Veterinary Integrative Sciences 2022; 20(1): 41 - 48. DOI; 10.12982/VIS.2022.004

Vet Integr Sci

Veterinary Integrative Sciences

ISSN; 2629-9968 (online)

Website; www.vet.cmu.ac.th/cmvj

Case report

HRM species identification of bone samples collected from snake feces

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Abstract

Species identification is essential and necessary in the forensic sciences. This case study aims to identify animal species using unidentified bone samples found in snake feces with the use of inter-simple sequence repeat markers coupled with high resolution melting analysis (ISSR-HRM). In this case study, six ISSR primers were used and compared with lemur blood. The results of this study indicate that the derivative melting curve established from two bones and the lemur blood sample displayed a similar melting temperature. Additionally, D-loop sequencing of the bones and blood samples were checked against the GenBank database. We found that the samples belonged to a black-and-white ruffed lemur (*Varecia variegata*) with percent identity values of 99.54 and 99.85, respectively. Thus, ISSR-HRM has been effectively used for species identification, particularly when results can be compared with the target species.

Keywords: Animal, Classification, Forensic science, Melting temperature, PCR

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Article history; *received manuscript: 27 August 2021,*
revised manuscript: 15 September 2021,
accepted manuscript: 29 September 2021,
published online: 1 October 2021
Academic editor; *Kittisak Buddhachat*

INTRODUCTION

Species identification is one of the most important issues in forensic science. There are two main techniques that can be used for species identification. Firstly, a species can be identified from certain external features of animal remains that include organs, meat, bones, hair, and blood stains (Kriangwanich et al., 2021a). However the effectiveness of this technique can be limited by the process of decomposition of the specimen or any incomplete morphological features. These factors can make identification very difficult (Cousins et al., 2012). The second technique employs various molecular markers such as the random amplification of polymorphic DNA (RAPD), mitochondrial DNA (mtDNA) genes, microsatellites, and inter-simple sequence repeats (ISSR) to successfully identify a species (Baker et al., 2007). High resolution melting analysis (HRM) is a rapid identification method that can be useful for genotyping any known variants and when scanning for unknown variants (Wittwer, 2009). This method is less time-consuming than other molecular marking methods (Jiang et al., 2019). Importantly, the melting curves of graph changes that are created by HRM can be sampled separately making them more easily observed (Jin et al., 2015). Moreover, HRM results reveal real-time measurements of different melting temperatures, thereby reducing the potential for human error (Erali and Wittwer, 2010; Power, 1996; Reed et al., 2007; Wittwer et al., 2003). ISSR markers coupled with HRM analysis (ISSR-HRM) have recently been recognized as the most up-to-date approach for the species identification of land and marine mammals (Kriangwanich et al., 2021a; Kriangwanich et al., 2021b). The objective of this study was to successfully identify animal species from unidentified bone samples found in snake feces with use of the ISSR-HRM technique.

CASE

In this case study, a black-and-white ruffed lemur (*Varecia variegata*) had recently disappeared from the feeding area of a containment area. Later, we found that the feces of a snake contained animal organs (Figure 1). The feces included animal bones, animal hair, and the shafts of feathers. The bones we found included the calcaneus bone, ribs, and vertebrae; however, species identification by morphology alone was unsuccessful. It was logically speculated that these bones likely belonged to the missing lemur.

Figure 1 Snake feces collected from the field (a) consisting of 3 bone types; calcaneus (b), rib (c) and vertebrae (d), shafts of feathers (e), and animal hair (f).

SPECIES IDENTIFICATION

DNA isolation

DNA was extracted from two unidentified bone samples and one lemur blood sample following the protocol described in an RBC Bioscience™ Real genomics DNA extraction kit for tissue samples (RBC Bioscience Corp., New Taipei, Taiwan) and an RBC Bioscience™ Real genomics DNA extraction kit for blood samples (RBC Bioscience Corp., New Taipei, Taiwan), respectively. The extraction process was performed at the Faculty of Veterinary Medicine, Chiang Mai University. DNA was then measured qualitatively and quantitatively using agarose gel electrophoresis and spectrophotometry, respectively (Poommouang et al., 2021).

ISSR-HRM

A total of 34 Inter Simple Sequence Repeat (ISSR) markers (Table 1) that had been established in a previous study (Kriangwanich et al., 2021a; Kriangwanich et al., 2021b) were screened in order to obtain a melting profile of the DNA fingerprints established by HRM analysis (Kriangwanich et al., 2020). ISSR-HRM was performed on PCRmax Eco 48 (PCRmax Limited, Staffordshire, UK) following the protocol established by (Kriangwanich et al., 2021a). This method was employed with a final volume of 10 µl that contained a 2X SensiFastTM HRM Mix (Bioline, Memphis, TN, USA), 10 µM ISSR primer, and a 10 ng DNA template. Deionized water was added instead of the DNA template in order to establish a negative control.

Table 1 Nucleotide sequences of inter-simple sequence repeat primers.

Primers	Sequence (5‘-3‘)	Length
UBC801	ATA TAT ATA TAT ATA TT	17-mer
UBC802	ATA TAT ATA TAT ATA TG	17-mer
UBC803	ATA TAT ATA TAT ATA TC	17-mer
UBC805	TAT ATA TAT ATA TAT AC	17-mer
UBC807	AGA GAG AGA GAG AGA GT	17-mer
UBC808	AGA GAG AGA GAG AGA GC	17-mer
UBC809	AGA GAG AGA GAG AGA GG	17-mer
UBC811	GAG AGA GAG AGA GAG AC	17-mer
UBC814	CTC CTC TCT CTC TCT A	16-mer
UBC817	CAC ACA CAC ACA CAC AA	17-mer
UBC818	CAC ACA CAC ACA CAC AG	17-mer
UBC822	TCT CTC TCT CTC TCT CA	17-mer
UBC823	TCT CTC TCT CTC TCT CC	17-mer
UBC824	TCT CTC TCT CTC TCT CG	17-mer
UBC825	ACA CAC ACA CAC ACA CT	17-mer
UBC826	ACA CAC ACA CAC ACA CC	17-mer
UBC827	ACA CAC ACA CAC ACA CG	17-mer
UBC835	AGA GAG AGA GAG AGA GYC	18-mer
UBC844	CTC TCT CTC TCT CTC TRC	18-mer
UBC845	TCT CTC TCT CTC TCT CRG	18-mer
UBC847	CAC ACA CAC ACA CAC ARC	18-mer
UBC848	CAC ACA CAC ACA CAC ARG	18-mer
UBC861	ACC ACC ACC ACC ACC ACC	18-mer
UBC866	CTC CTC CTC CTC CTC CTC	18-mer
UBC868	GAA GAA GAA GAA GAA GAA	18-mer
UBC869	GTT GTT GTT GTT GTT GTT	18-mer
UBC872	GATA GATA GATA GATA	16-mer
UBC874	CCCT CCCT CCCT CCCT	16-mer
UBC876	GATA GATA GACA GACA	16-mer
UBC880	GGA GAG GAG AGG AGA	15-mer
UBC881	GGG TGG GGT GGG GTG	15-mer
UBC892	TAG ATC TGA TAT CTG AAT TCC C	22-mer
UBC899	CAT GGT GTT GGT CAT TGT TCC A	22-mer
UBC900	ACT TCC CCA CAG GTT AAC ACA	21-mer

HRM analysis was carried out after 40 real-time PCR cycles at temperature increments of 0.1°C/cycle between 55°C and 95°C in order to generate high resolution melting curves. Eco software v5.2.12 (PCRmax) was used to generate melting curve profiles from ISSR-HRM analysis. Temperature boundaries were set by the pre- and post-melt normalization regions after HRM analysis. This was done to generate normalized melting curves (Wittwer et al., 2003). The melting temperature (Tm) was primarily established by the melting curve for a negative derivative of fluorescence (F) over temperature (T) with a normalized raw curve depicting a decreasing degree of fluorescence in accordance with any increase in temperature.

Control region (D-loop)

One pair of PCR primers, namely DugDLF (5′-CAT ATT ACA ACG GTC TTG TAA ACC-3′) and DugDLR (5′-GTC ATA AGT CCA TCG AGA TGT C-3′), that contained 615 bp long regions of the mtDNA D-loop was used for the purposes of amplification (Blair et al., 2014). Three samples were amplified individually by PCR and these consisted of 1X ViBuffer S (16 mM (NH2)4SO4, 50 mM Tris-HCl, 1.75 mM MgCl2, as well as 0.01% TritonTM X-100), 0.2 mM dNTP (Vivantis, Selangor Darul Ehsan, Malaysia), 0.2 µM in each forward and reverse primer, 1U Taq DNA polymerase (Vivantis, Selangor Darul Ehsan, Malaysia), and 10 ng DNA template with deionized water added to a volume of 25 µl. Deionized water was then used instead of the DNA template to serve as a negative control. PCR amplifications were performed in PTC-200 at DNA EngineThermal Cycler (Bio-Rad Laboratories, Inc., CA, USA) under the following conditions: pre-denaturation at 95°C for 5 min, 40 cycles consisting of a denaturation step at 95°C for 30 seconds, an annealing step at 50°C for 45 seconds, and an extension step at 72°C for 1 minute with a final extension step at 72°C for 10 minutes. Sanger direct sequencing was performed by Ward Medic Ltd. Bangkok, Thailand. Sequences were edited manually using the program MEGA-X version 10.1.818 (Kumar et al., 2018).

RESULTS

Only six suitable ISSR primers that offered distinct melting curve patterns were selected to create melting fingerprints using the ISSR-HRM technique.

Two primers (UBC818, UBC869) generated a Tm of 2, while 4 primers (UBC817, UBC847, UBC848, and UBC880) generated a Tm of 4. By comparing the derivative melting curve established from 2 bones and the lemur blood, we found that all 6 primers produced similar Tm values (Figure 2).

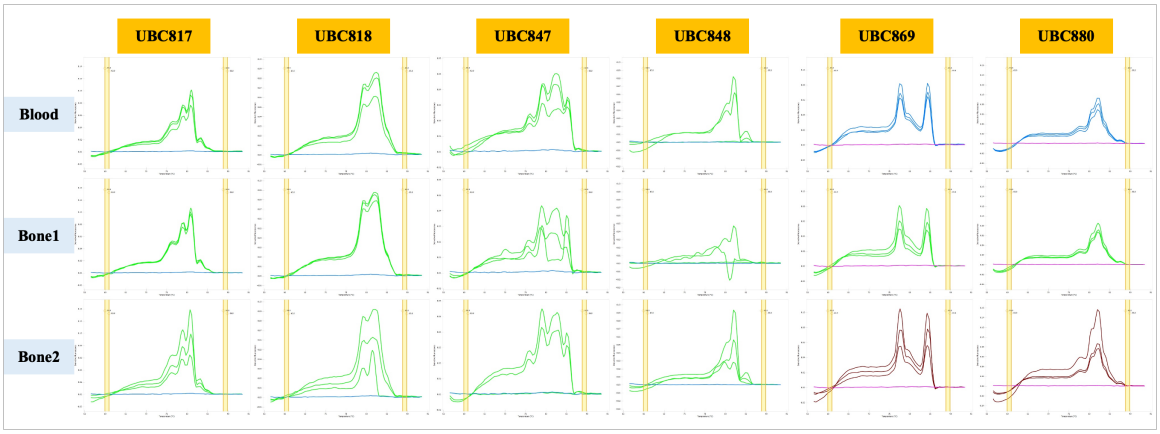

Figure 2 Comparative derivative melting curve of six primers for DNA from lemur blood and two bone samples.

The sequence of D-loop obtained from the bone and blood samples (Figure 3) was subjected to blasting (nucleotide blast) at the National Center for Biotechnology Information (NCBI) Genbank. It was found that both the bone and blood samples had the same nucleotide sequence as the black-and-white ruffed lemur (*Varecia variegata*) with percent identity values of 99.54 and 99.85, respectively.

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>Lemur_blood_662bp
ACCAAAAATGGAGAATTCCCCTCCCAAGGACATCTCAAGGAAGAAGCaTTAGCCCCACCTTCAGCACCCAAAGCTGACA
TTCTACTTAAACTACCCCTTGATGTATAATTTAAAATAGTAATTACTAATAAATTTAGTTCCATTTCTATGTACATCGT
GCATAATGTGCCTTTCCTCATTAACATGTACCTATATTATACATGTATAATTTTACATAAGACATAATATGTATATCGT
ACATACAGTTCTAGCCTGGCGCGCATATAAGCAAGTACTTAGTCCCTAAGTTGTACATAAAACACTCCATGTTATCGTT
CATTCAACTCTATTCCATACGCATATAAGCCAGTACATTACTCCATAATCGTGCATAGGCATTGTTCCTATAACTGTAC
ATAAAACATTCCTCAATTGATCGAACAATGGCGCATATCAGATCAGAACTACTTATTCGATTCCTGTCAATATGGATAT
CCACCTATACCAGATCGTAGCTTAATCTACCATCCTCCGTGAAACCAGCAACCCGCCCGCAGAATGCCTCTCTTCTTGC
TCTGAGCCCATTCAAACTTGGGGGTGTCTATACTGAAACTTTATCTGGCCTCTGGTTCTTACATCAGGGCCATGTGAGA
TATACCCGCTCACTCGTTCCCCTTAAATAA

>Lemur_bone_654bp
TGGAGAATTCCCCTCCCAAGGACATCTCAAGGATGAAGCATTAGCCCCACCTTCAGCACCCAAAGCTGACATTCTACTT
AAACTACCCCTTGATGTATAATTTAAAATAGTAATTACTAATAAATTTAGTTCCATTTCTATGTACATCGTGCATAATG
TGCCTTTCCTCATTAACATGTACCTATATTATACATGTATAATTTTACATAAGACATAATATGTATATCGTACATACAG
TTCTAGCCTGGCGCGCATATAAGCAAGTACTTAGTCCCTAAGTTGTACATAAAACACTCCATGTTATCGTTCATTCAAC
TCTATTCCATACGCATATAAGCCAGTACATTACTCCATAATCGTGCATAGGCATTGTTCCTATAACTGTACATAAAACA
TTCCTCAATTGATCGAACAATGGCGCATATCAGATCAGAACTACTTATTCGATTCCTGTCAATATGGATATCCACCTAT
ACCAGATCGTAGCTTAATCTACCATCTTCCGTGAAACCAGCAACCCGCCCGCAGAATGCCTCTCTTCTTGCTCTGAGCC
CATTCAAACTTGGGGGTGTCTATACTGAAACTTTATTTGGCCTCTGGTTCTTACCTCAGGGCCATGTGAGATATACCCG
CTCACTCGTTCCCCTTACTAAA
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Figure 3 D-loop sequence from lemur blood (662bp) and bone samples (654bp).

DISCUSSION

ISSR-HRM analysis serves as an effective alternative tool in the species identification process. In previous studies, the HRM technique was used to identify several species including mammalian species (Kriangwanicha et al., 2021a), marine mammal species (Kriangwanicha et al., 2021b), fish (Buddhachat et al., 2021), parasites (Buddhachat et al., 2020), and several breeds of dogs (Kriangwanicha et al., 2020). This case study utilized research data obtained from previous studies. The melting curve of six ISSR primers derived from the lemur blood sample was similar to that of the unidentified bones (speculated to be of a lemur), which was later confirmed using the standard technique. The advantage of the ISSR-HRM technique is that only a single primer is needed to produce multiple DNA bands from different loci. Therefore, this technique can be used to identify a wide variety of animal species using minute sample of DNA (Gupta et al., 1994; Ng and Tan, 2015). A limitation of this technique has been addressed in a number of previous research articles (Kriangwanich et al., 2021a; Kriangwanich et al., 2021b). This limitation is that we must have a standard Tm curve for many species that can be used for the purposes of comparison with the samples. Additionally, frequently changing PCR machines can also possibly influence the Tm value.

In conclusion, ISSR-HRM has been determined to be an adequate screening tool for species identification, particularly when known and unknown samples are used. Moreover, this technique can be used in cases where only a low quality DNA is available as the ISSR fragments are very short. Moreover, this technique is noticeably less time-consuming than other methods since the entire process can be finished within 5 days.

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How to cite this article;

Anocha Poommouang, Piyamat Kongtueng and Raksiri Nomsiri. HRM species identification of bone samples collected from snake feces. Veterinary Integrative Sciences. 2022; 20(1): 41 - 48.