Forensic Science International: Animals and Environments 5 (2024) 100080

Contents lists available at ScienceDirect

Forensic Science International: Animals and Environments

journal homepage: www.sciencedirect.com/journal/forensic-science-international-animals-and-environments

A guide for the validation of DNA based species identification in forensic casework

Lucy M.I. Webster [a,*], Tracey-Leigh Prigge [b], Greta J. Frankham [c,d]

[a] Wildlife DNA Forensic Unit, SASA, Edinburgh EH12 9FJ, UK
[b] School of Biological Sciences, University of Hong Kong, Hong Kong, China
[c] Australian Centre for Wildlife Genomics, Australian Museum Research Institute, 1 William Street, Sydney 2010, Australia
[d] Centre for Forensic Science, University of Technology Sydney, Ultimo, NSW 2007, Australia

ARTICLE INFO

Keywords:
Wildlife forensic science
Method validation
Guidance
Targeted species ID
Developing laboratories

ABSTRACT

Method validation is an essential step ahead of applying a method in forensic casework, to ensure the results will be admissible in court. However, unlike mainstream forensic disciplines, wildlife forensic labs often evolve from conservation-oriented units and may not have a strong foundation in generating data within a legal context. As such, the processes and principles of method validation may not be familiar or fully understood. In this paper we describe the process of method validation in a wildlife forensic science context. We provide guidance on the documentation required to take a DNA based method, which has been developed to identify a specific target species, through the validation process so that it is fit for use in forensic casework. This process has been agreed upon among members of the Society for Wildlife Forensic Sciences (SWFS) Technical Working Group (TWG) to illuminate the requirements for both practitioners and academics.

1. Introduction

There are a vast number of species of animals and plants which may be illegally traded [1] or involved as silent witnesses to crimes; and their biological traces can help investigators prove a crime has taken place or link a suspect to a crime (e.g. [2]). These traces should be analysed at a laboratory which is set up to forensic standards. The field of wildlife forensic science requires practitioners to use validated methods in forensic casework [3], however, for labs moving into this field or wishing to make a research method they have developed ready to use for casework, the process of method validation to forensic standards is not always obvious. Even the terminology may be unfamiliar -see Box 1 for definitions we will follow and Fig. 1 for an illustration of the process. The full process of method validation is rarely published in peer reviewed journals, and in-house validation documents are generally not accessible to those outside an organisation. Some aspects of routine laboratory work have already been extensively tested and only require verification within a new forensic lab (e.g. Sanger Sequencing [4]), whereas other novel aspects will require validation. This paper documents the method validation process agreed on by the SWFS TWG for a wildlife forensic laboratory, using DNA-based targeted species identification (ID) with a novel set of primers as an example. The method will define the "target" - it could be a single species or a range of related species - and the result interpretation will be based on DNA sequence data. Targeted species ID tests such as this are required if forensic samples are likely to contain DNA from more than one species, when general tests incorporating universal primers (e.g. [5,6]) would co-amplify from each species present and lead to a mixed, uninterpretable DNA sequence when using the traditional Sanger Sequencing method [4]. This example is currently a common task for a wildlife forensic laboratory carrying out DNA analysis, but the validation process may be applied to other methods and can also be adopted by research groups who wish to see their methods applied in criminal casework.

2. The validation plan

The validation plan (VP) is prepared before you start your method validation and should be reviewed by a colleague before the experiments begin. You should carefully consider exactly what needs to be validated. For simplicity, our example involves a targeted species ID test with new PCR primers, but does not involve validation of methods used before or after PCR. Aspects such as DNA extraction, PCR cleanup and data analysis are not included here but should still be verified or validated prior to use in casework. Your VP should include the following

* Corresponding author.
E-mail address: Lucy.Webster@sasa.gov.scot (L.M.I. Webster).

https://doi.org/10.1016/j.fsiae.2023.100080
Received 4 July 2023; Received in revised form 10 October 2023; Accepted 6 December 2023
Available online 7 December 2023

sections:

2.1. Background and scope

Include a short literature review of information relevant to the method. For our targeted species ID test: What gene region and species group is the method designed to target? Has the taxonomic status of target species been demonstrated using genetic methods and the specific gene region being used? Is hybridisation an issue? Do you have reference data from all congeneric species? It is also useful to document the enforcement need for the specific method (e.g. are all species from a genus included in CITES? If so, will a genus-level ID be enough or does legislation require a species-level identification?). This enforcement need will help you to define the scope of application for the method that you are validating. Also consider the potential reporting limitations for your method in relation to the enforcement requirements, as absence of a DNA sequence from a target does not equate to absolute evidence of absence of that species.

2.2. Details of the method

It is crucial to note that the method to be validated must have already been through a development phase and have been finalised. Specify full details of the finalised method to be validated, including characterisation of the target locus, primer sequences, PCR mastermix recipe (including manufacturer details for specific components), PCR cycling conditions, and any equipment in your laboratory that will be used in the method components of the validation. Parts of the process which are not included in this validation (e.g. DNA extraction, PCR cleanup and data analysis) should be referenced. Document any prior experimentation carried out in the method development phase to ensure that the method will meet the scope (e.g. preliminary specificity check), especially levels of intraspecific and interspecific diversity observed at the target locus. Also document any risks from either a health and safety perspective (e.g. zoonoses risk from animal tissue) or to the judicial system (e.g. how can you mitigate for errors?).

2.3. Validation parameters

Specificity, sensitivity, reproducibility and robustness are common parameters to clarify via the validation process and this section should document all relevant parameters and how they will be tested [7]. Importantly, this section will also determine what criteria your laboratory requires in order for a method to pass a validation and/or be accepted for use in casework. An example of this can be seen in Table 1.

2.4. Review

Once you have finalised the above sections, it's a good idea to have a colleague who is familiar with the type of method you are validating review your plan. This may lead to revisions before the validation experiments can begin. For some organisations this review will be a formal approval, with the plan becoming a controlled document and any alterations requiring a version change and re-approval.

Instead of separate VPs for each individual protocol within the lab, some labs produce generic VPs that can be applied to new methods of a particular kind such as the example described in this paper of a new primer set for a targeted species ID test. Therefore, once a generic VP has been written and approved, further VPs for this type of method type may not be required. In such cases, the detail noted in Section 2.1. and 2.2. above would instead be incorporated into the Validation Report (see Section 4).

3. Laboratory validation

In this step, the lab completes the experiments outlined in Section 2.3. "Validation parameters" of the VP, applying the method from start to finish for all experiments. It is crucial to keep careful records of your laboratory work during this process - for example, names of the analysts carrying out lab work, lot/batch numbers of critical reagents used, unique identifiers for pieces of equipment used, traceable IDs for samples/DNA extractions/sequences, and the use and results for the positive and negative controls used for the validation work. All reference material and DNA extractions used for the validation should be traceable back to specimens originally identified by someone who is competent in the relevant taxonomy, and wherever possible from a voucher specimen (Box 1, [3]).

During this process, you may realise that the method will not meet the criteria documented in the VP to pass the validation. For example, your method may amplify DNA from a non-target species that you have specified that it should not target. When this happens, it may be necessary to stop the validation process and re-enter the development phase (see Fig. 1). Once method refinement is complete, the relevant sections of the VP (e.g., Section 2.2. "Details of the method") should be updated to include details of the extra development experimentation, and then reviewed, prior to restarting the laboratory validation phase. If,

Box 1
Glossary.

Forensic casework: Analytical services carried out to forensic standards on items/evidence relating to a criminal investigation.
Forensic standards: Mandatory minimum practices necessary to ensure that analysts performing work for criminal casework produce accurate, precise analytical findings and convey these findings in an unbiased, objective manner. They can be modified in response to new information and innovations.
Internal validation: The accumulation of test data within the laboratory for developing the laboratory standard operating procedures and determining the limits of the method(s). Internal validation demonstrates that the established protocols for the technical steps of the test and for data interpretation perform as expected in the laboratory[21].
Method validation: The process of performing and evaluating a set of experiments that establish the efficacy, reliability, and limitations of a method, procedure or modification thereof; establishing recorded documentation that provides a high degree of assurance that a specific process will consistently produce an outcome meeting its predetermined specifications and quality attributes[21].
Reference material: Biological specimens of known identity or data derived from them[3].
Voucher sample: A type of reference material of known identity that is stored (e.g in a museum) with information, e.g. geographic origin, life history, stage and sex, on record, and which can be independently verified if required[3].
Wildlife forensic science: The application of a range of scientific disciplines to legal cases involving non-human biological evidence (Society of Wildlife Forensic Sciences).

however, a generic validation plan is being used, the additional development steps carried out should be documented, prior to commencing further validation. We would strongly recommend that any key requirements for a method are fully tested in the development phase wherever possible to avoid additional work to the VP.

4. Validation report

Once the experiments needed to address Section 2.3. of the VP have been completed, the results should be collated into a validation report (VR). This document should include all of the validation plan sections as well as the following:

4.1. Assessment of performance against criteria

Go through each of the experiments for the parameters (e.g., Table 1) and present data that supports your assertion that the method passes validation. These data may include gel images, DNA sequence traces and phylogenetic trees. Where there are parameters that are being characterised rather than having a specific pass/fail criteria, document what your experiments have found (e.g., acceptable temperature windows for PCR amplification).

4.2. Conclusions and recommendations

Has your method passed the validation exactly as documented in the VP? Does it meet the scope you defined? Or does the scope of application need to be altered? In some cases, there may be some limitations to the method identified during validation which do not cause the method to fail the validation, but they may add caveats on the application in casework. For example, your method may have been designed to target DNA from the canid family, however your specificity study has identified co-amplification with some bird of prey species. This may mean that, while the method is fit for purpose in most scenarios, it would not be appropriate in cases where there could be a mixture of canid and bird of prey DNA. Once the scope has been clarified and the method has passed validation, the method will then need to be incorporated into a Standard Operating Procedure, and staff training can begin.

4.3. Authorisation (technical review)

The VR should be reviewed by someone independent of the validation process but familiar with the methods - it could be the same person who reviewed the VP. This review may lead to amendments to the report but, if additional experiments are requested, this would need a further revision to the plan - and potential additional review with a further version number and re-authorisation as indicated in Fig. 1.

5. Publication

Together, the VP and VR document the overall validation process for a forensic laboratory's quality management system. These are invaluable internal documents, but making them accessible to the wider wildlife forensic community as-is is not straightforward. They are not considered sufficiently novel for some journals, and the format may require significant editing into a manuscript for publication. We recommend, however, that labs that are developing and validating new methods strive to publish their validation studies following similar examples in the literature (e.g., for some targeted species ID methods see [8–12]. Peer review and publication prior to application in casework is considered essential in some jurisdictions, such as with the Daubert standard in the USA [13] and the Forensic Science Regulator's codes in England and Wales [14]. Publication will enable other forensic practitioners to verify and swiftly adopt your method while avoiding duplication of effort whereby labs independently develop and validate different forensic methods to answer the same investigative question.

6. Discussion

Wildlife forensics has been a recognised forensic field for over a quarter of a century (e.g. [15]), and was formalised into a specialist discipline with the establishment of the Society of Wildlife Forensic

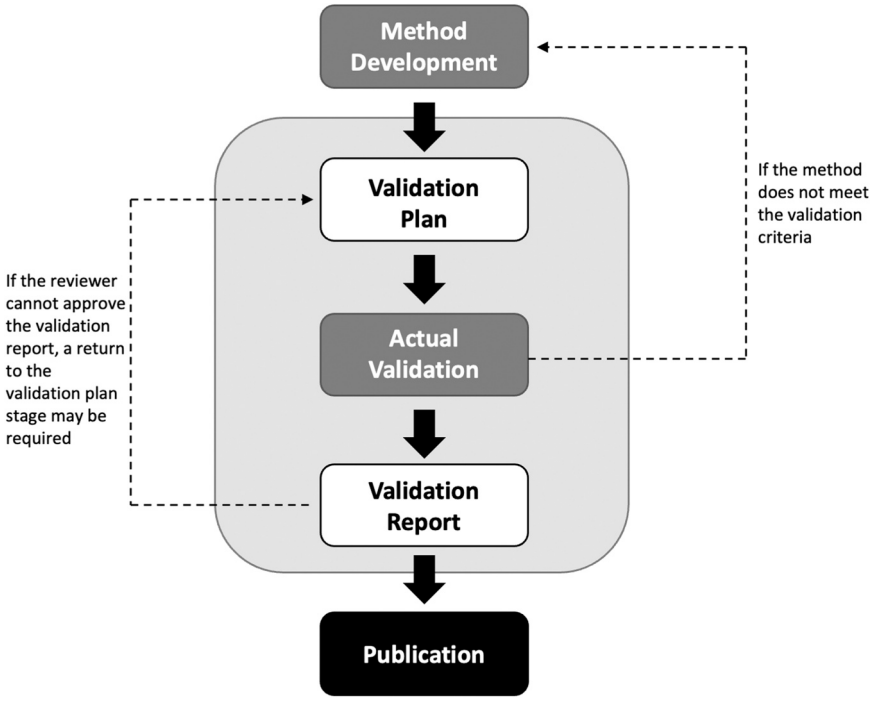

Fig. 1. Flow chart of the validation process. The steps in the light grey shaded box (validation plan, actual validation and validation report) are described in this paper. Method development and actual validation (dark grey) are experimental processes that are carried out in the laboratory. The validation plan and validation report (white) are internal laboratory quality documents. Publication (black) is highly recommended and would ideally be in an open access journal.

Table 1
Validation parameters to consider for a targeted test for DNA-based species identification. Sections in italics provide additional guidance to allow you to tailor this table to your laboratories requirements.

Parameter	Guidance	Example validation exercises	Criteria for pass / Characterisation
Specificity	You need to know that your method will work within the required scope - amplifying the targets and perhaps also not amplifying contaminant species (e.g. human DNA). Part of this may be a desk based in-silico study - using available online sequences - but a practical demonstration of specificity will be required for key species to be targeted, avoided and just evaluated for species regularly processed by the lab in case of cross contamination. Where potentially problematic co-amplifications are identified, mixture studies can help to characterise the impact of the issue in practice. It may be that if the target is present, even at low concentrations, the non-target is not amplified.	1. Apply method to DNA extracts of closely related species, and species which may co-occur or be substitutes of the target species. 2. In-silico study using available online sequences for relevant closely related species not available for 1. 3. Apply method to DNA extracts of species regularly encountered in the lab for potential cross-amplification - *include humans, pets, and species you frequently work with.* 4. Optional: Mixture studies - Different mixture ratios can be used to investigate the limits of detection and/or patterns of preferential amplification.	Species which must not co-amplify: *list all that are relevant to your scope* All of these exercises will help to characterise the method and what species may cross-amplify. Any mixture studies performed will clarify casework scope where the method should not be applied. *In some cases you may specify that it must not amplify certain species such as humans, in order to pass the validation. However, we would recommend that wherever you have these key requirements that you have tested them during the method development phase.* *The characterisation of the species-specificity could impact scope for method implementation.*
Sensitivity	This is where you establish how sensitive your method is from both a working analytical standpoint (i.e. concentration) and also for the casework-type samples that will be encountered (i.e. potentially degraded samples). The analytical sensitivity will require a dilution study. The casework type samples may incorporate suitable DNA extracts from previous casework or initiate experiments to generate these sample types. Where required this could include an inhibition study, but this will be entirely dependent on likely contaminants in casework.	1. Analytical sensitivity: Create a serial dilution of DNA from several target sources and apply the method. *Suggested minimum of 3 individuals input DNA from 1 ng - 10fg*	1. To characterise the sensitivity of the method, however it should work for at least the strongest X (suggest 3) dilutions in the series.
		2.Casework relevant sample types: Apply method to relevant DNA extracts from closed casework *(where available)* and/or a range of sample types known to occur in casework.	2. This experiment will characterise the success of the method on known casework samples and a range of sample types, where available.
		3. Optional: Casework relevant sample types: Set up an experiment using biological material from the species of interest to generate casework relevant sample types (e.g. blood on traps left outside, swabbed 2 weeks later). Or work with a local zoo or wildlife park to collect casework relevant samples for testing, e.g. shed skins, feathers, scats, swabs from enclosures).	3. If necessary, where samples for 2 are not available, this experiment will characterise the success of the method on casework-like samples.
		4.Optional: Inhibition study. Create experimental replicate samples with relevant contaminants likely to be encountered in casework for this species. Apply target DNA to a swab, and rub against the contaminant (e.g. soil, denim, soap, food additives). Extract DNA from replicate swabs and apply the standard method.	4. If necessary, this inhibition study will characterise the impact of likely contaminants. This will inform the interpretation of results where no amplification is observed, and also allow appropriate guidance when problematic contaminants are known to be present in case samples.
Robustness	These experiments will alter some of the details of your method to determine how robust it is to minor alterations. Consider which aspects reflect realistic changes or alterations that you may wish to make for different cases. This may include alterations to annealing temperatures, PCR cycle number and/or PCR reagents. A Temperature study will inform you whether your method is robust to minor alterations where the PCR machine may be out of calibration. If increasing PCR cycles can give a clean DNA sequence for analysis, it could be an option for a case where only very low amounts of DNA are likely to be present. The testing of alternative PCR reagents within the validation study will identify those which you will expect to work under standard conditions.	1. Temperature study - apply method using a gradient PCR program from -2^{o}C to $+2^{o}$C across all cycling steps using both limiting and adequate DNA input *(from results in Sensitivity study 1).*	1. To characterise whether slight changes in temperature will cause the PCR amplification to fail
		2. PCR cycle increase - apply the method at + 5, + 10 and + 15 cycles using both limiting and adequate DNA input.	2. To characterise the effect of over-cycling.
		3. Optional: PCR reagents - test the method with alternative PCR reagents (e.g. Taq mastermix) using at least 3 DNA extracts at both limiting and adequate DNA input.	3. To identify other reagents which may work with the method, and could be used if the original product is no longer available. There is a possibility that this will also identify reagents that work better than the original.

(*continued on next page*)

Table 1 (*continued*)

Parameter	Guidance	Example validation exercises	Criteria for pass / Characterisation
Repeatability	These experiments should demonstrate that your results are repeatable on different days and using the same protocol on different instruments. It also demonstrates that the method gives the same results using different samples of the same species. Where there is any variation in your results this can be used to provide documented, quantifiable error rates in your validation report which will be of value to the court. It can be difficult to source multiple voucher samples (see Box 1) for validation experiments. Provided the DNA sequence is consistent with other examples from this species that can be considered validated it is acceptable to use non vouchered DNA extracts (e.g. from research or case samples) to increase the number of different individuals that can be represented in the repeatability study.	DNA extracts from at least five different individuals from the target species, ideally from different parts of their range should be used. The DNA sequence from each individual should already be recorded, and the method should be applied on four different occasions over the course of a month, using different PCR machines and changing the position of the extract on the plate or tube placement for each replicate.	The DNA sequences produced should be identical to the known sequence, and of similar quality. Where there are sequence differences this allows quantification of the sequencing error rate. *For short sequences there are unlikely to be any differences in sequence, and published estimates of Sanger sequencing error can be used* (e.g. *error rate of 0.001%;*[17]*).*
Reproducibility	These experiments should demonstrate that your results are reproducible among analysts and, if possible, among laboratories using the same method. We would recommend that a pre-test should be carried out at a new lab before running this validation experiment, fully documenting all of the instruments and reagents to confirm that a reproducible result should be achievable.	1. Within-lab: Two different analysts should each run two replicates of the repeatability experiment. 2. Optional: Between-lab. Exchange known samples amongst labs for an additional replicate of the repeatability study to be carried out at a different laboratory.	The DNA sequences produced should be identical to the known sequence, and of similar quality.
Quality controls	Negative controls should be included in all validation experiments to confirm that contamination has not influenced any of the interpretation of the results. Any contamination should be identified to establish the source. Positive controls should also be included as they provide an independent evaluation of the success of the PCR and sequencing reactions under the same conditions.	Include negative and positive controls in all experiments. Ideally, positive controls should be from a non-target species source. If the primers are so specific that a non-target species cannot be used, effort should be made to use a sequence haplotype that is unique and recognizable, or to design an artificial control *(for an example see*[22]).	All controls should work as expected across all validation experiments.

Sciences in 2009 and the European Network of Forensic Science Institutes' Animal, Plant and Soil Traces expert working group in 2010. However, the community is still in a transitional phase globally with many wildlife forensic labs establishing across the world, and other labs are keen to venture into this arena. The impetus behind this paper is to demystify the necessary forensic validation process, which goes beyond the development of a method in a research setting to further demonstrate its suitability for application in criminal casework. Our aim is also to give practical tips on the experiments to run and the parameters to consider. Method validation is an essential step towards meeting the standards required for admissibility of expert testimony [13,14]. It is important to note that if your laboratory is working towards carrying out casework there are many aspects of the laboratory, aside from just method validation, that need to be carefully considered, and put in place prior to or in parallel with method development and validation (see [3, 16]). While we have used the development of a targeted species ID method as an example, we hope the framework of the VP and VR can be used across the spectrum of scientific fields currently being developed for wildlife forensic applications.

The example used in this paper to outline the sorts of experiments required in Table 1 is a common validation process among wildlife DNA forensic labs. While newer mass parallel sequencing methods hold the potential to deconvolute all constituent species from a mixed sample, validations of such methods are yet to be published. The experiments we describe in Table 1 are recommendations for how a lab could validate the different parameters listed for this type of method, but they are not fixed. As the saying goes, there is more than one way to skin a cat; to expand on that, there is more than one way to validate a method to determine what kind of cat was skinned. For example, ideally casework-relevant samples from closed cases will be included in the validation (e.g. [9,12]) but in practice labs may not have relevant closed cases at the point of validating a new method. In these instances, samples which reflect the type of sample likely to occur in casework can be used (e.g. [10,11]) or created via experimentation.

Specificity studies are crucial to characterise the method and the scope of its application in casework. As such, the species which must not co-amplify with the target should be tested during the method development phase, rather than waiting until actual validation (Fig. 1). This is why consideration of the enforcement question and context for developing the test in the first place is important to document. This process should identify which species need to be included in specificity studies, e.g., closely related species in the same genus, co-occurring species, or species commonly trafficked together, as well as common contaminants (e.g., DNA from Humans, detector dogs, or other domestic animals). Failure to include these species during method development could lead to a failure at the validation stage, and require the development of a new method and additional validation studies. It is, however, impossible to test all non-targets, and if non-target amplification occurs with species unlikely to co-occur with the target in casework, the validation can still be considered successful. For example, a test developed to target a fragment of the cytochrome b gene in American mink (*Mustela vison*), was found to amplify a non-specific product of a similar molecular length when applied to sheep (*Ovis aries*) DNA extracts (SASA, unpublished data). In casework, mixtures between these two species will be rare, thus the test will still pass the validation, while highlighting the need to sequence the amplicons to avoid false positives, rather than relying on the presence/absence of amplicons.

The sensitivity experiments described in Table 1 will illustrate the limit of detection in ideal, single-source extracts, but they will not translate to a quantitative limit of copy number, as this is not a quantitative PCR method. In casework, where non-ideal, "dirty," and mixed-source sample types are the norm, the investigative question is usually to identify the presence of biological traces from a particular target species. Standard PCR followed by DNA sequence analysis to confirm the species present is more common than quantitative PCR in wildlife forensic labs, negating the need for quantitative interpretation of the DNA present. It is important to make it clear, however, that absence of amplifiable DNA does not equate to proof of absence of the species, and wording for negative results should take into account detection limits established from the validation experiments. Extract quantification using spectrophotometry or fluorometry is encouraged, however it is not a necessary step. Quantification may indicate where a sample should be diluted prior to application of a targeted species ID test if the original sample is single source to avoid possible PCR inhibition. For example, bone extracts may have a much higher concentration of DNA than ivory extracts, and so the input of DNA extract into the PCR reaction should be based on concentration rather than a standardised volume from an undiluted extract (M. Burnham Curtis, personal communication).

Precision and accuracy are parameters we have not included but are often used and quantified in validation studies for methods involving a measurement, to assist with the calculation of error rates. For this example, the DNA sequences produced do not provide a relevant measurement for this parameter to be assessed, however sequencing error rates can still be calculated based on results from the repeatability experiments (Table 1) and published estimates are also available for Sanger sequencing [17]. An appropriate statistical method has not yet been established for the interpretation of DNA sequence data for taxon classification due to the variation in species divergence times and rates of evolution across the evolutionary tree of life. During the method development phase, however, it should be established that there is sufficient genetic distance between the target and closely-related species for the level of identification required. This level may be species, genus or even family-level identification, depending on the law enforcement need, which can vary between countries. The validation planning stage is therefore crucial to document the purpose of the method and how it can be used to identify the taxon of interest and accurately exclude others.

Many publications in the conservation field include the tantalising words "this method could be applied in forensic casework" (e.g. [18–20]) but in practice, without forensic validation they cannot be applied. We would like to encourage labs who develop methods for other purposes such as conservation, and consider them suitable for forensic application, to go through this validation process and publish validation studies alongside the method so that they can be swiftly adopted by the wildlife forensic community.

Ethical statement

This is a guidance document, and no animal samples, or ethics approval has been required from the authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank the Society for Wildlife Forensic Sciences (SWFS) Technical Working Group for their discussion on this topic to make it as applicable as possible to a range of countries. In particular, Kathy Moore, Mary Burnham Curtis and Kim Frazier and two anonymous reviewers for their invaluable comments which have improved this manuscript.

References

[1] UNODC, Trafficking in Protected Species, World Wildlife Crime Report (2020).
[2] J.L. Halverson, C. Basten, Forensic DNA identification of animal-derived trace evidence: tools for linking victims and suspects, Croat. Med. J. 46 (2005) 598–605.
[3] M.K. Moore, B.W. Baker, T.L. Bauman, M.K. Burnham-Curtis, E.O. Espinoza, C. S. Ferrell, G.J. Frankham, K. Frazier, J.L. Giles, D. Hawk, J.J. Rovie-Ryan, R.

N. Johnson, T. Knott, I.L. Kornfield, C. Lindquist, W.D. Lord, K.L. Morgan, R. C. O'Brien, R. Ogden, T.-L. Prigge, P. Schwenke, F.T. Sitam, P. Trail, E. Wictum, P. J. Wilson, B.C. Yates, L.M.I. Webster, The society for wildlife forensic science standards and guidelines, Forensic Sci. Int. Anim. Environ. 1 (2021) 10015, https://doi.org/10.1016/j.fsiae.2021.100015.

[4] M.R. Wilson, J.A. DiZinno, D. Polanskey, J. Replogle, B. Budowle, Validation of mitochondrial DNA sequencing for forensic casework analysis, Int. J. Leg. Med. 108 (1995) 68–74, https://doi.org/10.1007/BF01369907.

[5] O. Folmer, M. Black, W. Hoeh, R. Lutz, R. Vrijenhoek, R, DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates, Mol. Mar. Biol. Biotechnol. 3 (1994) 294–299, https://doi.org/10.1371/journal.pone.0013102.

[6] S.K. Verma, L. Singh, Novel universal primers establish identity of enormous number of animal species for forensic application, Mol. Ecol. Notes 3 (2003) 28–31, https://doi.org/10.1046/j.1471-8286.2003.00340.x.

[7] ANSI/ASB Standard 047 Wildlife Forensics Validation Standard-Validating New Primers for Sequencing. (2019), First Edition.

[8] A.E. Summerell, G.J. Frankham, P. Gunn, R.N. Johnson, DNA based method for determining source country of the short beaked echidna (*Tachyglossus aculeatus*) in the illegal wildlife trade, Forensic Sci. Int. 295 (2019) 46–53, https://doi.org/10.1016/j.forsciint.2018.11.019.

[9] K.M. Ewart, A.L. Lightson, F.T. Sitam, J. Rovie-Ryan, S.G. Nguyen, K.I. Morgan, A. Luczon, E.M.S. Anadon, M. De Bruyn, S. Bourgeois, K. Ouitavon, A. Kotze, M.S. A. Bakar, M. Salgado-Lynn, R. McEwing, DNA analyses of large pangolin scale seizures: species identification validation and case studies, Forensic Sci. Int. Anim. Environ. 1 (2021), 100014, https://doi.org/10.1016/j.fsiae.2021.100014.

[10] K.I. Morgan, K.M. Ewart, T.Q. Nguyen, F.T. Sitam, K. Ouitavon, A.L. Lightson, A. Kotze, R. McEwing, Avoiding common numts to provide reliable species identification for tiger parts, Forensic Sci. Int. Rep. 3 (2021), 100166, https://doi.org/10.1016/j.fsir.2020.100166.

[11] A.O. Brown, M. Ueland, B.H. Stuart, G.J. Frankham, A forensically validated genetic toolkit for the species and lineage identification of the highly trafficked shingleback lizard (*Tiliqua rugosa*), Forensic Sci. Int. Genet. 62 (2023), 102784, https://doi.org/10.1016/j.fsigen.2022.102784.

[12] C.E.R. Hatten, Y.S. Fitriana, T.-L. Prigge, M. Irham, H. Sutrisno, C. Abinawanto, C. Dingle, DNA analysis and validation for species identification of seized helmeted hornbill (Rhinoplax vigil) casques, Forensic Sci. Int. Anim. Environ. 3 (2023), 100058, https://doi.org/10.1016/j.fsiae.2022.100058.

[13] H.A. Blackmun and Supreme Court Of The United States. U.S. Reports: Daubert v. Merrell Dow Pharmaceuticals, Inc., 509 U.S. 579. 1992. Periodical. https://www.loc.gov/item/usrep509579/.

[14] Forensic Science Regulator, Codes of Practice and Conduct for forensic science providers and practitioners in the Criminal Justice System - Issue 7.

[15] G. Shorrock, The success of DNA profiling in wildlife law enforcement, Int. J. Biosci. Law 1 (1998) 327–342, https://doi.org/10.3354/esr00144.

[16] R. Ogden, Forensic science, genetics and wildlife biology: getting the right mix for a wildlife DNA forensics lab, Forensic Sci. Med. Pathol. 6 (3) (2010) 172–179, https://doi.org/10.1007/s12024-010-9178-5.

[17] J. Shendure, H. Ji, Next-generation DNA sequencing, Nat. Biotechnol. 26 (2008) 1135–1145, https://doi.org/10.1038/nbt1486.

[18] P.R. Meganathan, B. Dubey, I. Haque, Molecular identification of crocodile species using novel primers for forensic analysis, Conserv. Genet. 10 (2009) 767–770, https://doi.org/10.1007/s10592-008-9658-2.

[19] A.S. Glen, O. Berry, D.R. Sutherland, S. Garretson, T. Robinson, P.J. de Tores, Forensic DNA confirms intraguild killing of a chuditch (*Dasyurus geoffroii*) by a feral cat (*Felis catus*), Conserv. Genet. 11 (2010) 1099–1101, https://doi.org/10.1007/s10592-009-9888-y.

[20] S. Vargheese, L.M. Chowdhury, S. Ameri, A. Mercy, A. Kathirvelpandian, Character based identification system for Elasmobranchs for conservation and forensic applications, Mitochondrial DNA A DNA Mapp. Seq. Anal. 30 (4) (2019) 651–656, https://doi.org/10.1080/24701394.2019.1611799.

[21] ANSI/ASB Standard 038, Standard for Internal Validation of Forensic DNA Analysis Methods. (2020), First Edition.

[22] L.M.I. Webster, R. McEwing, Resurrecting the Dodo: positive control DNA for species identification, Forensic Sci. Int. Genet. Suppl. Ser. 4 (1) (2013) e140–e141, https://doi.org/10.1016/j.fsigss.2013.10.071.