	82.3
SP7	18:00	82.3
SP8	–	82.3
SP9	21:15	82.5
SP10	20:30	82.6

Reactions were run on a Genie II using the six primers described in Table 1

Bst DNA Polymerase was the first strand displacement enzyme used for LAMP reactions. However, it is not thermostable above 70 °C, which precludes a denaturation step prior to amplification. Here, I evaluated three polymerases that are thermostable up to 94 °C and operate at optimum temperatures of 65–70 °C: OmniAmp (Lucigen), ISO-001/TIN (Optigene Ltd.), and SD polymerase (Bioron). Reactions were performed in 12.5 µl volumes with final primer concentrations of 0.2 µM F3, 0.1 µM B3, 1.6 µM Loop F, 0.8 µM Loop B, 1.6 µM FIP, and 1.6 µM BIP (Table S2). Reaction conditions were as follows: denaturation at 92 °C for 30 s, amplification at 66 °C for 28 min, then an annealing curve from 98 to 78 °C. Genie Explorer software (Optigene Ltd.) was used to confirm results with the following threshold values: normalisation period of 300 s, positive amplification scored as a fluorescence ratio above 0.01, and anneal peaks scored as a fluorescence derivative above 10,000 units. A total of one NADH five primer set and five Cytochrome b primer sets were tested for specificity using DNA from chital, sika deer, sambar deer, roe deer, red deer, muntjac, chinese water deer, rusa, fallow deer, barasingha, human, cow, chicken, sheep, goat, domestic dog, and domestic cat. Genomic DNA was extracted from buccal swabs or deer ear tips using the Qiagen DNA Investigator kit (Qiagen), PrepMan Ultra (Life Technologies), and MightyPrep (Takara Clontech). DNA extraction using PrepMan and MightyPrep involved hand mixing then heating the sample to 95 °C for 10 min.

Results and discussion

One primer set targeting a 292 bp Cytochrome b fragment was found to be the most specific for chital (Table 1). All three enzymes performed well, with SD polymerase producing the least amount of background amplification. Across 11 reference chital samples the average time to peak fluorescence was ~19 min with an average annealing

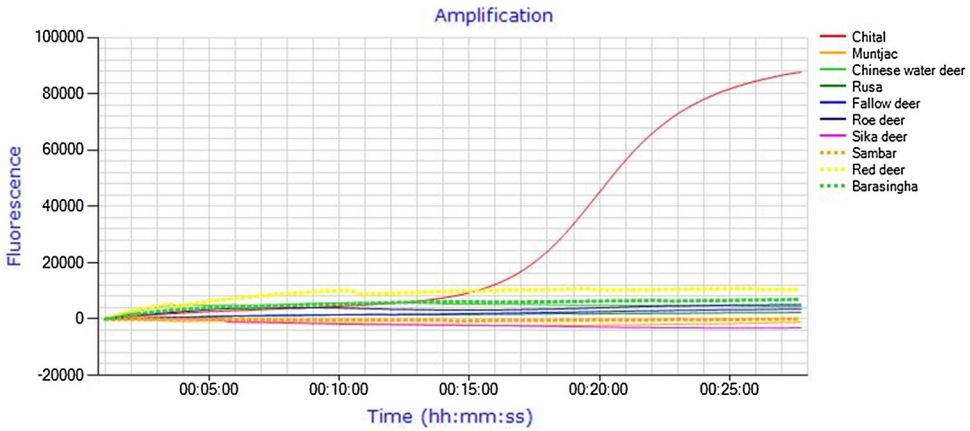

Fig. 1 Fluorescence curve showing the specificity of the Cytochrome b chital LAMP primers when tested against muntjac, chinese water deer, rusa, fallow deer, roe deer, sika deer, sambar deer, red deer and barasingha. Only the chital sample (well number 1) generated an increase in fluorescence

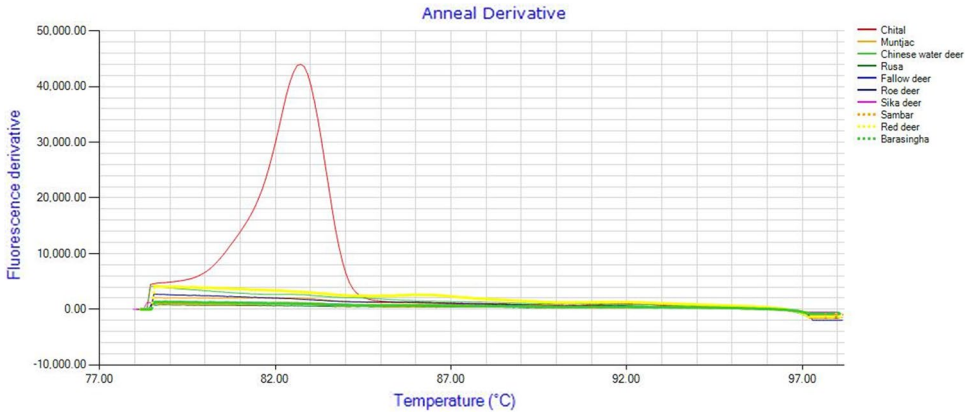

Fig. 2 Anneal curve confirming the presence of a LAMP amplification product for chital. A single amplification product with an annealing temperature of ~82.7 °C was recorded for the positive control (chital). No amplification products were detected for the other species tested (muntjac, chinese water deer, rusa, fallow deer, roe deer, sika deer, sambar deer, red deer and barasingha)

temperature of ~82.4 °C, though reaction times of up to 28 min were required to achieve the fluorescence ratio of >0.01 (Table 2). When tested on DNA samples from the non-target species listed above, the Cytochrome b primer set showed no cross-species amplification (Figs. 1, 2). Chital DNA could also be detected in the aliquots from the PrepMan and MightyPrep extractions, though the time to peak fluorescence was increased by ~5–10 min.

This LAMP assay is suited to field use as reactions can be monitored in real time with an annealing curve used to confirm the presence of amplification products, enzymes and reagents can be lyophilised to increase stability in tropical environments, and crude DNA extracts can be used. Most field applications can therefore be performed with only a Genie II, or similar machine, and a heat block. Though not tested in this study, LAMP is also effective with low quality samples such as faeces (Aikawa et al. 2015) and I have successfully amplified faecal DNA using primers designed for *Panthera tigris* (data not shown). However, the limitations of LAMP include the difficulty in designing suitable primer sets and the potential for inhibition with samples such as tanned hides, which require amplification with inhibition-resistant polymerases such as KAPA2G Robust (KAPA Biosystems) (O. Smith, unpublished data).

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