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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

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
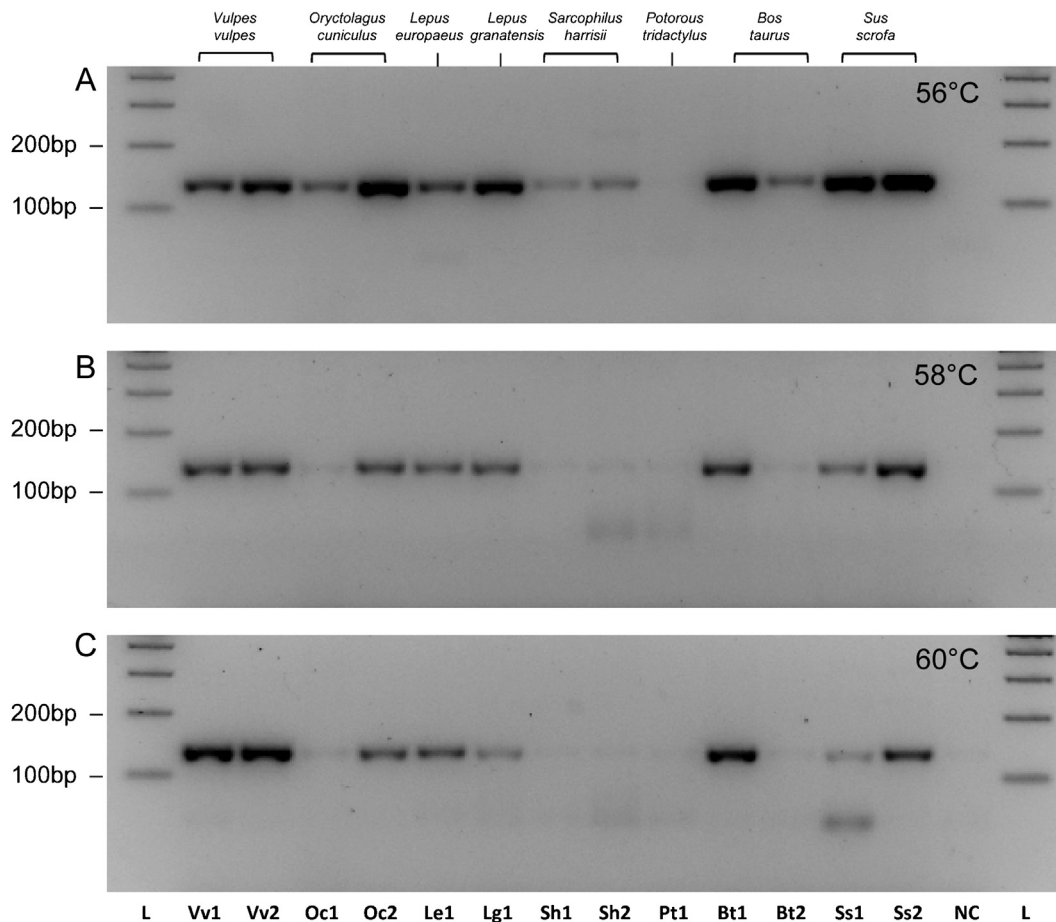
Fig. 1. Amplification of DNA from several species using the putatively *CYTB* fox-specific *VV-cytb F* and *VV-cytb R* primers designed by Berry et al. [16]. Agarose gel (2%) showing the amplification products obtained at annealing temperatures of 56 °C (A), 58 °C (B) and 60 °C (C). Legend: Vv1 and Vv2, 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.

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.

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Received 7 March 2014