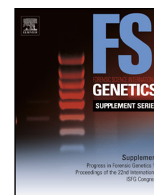
Forensic Science International: Genetics Supplement Series 5 (2015) e116–e117

Contents lists available at ScienceDirect

Forensic Science International: Genetics Supplement Series

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



Identification of big game species by a universal cytochrome B primer pair through High-Resolution Melting

Andrés Lopez-Oceja[a], David Gamarra[a], Susana Jiménez-Moreno[b,*], Marian M. de Pancorbo[a]

[a] BIOMICs Research Group, University of the Basque Country (UPV/EHU), Vitoria, Spain
[b] Área Medicina Legal y Forense, Dpto. Patología y Cirugía, Universidad Miguel Hernández, Elche, Alicante, Spain

ARTICLE INFO

Article history:
Received 1 September 2015
Accepted 14 September 2015
Available online 1 October 2015

Keywords:
Cytochrome B
Big game species
High Resolution Melting

ABSTRACT

Species identification by DNA barcodes has become in recent years a very useful tool in molecular biology. But the degradation level shown in some samples, makes regular barcodes impractical for cases in which DNA is fragmented as it happens commonly in forensic casework. Therefore, the use of mini barcodes with sizes below 200 bp is recommended to achieve optimal amplifications. The present study is focused on the identification of five Iberian Peninsula big game species, Red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), brown bear (*Ursus arctos*) and wolf (*Canis lupus*), using a pair of universal primers that amplify a 148 bp Cytochrome B mini barcode. The analysis of amplicons was made using High-Resolution Melting and the results have shown a correct identification of the five big game species analyzed even in degraded samples.

© 2015 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Levels of monitoring and control of poaching have been increased during last years, reducing the number of cases [1] but it still requires new tools for genetic identification of species in forensic casework.

DNA of forensic evidences from cinegetic samples is commonly highly degraded [2]. The small size of the fragments of the degraded DNA requires amplicons less than 200 bp for optimum sample amplification [3]. The analysis of reduced amplicons can be carried out by High-Resolution Melting (HRM). This technique has advantages such as low cost and short time, bringing the same level of discrimination of species that analytical methods based on Sanger sequencing.

The Cytochrome B (CytB) is a mitochondrial gene that has been widely used in taxonomic and forensic studies [4,5]. The CytB provides an accurate reconstruction of the phylogeny of mammals, in the levels of super-order, order and family [6]. In addition, this locus maintains a high level of phylogenetic discrimination even when short amplicons are used.

The aim of this study was to design a pair of primers able to amplify a highly variable small region of 148 bp from the CytB gene for making a panel able to identify five game species by HRM technique.

2. Material and methods

Individuals from a total of seven species belonging to four families of two different orders were analyzed (Table 1). Muscle tissues and stool samples were obtained from several hunting associations and Cabarceno Nature Park (Cantabria, Spain). For the extraction of muscle tissues, 5 mg were taken and DNA was extracted by the salting out method, following the protocol of the Gentra Puregene tissue kit (Qiagen). 200 mg of stool samples were taken for DNA extraction using the Stool QIAmp DNA Mini kit (Qiagen).

To perform primers design, CytB gene sequences of each of the seven analyzed animal species were obtained from GenBank. The sequences were aligned using the ClustalX software v.2.0.11 [7]. The variable regions flanked by highly preserved sequences were searched manually. After selecting the flanking regions we proceeded to design a pair of primers using the PerlPrimer v.1.1.21 software [8].

Purified DNA templates were amplified in a BioRad CFX-96 real time system (BioRad, USA). The following reagents were used: 0.15 μM of each primer, 2.5 μL of SsoFastTM EvaGreen® supermix and 5 μL of DNA. The PCR conditions were as follows: Initial denaturation at 98 °C for 2 min; 32 cycles at 98 °C for 5 s and 50 °C for 30 s. The plate read was taken after an initial step of 95 °C 30 s

* Corresponding author at: Ctra. Alicante—Valencia KM. 8, 7. 03550 Sant Joan d'Alacant, Alicante, Spain.
E-mail address: susana.jimenez@umh.es (S. Jiménez-Moreno).

http://dx.doi.org/10.1016/j.fsigss.2015.09.047
1875-1768/© 2015 Elsevier Ireland Ltd. All rights reserved.

Table 1
Species analyzed in this study. N indicates the number of individuals analyzed for each species. Ti indicates muscle tissue; Fe indicates feces.

Order	Family	Common name	Scientific name	N	Matrix
Artiodactyla	Cervidae	Red deer	*Cervus elaphus*	10	Ti
		Roe deer	*Capreolus capreolus*	10	Ti
	Suidae	Wild boar	*Sus scrofa*	10	Ti
		Pig	*Sus scrofa domesticus*	10	Ti
Carnivora	Canidae	Wolf	*Canis lupus*	3	Fe
		Dog	*Canis lupus familiaris*	3	Fe
	Ursidae	Brown bear	*Ursus arctos*	3	Fe

and 60 °C 2 min. The melt curve was from 65 °C to 95 °C with an increment of 0.5 °C each after 5 s. Melting profiles were analyzed with the Bio-rad Precision Melt Analysis Software v1.0 (BioRad, USA).

3. Results and discussion

The selection of species was performed taking into account sensitive big game species. On the one hand the three species most hunted by number of individuals in the Iberian Peninsula were chosen, red deer, roe deer and wild boar [1]. On the other hand, protected species because of their small number of individuals were included such as the brown bear and the wolf. Samples of two domestic species, dog and pig, were included to verify that different HRM profiles were obtained in wild and domestic species and verify that it is possible to discriminate correctly these nearby species.

CytB gene primers designed in this study were able to amplify the seven analyzed species. Both normalized melt curve as the difference curve, showed a clear differentiation of each species obtaining very different HRM profiles for each one (Fig. 1). No overlapping HRM-clusters were shown among the different species. While the pig and roe deer clusters are very close to each other, all individuals are within their respective cluster, without any kind of mixture. Therefore has been achieved a clear identification of the seven species here analyzed.

This primer design, for amplify a small region of 148 bp, was specifically designed to be used in degraded samples. This study has proven the usefulness of the primers in stool samples containing degraded DNA to obtain HRM profiles.

4. Conclusion

A pair of primers of Cytb gene that discriminated by HRM five game species of high economic and ecological value has been obtained. These primers amplify a small region of 148 bp and have been shown equally useful when amplify degraded samples.

Acknowledgements

A. Lopez-Oceja and D. Gamarra thank the Department of Economic Development and Competitiveness of the Government of the Basque Country for their predoctoral fellowships and the IT-833-13 Consolidated Group and UFI11/32 UPV/EHU for funding. Special thanks to Cabarceno Nature Park (Cantabria, Spain).

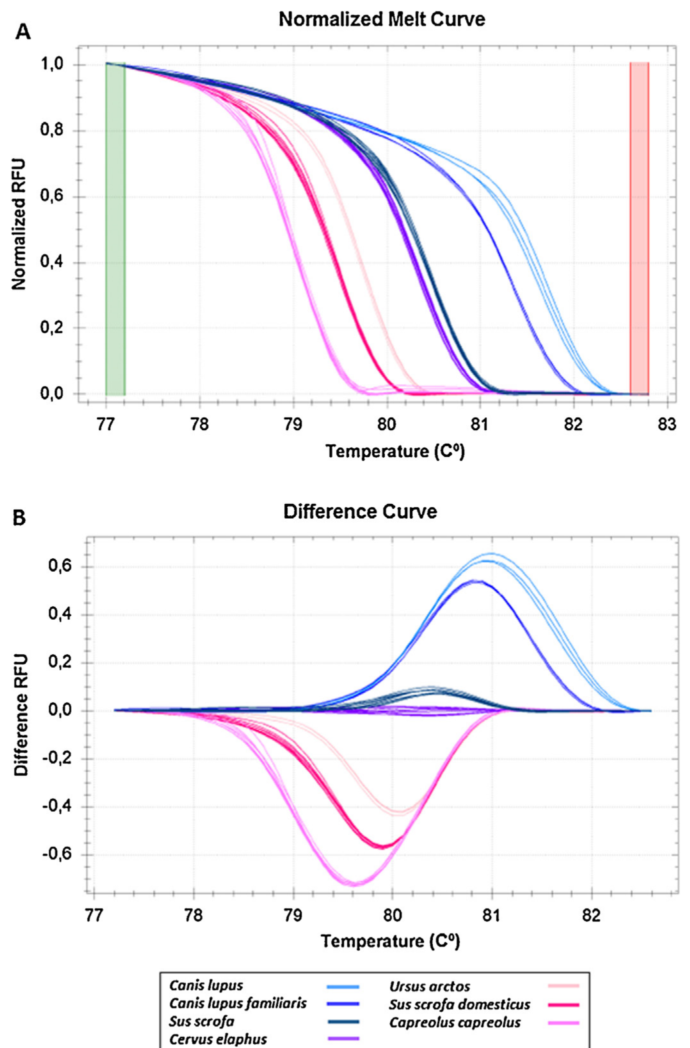


Fig. 1. Genotyping of CytB gene by Precision Melt Analysis Software: (A) normalized HRM melt curves. (B) Difference HRM curves.

References

[1] M. Pita, S. Casas, J. Herrero, et al., Game reserves in Spain: the public management of hunting, For. Syst. 21 (3) (2012) 398–404.
[2] J.B. Whitfield, Destructive sampling and information management in molecular systematic research: an entomological perspective.
[3] P.M. Schneider, K. Bender, W.R. Mayr, et al., STR analysis of artificially degraded DNA—results of a collaborative European exercise, Forensic Sci. Int. 139 (2) (2004) 123–134.
[4] H.M. Hsieh, H.L. Chiang, L.C. Tsai, et al., Cytochrome b gene for species identification of the conservation animals, Forensic Sci. Int. 122 (1) (2001) 7–18.
[5] W. Parson, K. Pegoraro, H. Niederstätter, et al., Species identification by means of the cytochrome b gene, Int. J. Legal Med. 114 (1–2) (2000) 23–28.
[6] S.S. Tobe, A.C. Kitchener, A.M. Linacre, Reconstructing mammalian phylogenies: a detailed comparison of the cytochrome b and cytochrome oxidase subunit I mitochondrial genes, PLoS One 5 (11) (2010) e14156.
[7] M.A. Larkin, G. Blackshields, N.P. Brown, et al., Clustal W and Clustal X version 2.0, Bioinformatics 21 (23) (2007) 2947–2948.
[8] O.J. Marshall, PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR, Bioinformatics (2004) .