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## Letter to the Editor

**Defining specificity in DNA detection of wildlife: Response to Gonçalves et al. “The risks of using “species-specific” PCR assays in wildlife research: The case of red fox (*Vulpes vulpes*) identification in Tasmania”**



Dear Editor,

Gonçalves et al. [1] report an examination of the PCR-based species identification test used to detect foxes from faecal material in the ongoing fox eradication programme in Tasmania. The authors rightly point out that poor design in screening methods may lead to false negative or false positive results and they sought to “better evaluate the specificity of the putatively fox-specific pair of PCR primers” as reported in Berry et al. [2] and applied by Sarre et al. [3]. We welcome the scrutiny of the test as DNA-based tests are being used more frequently in wildlife detection [4] and yet estimation of test specificity and sensitivity is rarely attempted. Unfortunately, Gonçalves et al. [1] have undertaken an analysis of an incomplete imitation of the test we applied. In doing so, they reach conclusions that do not apply to the test as it is conducted in practice nor do they actually estimate test specificity. Taken at face value, the findings by Gonçalves et al. [1] could lead to erroneous interpretations, as was the case in a recent publication in which their findings were cited as support for claims of a high rate of false positives in the detection of Tasmanian foxes [5].

The test for fox DNA [2,3] uses a sequential two phase approach aimed at determining, with acceptable probability (Ramsey et al. in review), whether a scat contains fox DNA or not. The first phase of the test comprises an initial screening of DNA through amplification with the primers *VV-cytb F* and *VV-cytb R* [2]. These primers preferentially amplify fox mitochondrial cytochrome *b* DNA and are used to identify samples that are likely to contain fox DNA on the basis of the size of the fragment amplified. They are used as part of a multiplex PCR incorporating a second ‘universal’ primer pair targeting mammalian mitochondrial 12s ribosomal DNA as a positive test for PCR amplification. If the ‘universal’ primers amplify a product but the primers targeting fox cytochrome *b* DNA do not, then the lack of amplification of possible fox DNA cannot be attributed to PCR failure and is most parsimoniously interpreted as meaning that fox DNA has not been detected. The second phase of the fox DNA detection test is conducted only if a PCR product of the size of the target cytochrome *b* fragment is detected in the multiplex reaction and involves direct sequencing of the PCR product obtained from a second amplification using the *VV-cytb F* and *VV-cytb R* primers only. A scat is considered to contain fox DNA only when a direct match is found between the sequence obtained from the fragment amplified and published fox sequences [2,3,6]. This sequential two phase approach, which is described explicitly in Sarre et al. [6], was adopted to make cost effective the screening of predator scats in the large numbers typically required for the

detection of rare fauna. The specificity of this approach is a product of the combination of the two phases of the test and cannot be meaningfully estimated without considering both phases.

In undertaking their evaluation, Gonçalves et al. [1] chose to examine only the first PCR product size screening phase (and then only partially as they analysed the universal primers in a separate PCR, not as part of a multiplex) and did not include the second, critical, sequencing step. Their assessment therefore is not as they imply, an evaluation of the test, and can be considered as only an evaluation of part of the test. Even then their failure to adequately replicate our published experimental design makes their evaluation deeply flawed. Specifically, they extracted DNA from a series of domestic and wild species likely to be found in Tasmania and tested the ability of the *VV-cytb F* and *VV-cytb R* primers to amplify a PCR product at annealing temperatures ranging from 51 °C to 62 °C. Under these temperatures and their own PCR conditions, they amplified product from DNA extracted from a number of species including foxes, rabbits, hares, cows, pigs, and Tasmanian devils. We have previously shown that the fox primers amplify rabbit and hare [3] so their finding of amplification from these species is not new. Nevertheless, the amplification of other taxa under different amplification conditions is worthy of comment. We recently conducted blind trials of 527 scats of known origin including foxes, cats, dogs, Tasmanian devils, and two species of quoll (Ramsey et al. in review). On no occasion in these blind trials did we amplify DNA from the non-fox carnivores; findings that are in total agreement with the extensive cross-species testing on high quality DNA conducted in our original experiments [2]. Our data therefore run counter to the findings of Gonçalves et al. [1] who amplified DNA from Tasmanian devil tissue as well as non-marsupial species.

So, how might these disparate results be explained? We think that the most likely explanation is that variations in PCR conditions used by Gonçalves et al. [1] including differences in polymerase enzyme and buffer systems (they used Thermo Scientific PCR Master Mix compared with Bioline’s Biotaq Red), cycling conditions, reaction volumes, quantity and quality of template DNA, and the absence of universal primers make for different amplification conditions and are likely to produce differences in PCR amplification. It has long been known that PCR specificity is influenced by each of these components and that optimisation is required if specificity is to be maximised accordingly [7,8]. It is not clear from their publication [1] that a full optimisation process for maximising specificity has been undertaken and we contend that insufficient optimisation by Gonçalves et al. [1] is the likely cause of the disparity between their results and ours.

Contamination, most commonly from aerosol PCR product, is an additional factor that needs consideration. Unless appropriate precautions are taken, contamination of PCR amplifications from mtDNA target sequences is extremely likely in situations where multiple PCRs are conducted over time [9,10]. Precautions that we

take in any study of trace DNA include the extraction of DNA and setup of PCRs in a dedicated Trace DNA facility located in a separate building not subject to potential airborne PCR product, rigorous application of sterile techniques, one-way flow of personnel and materials from PCR reagent setup to DNA extraction to PCR amplification, and the running of multiple negative controls with each PCR.

Irrespective of the underlying cause of the false positives identified by Gonçalves et al. [1] what they have been able to show is that in their laboratory, under their conditions, they produced a high rate of false positive PCR products.

On the basis of the analysis described above, Gonçalves et al. [1] make the astonishing claim that “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”. It is not relevant to make this claim given that sequencing (post-PCR phase) has already been identified as critical in the classification of samples as positive for fox DNA [2,3,6].

Any diagnostic test will include the possibility of false positives. The risk of such false positives must always be weighed against the risk of ignoring positive results, and as a consequence, taking no management action. Foxes have decimated native wildlife on the Australian mainland [11,12], with many affected species now restricted to Tasmania. It is likely that were foxes to become established in Tasmania, they would do the same to wildlife there [13]. Ignoring the evidence of foxes in Tasmania risks a catastrophic and irreversible change to native fauna in that state, and for some species, extinction in the wild.

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