Conservation Genet Resour
DOI 10.1007/s12686-014-0343-0

TECHNICAL NOTE

A multiplex PCR assay for identification of the red fox (*Vulpes vulpes*) using the mitochondrial ribosomal RNA genes

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Received: 7 May 2014 / Accepted: 27 September 2014

Abstract The European red fox (*Vulpes vulpes*) is a highly adaptable predator indigenous to the northern hemisphere. However, in Australia the red fox is a widespread exotic predator that has contributed to the decline and extinction of several native species. Here we describe a multiplex PCR assay for the molecular identification of the red fox. The identification is achieved by the generation of a diagnostic profile combining the lengths of mitochondrial ribosomal RNA (rRNA) gene regions amplified using highly conserved PCR primers. The method was tested in DNA samples from 17 species, including in mixtures. Our results demonstrate that the red fox has a unique combination of fragment lengths determined by capillary electrophoresis that can be used for its unambiguous discrimination from common domestic and wild species.

Keywords Red fox (*Vulpes vulpes*) · Species identification · Mitochondrial DNA · Ribosomal RNA · Multiplex PCR

Electronic supplementary material The online version of this article (doi:10.1007/s12686-014-0343-0) contains supplementary material, which is available to authorized users.

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The red fox (*Vulpes vulpes*) is the most geographically widespread member of the Carnivora, being distributed from the Arctic Circle to North Africa, Central America and Asia (Sillero-Zubiri et al. 2004). It was introduced to Australia and now contributes to the decline of many small to medium sized vertebrate populations (Abbott 2011). Several DNA-based approaches have been developed to identify foxes, including some using DNA isolated from field-collected scats [e.g., (Paxinos et al. 1997; Berry et al. 2007; Livia et al. 2007; O'Reilly et al. 2008; Fernandes et al. 2008; Tobe and Linacre 2008; Pun et al. 2009; Weissenberger et al. 2011)]. Here we present the results of the application of an alternative assay (named SPInDel) for identification of the red fox based on the multiplex PCR analysis of mitochondrial 12s and 16s rRNA gene regions (Pereira et al. 2010; Carneiro et al. 2012). Briefly, its rationale involves the design of a set of primers complementary to highly conserved regions that delimitate segments of variable length resulting from multiple insertion/deletion polymorphisms (indels). Therefore, each species is identified by a unique numeric profile of fragment lengths resulting from the combination of the length of hypervariable regions.

We collected samples from 17 different species, including foxes, humans, common domestic livestock and Australian endemic wildlife species (Tables 1 and S1) where the procedures for sample collection and DNA

Published online: 04 October 2014

Table 1 Profiles of 22 samples determined with the SPInDel multiplex PCR

Sample	Species	Common name	Length of SPInDel hypervariable regions (nucleotides)							
			SPID2716	SPID1350	SPID639	SPID1051	SPID2975	SPID2173	SPID1051–SPID1350	SPID2716–SPID2975
Vv1	*V. vulpes*	Red fox	**117.41**	**146.35**	**179.33**	**233.58**	**290.18**	**340.97**	**450.97**	**559.11**
Vv2	*V. vulpes*	Red fox	**117.16**	**146.36**	**179.22**	**233.65**	**290.19**	**341.06**	**450.87**	**559.74**
Oc1	*O. cuniculus*	Rabbit	115.72	144.96	**178.74**	**233.19**	284.83	344.86	448.47	555.71
Oc2	*O. cuniculus*	Rabbit	115.94	145.13	**178.75**	**233.41**	284.93	344.92	448.49	555.64
Le1	*L. europaeus*	European hare	114.36	145.37	177.96	**233.45**	284.02	344.55	448.69	553.97
Lg1	*L. granatensis*	Iberian hare	114.34	145.33	178.23	**233.24**	284.02	343.72	448.14	554.68
Sh1	*S. harrisii*	Tasmanian devil	113.90	143.10	–	–	282.84	337.37	–	–
Sh2	*S. harrisii*	Tasmanian devil	113.32	143.18	–	–	282.85	337.43	–	–
Pt1	*P. tridactylus*	Long-nosed potoroo	112.19	148.28	178.30	–	–	343.66	–	–
Bt1	*B. taurus*	Cattle	110.37	150.47	176.87	234.31	279.92	342.10	454.73	541.43
Bt2	*B. taurus*	Cattle	110.37	150.47	176.72	234.53	279.85	343.02	454.67	540.51
Ss1	*S. scrofa*	Pig	112.84	151.15	180.07	**233.99**	284.94	337.80	455.12	551.77
Ss2	*S. scrofa*	Pig	113.52	150.92	179.88	**234.12**	285.09	337.81	455.05	551.92
Fc1	*F. catus*	Cat	114.48	147.33	181.19	235.31	288.47	340.44	454.28	552.44
Tv1	*T. vulpecula*	Brushtail possum	112.31	**146.23**	**179.33**	–	–	339.08	–	–
Tb1	*T. billardierii*	Tasmanian pademelon	113.34	147.57	177.73	–	284.33	343.41	–	549.62
Dv1	*D. viverrinus*	Eastern quoll	114.29	143.67	181.31	–	283.85	339.70	–	–
Mrr1	*M. r. rufogriseus*	Red necked wallaby	113.58	**146.51**	**179.78**	–	283.89	345.15	–	549.11
Dm1	*D. maculatus*	Spotted-tail quoll	114.21	143.42	–	–	–	–	–	–
Cf1	*C. familiaris*	Dog	**117.69**	**145.99**	**179.11**	**233.26**	288.54	342.86	445.25	–
Hs1	*H. sapiens*	Human	109.32	144.35	178.71	230.11	278.88	339.46	445.25	540.63
Ggf1	*G. g. familiaris*	Chicken	115.11	151.12	–	229.66	–	–	458.76	–

The fragment lengths are for the six target regions (the larger two extra regions result from the amplification with primers of different target regions). The bold underlined values differ by <0.5 from the lengths obtained in the *V. vulpes* samples

extraction were previously described (Gonçalves et al. 2014). We modified the multiplex PCR described in Pereira et al. (2010) by replacing the two largest markers named 'FG' and 'AC' by a new shorter marker (SPID639) to facilitate the analysis of samples with degraded DNA. We have also labelled primers only with the 6-Carboxyfluorescein dye (Fig. S1; Table S2). The multiplex PCR was used with the conditions previously described (Pereira et al. 2010) and detailed in Table S3.

The 22 samples were successfully amplified with the multiplex PCR and despite the non-amplification of a few markers in a small number of cases, the profiles were informative enough for the unambiguous identification of the red fox (Table 1; Fig. S2). The fox samples had unique numeric profiles of fragment lengths that diverge from all other species in at least two hypervariable regions. The most similar profiles to the fox were observed in domestic dogs that had length differences in regions SPID2975 and SPID2173. Accordingly, foxes could be discriminated in this panel of samples by using only the SPID2975 or SPID2173 regions, since the sequence length determined for foxes is unique for each of these regions. The fox profile was clearly different in the six target regions from cattle, cats and humans, and diverged by five regions compared to the two hare species, pigs, Tasmanian pademelon and the Eastern quoll (Table 1). In addition to the peaks corresponding to the six target regions, two extra peaks with larger sizes (~450 and ~550 nucleotides) resulting from the amplification between primers of different target regions were observed in some samples (Figs. 1 and S1; Tables 1 and S2). These extra peaks can also be used to support species identification. Because the number of species that might potentially be found in environmental samples is high, caution is required in the interpretation of results using any species identification approach. For instance, species from the genus *Vulpes* are expected to have more similar SPInDel profiles due to their shared recent evolutionary history. However, our previous investigations showed that equal SPInDel profiles in different species are very rare (Pereira et al. 2010; Carneiro

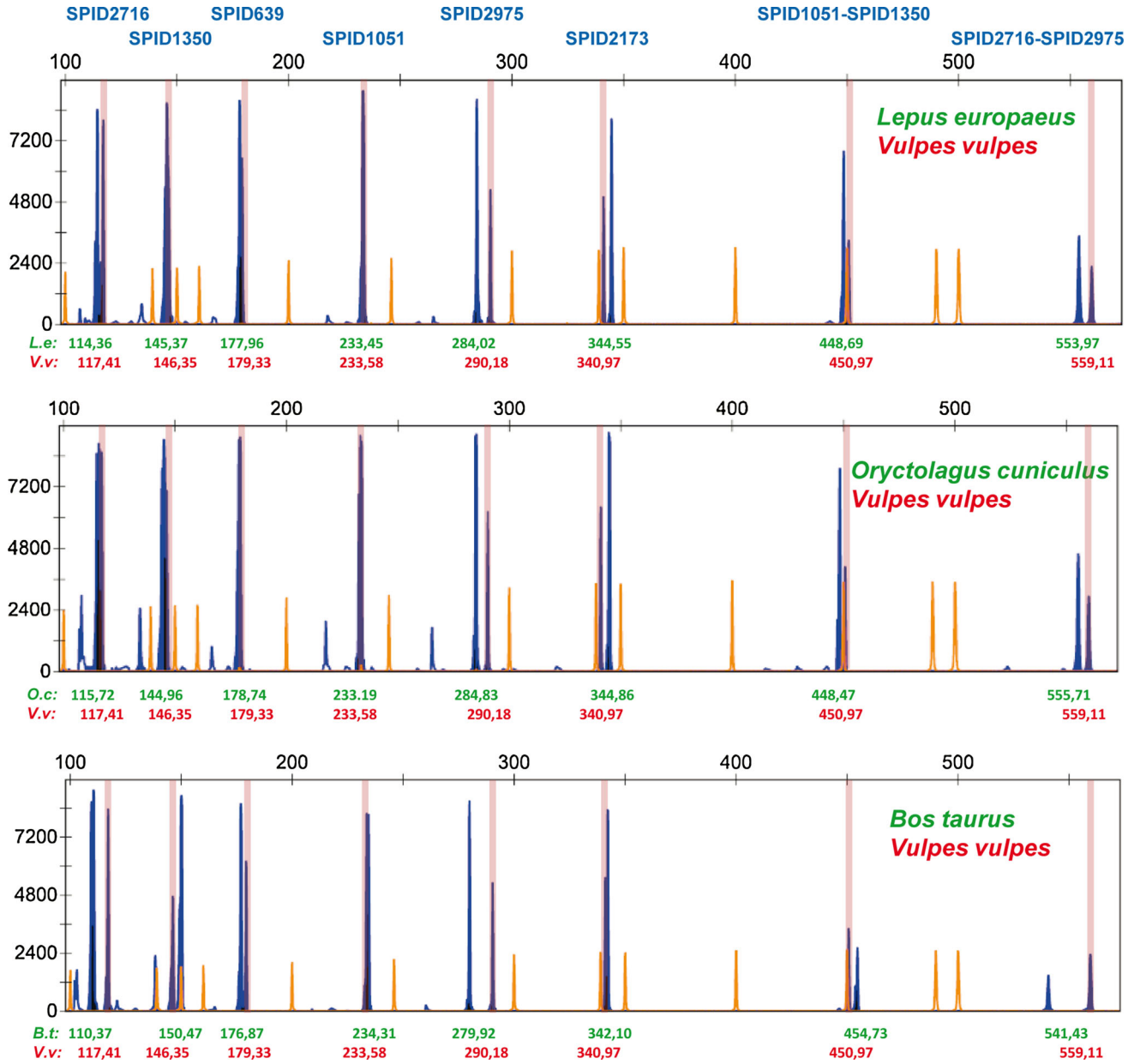

Fig. 1 The use of the SPInDel multiplex for the identification of fox DNA in mixtures. Each electropherogram was obtained using 0.5 μL of PCR product from two different species. The *blue peaks* represent the amplified target regions (names above the graphs), with their lengths indicated by *green* and *red* numbers. The peaks for the fox are highlighted by a *red bar*. The *orange peaks* correspond to the size marker. (Color figure online)

et al. 2012), and that species from other classes (e.g., birds, reptiles, fishes) have very different electrophoretic profiles, as shown here for the chicken sample (Fig. S3). It is unlikely that species from unrelated taxonomic groups will present equal profiles to the red fox considering the high number of possible numeric combinations from just a few target regions (Pereira et al. 2010). One advantage of the SPInDel method is that amplified products used for length analyses can be sequenced with the conserved primers, allowing further confirmatory tests.

Our method is suitable for detection of fox DNA in mixed samples by allowing a clear distinction between profiles of different species. For example, fox and hare DNA can be easily discriminated since both species yield amplicons with different lengths in five target regions (Figs. 1 and S2). Moreover, the SPInDel method allows the simultaneous analysis of multiple loci, which provides a clear advantage over methods targeting a single locus (Darling and Blum 2007; Pereira et al. 2008). In cases where one (or more) hypervariable region(s) have the same

length for two species, or fail to amplify by intra-species polymorphisms, a correct identification is still possible based on the information from the remaining target regions. The use of multiple loci also decreases the likelihood of false negatives because the probability of six target regions all failing to amplify by PCR due to polymorphisms is low, and certainly much lower than in methods using a single pair of PCR primers. The occurrence of false-positives caused by intra-species polymorphism is unlikely using our approach because most species diverge by several target regions, as we have previously shown (Pereira et al. 2010). Moreover, the frequency of intra-specific indels that may cause length differences is low even in non-coding mtDNA regions (Pearce 2006). Even if polymorphisms change the length of some target regions, the resulting profile will resemble more the correct species than any other.

Our method is potentially suitable for low-quantity and/or degraded DNA samples (e.g., faeces, hair and urine) by including primers for three regions that are shorter than 200 nucleotides (Hajibabaei et al. 2006; Eichmann and Parson 2008). The two markers that were amplified in all samples (SPID2716 and SPID1350) were those with the shortest sequence lengths (<152 nucleotides) and are likely to be most viable in species discrimination using degraded DNA. We have previously shown that our multiplex PCR approach works well on samples with degraded DNA by using highly processed food products (Pereira et al. 2010). Nevertheless, validation studies using fox samples are recommended to assess the level of sensitivity of the assay in the presence of highly degraded DNA collected in the field. Furthermore, the location of primers in highly conserved regions of rRNA genes permits successful PCR amplifications in very divergent species. Overall, we show that our multiplex PCR is sufficiently informative for unambiguous identification of red foxes.

Acknowledgments This work was supported by the Portuguese Foundation for Science and Technology (FCT), Fundo Social Europeu and Programa Operacional Potencial Humano [Investigator FCT program to FP]. CIIMAR was partially supported by the European Regional Development Fund through the COMPETE and national funds through FCT, under the project PEst-C/MAR/LA0015/2013. We thank Colette Harmsen, James Harris, Jack West and Eddie Juras for their assistance.

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