Forensic Science International: Genetics 11 (2014) e9–e11

Contents lists available at ScienceDirect

Forensic Science International: Genetics

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

Letter to the Editor

The risks of using "species-specific" PCR assays in wildlife research: The case of red fox (*Vulpes vulpes*) identification in Tasmania

Keywords:
Species-specific PCR
Species identification
Red fox
mtDNA
Scats
Tasmania

Dear Editor,

The objective of an ideal species-specific PCR assay (also known as allele-specific PCR) is to always provide a positive result whenever DNA from the target species is present and a negative outcome when it is absent, even in the presence of DNA from other species [1–3]. PCR primers must be designed in DNA regions where the sequence varies significantly between the target and other species, reliably excluding those that might contaminate casework samples. Under suitable PCR conditions, such primers generate a fragment that can be visualized by conventional gel or capillary electrophoresis. However, poor design of the screening method may lead either to a failure in the PCR in the presence of DNA from the target species (a false negative or Type II error) or positive PCR amplification in the absence of the target species' DNA (false positive or Type I error). In general, an incorrectly assigned result may occur if the PCR primers are not sufficiently selective, the annealing temperature is not adequately stringent or too many amplification cycles are used in the PCR [4].

The need to develop effective methods for correct identification of species is of growing importance in wildlife management and forensics [5–8]. A good case study of how data from species-specific PCR assays has been used to inform the management of an important invasive species relates to the detection of red fox (*Vulpes vulpes*) incursions on the island state of Tasmania (Australia) [9]. After anecdotal reports claimed that red foxes had been deliberately released into the Tasmanian environment [10–13], a fox eradication programme based upon widespread 1080 (fluoroacetic acid) poison baiting began in 2002 [13]. In order to detect the presence of foxes a large pool of predator faeces (scats) was collected predominantly between 2003 and 2012 by co-ordinated searches using volunteers [14] and routine searches with trained scat detection dogs [15]. By late 2012, 56 of 7658 were assigned as 'fox positive' using a fox-specific PCR assay targeting mitochondrial *cytochrome b* (*CYTB*), either with [14] or without [16] subsequent DNA sequencing. However, it was reported that 130 of 186 (70%) samples with positive PCR amplifications for fox produced incomplete sequences of poor quality not corresponding to the fox reference sequence and only 56 of 186 (30%) cases were identified as fox [14]. Moreover, it was also revealed that DNA from European rabbit (*Oryctolagus cuniculus*) and European hare (*Lepus europaeus*) amplified with the fox-specific PCR assay [14].

We sought to better evaluate the specificity of the putatively fox-specific pair of PCR primers (*VV-cytb F* and *VV-cytb R*) designed by Berry et al. [16] using DNA from a wider range of species (Table S1; a detailed description of all experiments can be found on the Supplementary Material). After verifying the quality of the DNA extractions using 'universal' primers (Fig. S1), we tested the *VV-cytb F* and *VV-cytb R* primers in gradient PCRs with annealing temperatures between 51 °C and 62 °C. Foxes and several other species yielded positive amplifications at different annealing temperatures (e.g. Fig. S2). Additionally, separate PCRs (not in a gradient) using annealing temperatures of 56 °C, 58 °C (used by Berry et al. [16]) and 60 °C (used by Sarre et al. [14]) produced clear amplifications for fox, European rabbit, European hare, Iberian hare (*Lepus granatensis*), cattle (*Bos taurus*) and pig (*Sus scrofa*) DNA (Fig. 1, Tables S1 and S2). PCR products were also obtained for the Tasmanian devil (*Sarcophilus harrisii*) and a long-nosed potoroo (*Potorous tridactylus*) with weaker intensity. At an annealing temperature of 56 °C, a single cattle sample (Bt1) and one rabbit (Oc2), the Iberian hare (Lg1) and both pig DNA samples yielded a larger quantity of amplified PCR product than either fox sample, suggesting that in some cases the primers designed by Berry et al. [16] amplified non-fox DNA more effectively. DNA from rabbit, hare, cattle and pig are readily amplified even at 60 °C (Fig. 1). Because DNA from hare is amplified at 62 °C (Fig. S2), non-specific DNA synthesis occurs at even higher annealing temperatures than those reported by Berry et al. [16] and Sarre et al. [14].

The selectivity of a species-specific primer depends on the number of base pair mismatches in relation to other non-target species, with mismatches at the 3′-end of the primer having a larger impact on PCR yield than those near the 5′-end [8,17,18]. Under less stringent conditions, a single mismatch in the 3′-end of each primer might not be sufficient to completely abrogate the PCR amplification [17,18] and the presence of up to four mismatches in the primer–template duplexes may not significantly affect product yield [19]. Using the Berry et al. [16] assay, up to nine mismatches in both primers were insufficient to avoid strong PCR amplifications with DNA from other species, as were demonstrated in the pig DNA samples (Figs. 1 and S3). A low number of mismatches in the putative fox-specific primers relative to other species were evident from the alignment of 33 *CYTB* sequences (Fig. S4). Therefore, the putatively fox-specific primers *VV-cytb F* and *VV-cytb R* did not specifically hybridize to fox DNA, despite being designed with the intention to specifically exclude species with morphologically similar scats to the fox [20].

Scats of omnivores with a highly varied diet, such as foxes, quolls (*Dasyurus* sp.), bandicoots (Peramelidae) as well as scavengers such as the Tasmanian devil [21] contain a complex

http://dx.doi.org/10.1016/j.fsigen.2014.03.009
1872-4973/

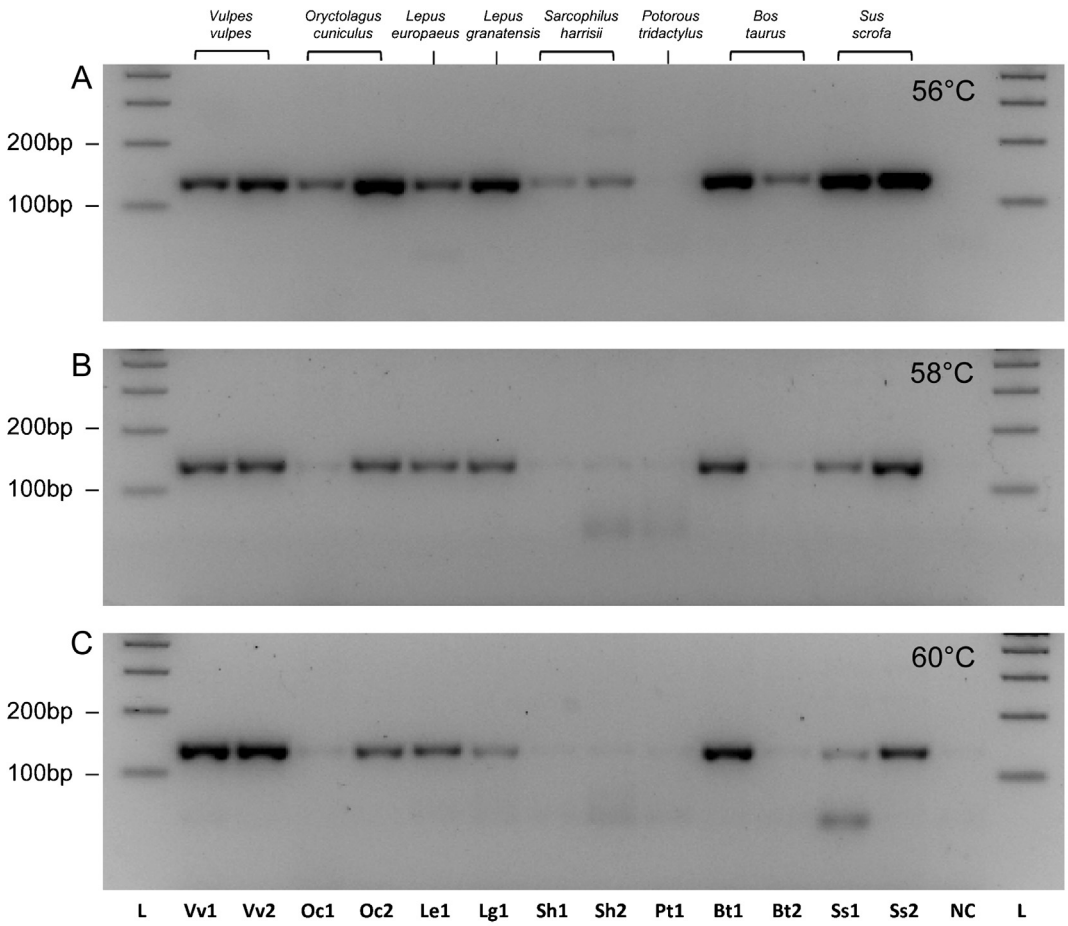

Fig. 1. Amplification of DNA from several species using the putatively *CYTB* fox-specific *VV-cytb F* and *VV-cytb R* primers designed by Berry et al. [16]. Agarose gel (2%) showing the amplification products obtained at annealing temperatures of 56 °C (A), 58 °C (B) and 60 °C (C). Legend: Vv1 and Vv2, red fox (*Vulpes vulpes*); Oc1 and Oc2, rabbit (*Oryctolagus cuniculus*); Le1, European hare (*Lepus europaeus*); Lg1, Iberian Hare (*Lepus granatensis*); Sh1 and Sh2, Tasmanian devil (*Sarcophilus harrisii*); Pt1, long-nosed potoroo (*Potorous tridactylus*); Bt1 and Bt2, cattle (*Bos taurus*); Ss1 and Ss2, pig (*Sus scrofa*); NC, negative control; L, 100-bp DNA ladder.

mixture of partially degraded biological material, including host-derived epithelial cells from the colon wall, undigested prey, enteric microorganisms and parasites. Even if obvious prey contents are separated in the scat prior to analysis, the possibility of contamination throughout the faecal material must be assumed. Our results demonstrate that strong amplifications using the putatively fox-specific assay are obtained with DNA from widely distributed prey species such as the European rabbit, European hare as well as that from common agricultural species such as cattle and pigs. European rabbits are particularly common in the diet of feral cats and foxes [22] and rabbits and hares are common as road kill [23] that is known to be scavenged as a food resource by marsupial carnivores such as Tasmanian devils and quolls [24]. Other common domestic livestock species appear to constitute a greater risk of sample contamination from multiple routes. Meat carryover in human faeces in sewage as well as waste from kitchens, abattoir and food manufacturing processes are therefore potentially significant environmental source of cattle and pig DNA [25]. Commercial dog foods may also contain a wide range of cattle meat and by products such as hooves and horns [26] and both cattle and pig products are components of a range of fox baits [27].

Consequently, the species-specific assay proposed by Berry et al. [16] must contend with the likelihood of strong PCR amplification arising from extremely abundant forms of non-fox DNA. Irrespective of what post-PCR laboratory methods are used, *VV-cytb F* and *VV-cytb R* do not permit sufficient specificity for the unequivocal determination of fox DNA. The fox-specific assay was developed without adequate cross-reactivity studies to exclude unspecific amplifications. Because the generally low quality and quantity of DNA found in scats typically requires less stringent PCR conditions, such as decreased annealing temperature and increased number of amplification cycles [14], there may be a higher risk of amplifying nonspecific sequences [4,28–30]. Therefore, this diagnostic assay alone cannot be relied upon to detect red foxes because of the high likelihood of false positives. Because wildlife management decisions may be increasingly influenced by data generated by molecular technologies, there is a need to adopt best practice guidelines for forensic genetic investigations [1,6,31–33] especially under conditions when the detection of a unique invasive species may be considered sufficient justification to initiate a costly eradication programme. This case is a practical illustration of the challenges associated with the design of reliable species-specific PCR assays and the need to better standardize practices used for taxonomic identifications in wildlife and forensic genetic investigations.

Acknowledgements

This work was supported by the Portuguese Foundation for Science and Technology (FCT), Fundo Social Europeu and Programa Operacional Potencial Humano [PTDC/CVT/100881/2008 and Investigator FCT program]. CIIMAR was partially supported by the European Regional Development Fund (ERDF) through the COMPETE – Operational Competitiveness Programme and national funds through FCT, under the project PEst-C/MAR/LA0015/2013. IPATIMUP is an Associate Laboratory of the Portuguese Ministry of

Education and Science and is partially supported by FCT. All research procedures were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997). Tasmanian samples were obtained as part of routine veterinary examinations and archival material collected under AEC protocols A009215 or approved by the Metropolitan AEC as protocol 2649. We thank Dr Colette Harmsen and Dr James Harris for their assistance and we extend our thanks to Mr. Jack West and Mr. Eddie Juras for the provision of the fox specimens used in this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2014.03.009.

References

[1] J.A. Darling, M.J. Blum, DNA-based methods for monitoring invasive species: a review and prospectus, Biol. Invasions 9 (2007) 751–765.
[2] R. Ogden, N. Dawnay, R. McEwing, Wildlife DNA forensics – bridging the gap between conservation genetics and law enforcement, Endanger. Spec. Res. 9 (2009) 179–195.
[3] F. Pereira, J. Carneiro, A. Amorim, Identification of species with DNA-based technology: current progress and challenges, Recent Pat. DNA Gene Seq. 2 (3) (2008) 187–200.
[4] E. van Pelt-Verkuil, A. Van Belkum, J.P. Hays, Principles and Technical Aspects of PCR Amplification, Springer, Amsterdam, 2008.
[5] M.L. Coghlan, N.E. White, L. Parkinson, J. Haile, P. Spencer, M. Bunce, Egg forensics: an appraisal of DNA sequencing to assist in species identification of illegally smuggled eggs, Forensic Sci. Int. Genet. 6 (2012) 268–273.
[6] R.N. Johnson, L. Wilson-Wilde, A. Linacre, Current and future directions of DNA in wildlife forensic science, Forensic Sci. Int. Genet. 10 (2013) 1–11.
[7] A. Sanches, P.M. Tokumoto, W.A. Peres, F.L. Nunes, M.S. Gotardi, C.S. Carvalho, et al., Illegal hunting cases detected with molecular forensics in Brazil, Invest. Genet. 3 (2012) 1–5.
[8] A. Ramón-Laca, A.M. Linacre, D.M. Gleeson, S.S. Tobe, Identification multiplex assay of 19 terrestrial mammal species present in New Zealand, Electrophoresis 34 (2013) 3370–3376.
[9] I. Abbott, The importation, release, establishment, spread, and early impact on prey animals of the red fox *Vulpes vulpes* in Victoria and adjoining parts of south-eastern Australia, Aust. Zool. 35 (2011) 463–533.
[10] C. Dennis, Baiting plan to remove fox threat to Tasmanian wildlife, Nature 416 (2002) 357.
[11] J. Marshall, 'Fox in the Henhouse': The Introduction of the European Red Fox (*Vulpes vulpes*) into Tasmania, and the Potential Threat to the Fauna Biodiversity it Represents. Online Undergraduate Review of Geography and Environmental Studies, Flinders University, Adelaide, 2011.
[12] S.D. Sarre, R. Walsch, N. Aitken, A. Foster, N. Mooney, DNA detection of foxes to prevent establishment in Tasmania, in: G.W. Witmer, W.C. Pitt, K.A. Fagerstone (Eds.), Managing Vertebrate Invasive Species: Proceedings of an International Symposium, USDA/APHIS Wildlife Services, National Wildlife Research Center, Fort Collins, Colorado, 2007, p. 44.
[13] G. Saunders, C. Lane, S. Harris, C. Dickman, Foxes in Tasmania, Invasive Animals Cooperative Research Centre, Canberra, 2006.
[14] S.D. Sarre, A.J. MacDonald, C. Barclay, G.R. Saunders, D.S.L. Ramsey, Foxes are now widespread in Tasmania: DNA detection defines the distribution of this rare but invasive carnivore, J. Appl. Ecol. 50 (2012) 459–468.
[15] D.A. Smith, K. Ralls, A. Hurt, B. Adams, M. Parker, B. Davenport, et al., Detection and accuracy rates of dogs trained to find scats of San Joaquin kit foxes (*Vulpes macrotis mutica*), Anim. Conserv. 6 (2003) 339–346.
[16] O. Berry, S.D. Sarre, L. Farrington, N. Aitken, Faecal DNA detection of invasive species: the case of feral foxes in Tasmania, Wildl. Res. 34 (2007) 1–7.
[17] S. Kwok, S.Y. Chang, J.J. Sninsky, A. Wang, A guide to the design and use of mismatched and degenerate primers, Genome Res. 3 (1994) S39–S47.
[18] S. Kwok, D. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, et al., Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies, Nucleic Acids Res. 18 (1990) 999–1005.
[19] C. Christopherson, J. Sninsky, S. Kwok, The effects of internal primer-template mismatches on RT-PCR: HIV-1 model studies, Nucleic Acids Res. 25 (1997) 654–658.
[20] O. Berry, S.D. Sarre, Gel-free species identification using melt-curve analysis, Mol. Ecol. Notes. 7 (2007) 1–4.
[21] R. Strahan, Complete Book of Australian Mammals, Angus and Robertson, Sydney, 1991.
[22] R.P. Pech, A. Sinclair, A. Newsome, P. Catling, Limits to predator regulation of rabbits in Australia: evidence from predator-removal experiments, Oecologia 89 (1992) 102–112.
[23] A.J. Hobday, M.L. Minstrell, Distribution and abundance of roadkill on Tasmanian highways: human management options, Wildl. Res. 35 (2008) 712–726.
[24] M.E. Jones, Road upgrade, road mortality and remedial measures: impacts on a population of eastern quolls and Tasmanian devils, Wildl. Res. 27 (2000) 289–296.
[25] J.M. Caldwell, J.F. Levine, Domestic wastewater influent profiling using mitochondrial real-time PCR for source tracking animal contamination, J. Microbiol. Methods 77 (2009) 17–22.
[26] S. Murray, A. Patil, G. Fahey, N. Merchen, D. Hughes, Raw and rendered animal by-products as ingredients in dog diets, J. Anim. Sci. 75 (1997) 2497–2505.
[27] G. Saunders, L. McLeod, Improving Fox Management Strategies in Australia, Bureau of Rural Sciences, Canberra, Australia, 2007.
[28] M.H. Kohn, R.K. Wayne, Facts from feces revisited, Trends Ecol. Evol. 12 (1997) 223–227.
[29] F. Pompanon, A. Bonin, E. Bellemain, P. Taberlet, Genotyping errors: causes, consequences and solutions, Nat. Rev. Genet. 6 (2005) 847–856.
[30] P. Taberlet, L.P. Waits, G. Luikart, Noninvasive genetic sampling: look before you leap, Trends Ecol. Evol. 14 (1999) 323–327.
[31] B. Budowle, P. Garofano, A. Hellman, M. Ketchum, S. Kanthaswamy, W. Parson, et al., Recommendations for animal DNA forensic and identity testing, Int. J. Legal Med. 119 (2005) 295–302.
[32] A. Linacre, L. Gusmão, W. Hecht, A. Hellmann, W. Mayr, W. Parson, et al., ISFG: recommendations regarding the use of non-human (animal) DNA in forensic genetic investigations, Forensic Sci. Int. Genet. 5 (2011) 501–505.
[33] M.K. Moore, I.L. Kornfield, Best practices in wildlife forensic DNA, in: J.E. Huffman, J.R. Wallace (Eds.), Wildlife Forensics: Methods and Applications, First edition, John Wiley & Sons, Ltd, Chichester, 2012, pp. 201–236.

Joana Gonçalves
Institute of Molecular Pathology and Immunology of University of Porto (IPATIMUP), Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal

Clive A. Marks**
Nocturnal Wildlife Research Pty Ltd, P.O. Box 2126, Wattletree Road RPO, East Malvern 3144, Australia

David Obendorf
7 Bonnington Road, West Hobart, Tasmania 7250, Australia

António Amorim[a,b]
[a]*Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal*
[b]*Faculty of Sciences, University of Porto, Rua do Campo Alegre, s/n, 4169-007 Porto, Portugal*

Filipe Pereira[a,b,*]
[a]*Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal*
[b]*Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal*

*Corresponding author at: Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal. Tel.: +351 22 340 18 00
E-mail addresses: fpereirapt@gmail.com (F. Pereira).
**Corresponding authorcliveamarks@gmail.com (C.A. Marks), jcoegoncalves@gmail.com (J. Gonçalves), dobendor@iinet.net.au (D. Obendorf), aamorim@ipatimup.pt (A. Amorim).

Received 7 March 2014