eDNA detection of alpine newts (*Ichthyosaura alpestris*): Assay design and preliminary testing.

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Foreword

This NECR details work completed by Cellmark for Natural England's District Level Licencing team, who commissioned the work as a link to their great crested newt work. We acknowledge further testing and validation of the assay will be required before it can be used, with confidence, for monitoring, and this report details the initial steps of assay design and testing. Further development of this assay should cite the work done in this report appropriately. Once fully developed the assay will enable the detection of the invasive Alpine newt from a water sample.

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Executive summary

The alpine newt (*Ichthyosaura alpestris*) is an invasive species that poses a risk to the great crested newt (*Triturus cristatus*) in the UK. Early detection of invasive species is key to controlling populations and environmental DNA methods provide the opportunity detect rare species without using traditional monitoring methods. This project developed a single species assay for the targeted detection of alpine newt DNA from environmental samples. The designed assay consists of primer and probe sequences for detection using quantitative PCR.

The assay was tested on environmental samples with positive detection in six out of seven ponds where alpine newt had been confirmed through visual monitoring.

Contents

1. Introduction

1.1 Purpose of the work

Design and development of an assay for the detection of alpine newt (*Ichthyosaura alpestris*) DNA in pond water samples was commissioned by Natural England due to interest in understanding the distribution of this non-native species.

The methodology used for the alpine newt detection assay is the same as the current great crested newt (*Triturus cristatus*) protocol (Biggs and others. 2014) which consists of DNA extraction from pond water samples followed by qPCR.

2. Methods

The target of the assay is an 82 bp fragment of the mitochondrial cytochrome C oxidase 1 (CO1) gene. The target was chosen based on sequence conservation within alpine newt (*Ichthyosaura alpestris*) DNA sequences available in GenBank and sufficient sequence differences to prevent targeting of unintended species. All gene sequences used for designing the primers and probe were retrieved from GenBank (Appendix 1).

An alignment of the target region of the assay including the primers and probe is shown in Figure 1. Due to sequence variability in *I. alpestris*, the forward primer was designed with a degenerate base (M=A/C) as the 8th position from the 5' end. The primers and probe designed for detecting alpine newt DNA are (5'-3'):

Forward primer: GCACTGGMTGAACAGTATACC

Reverse primer: AGTGAAAAGATTGTTAAGTCAACG

Probe: 6-Fam-ACTAGCTGGTAATTTAGCCCACGCTG-BHQ1

DNA sequences of non-target amphibians that are found in the UK or have a high degree of sequence similarity to *I. alpestris* are also included in the alignment (Figure 1). One *Ommatotriton nesterovi* sequence (haplotype A in Figure 1; GenBank accession EF526034.1) was identical to one of the alpine newt haplotypes. Given the sequence differences between this specimen and other *O. nesterovi* samples, it seems likely that this specimen has been misclassified (Appendix 1).

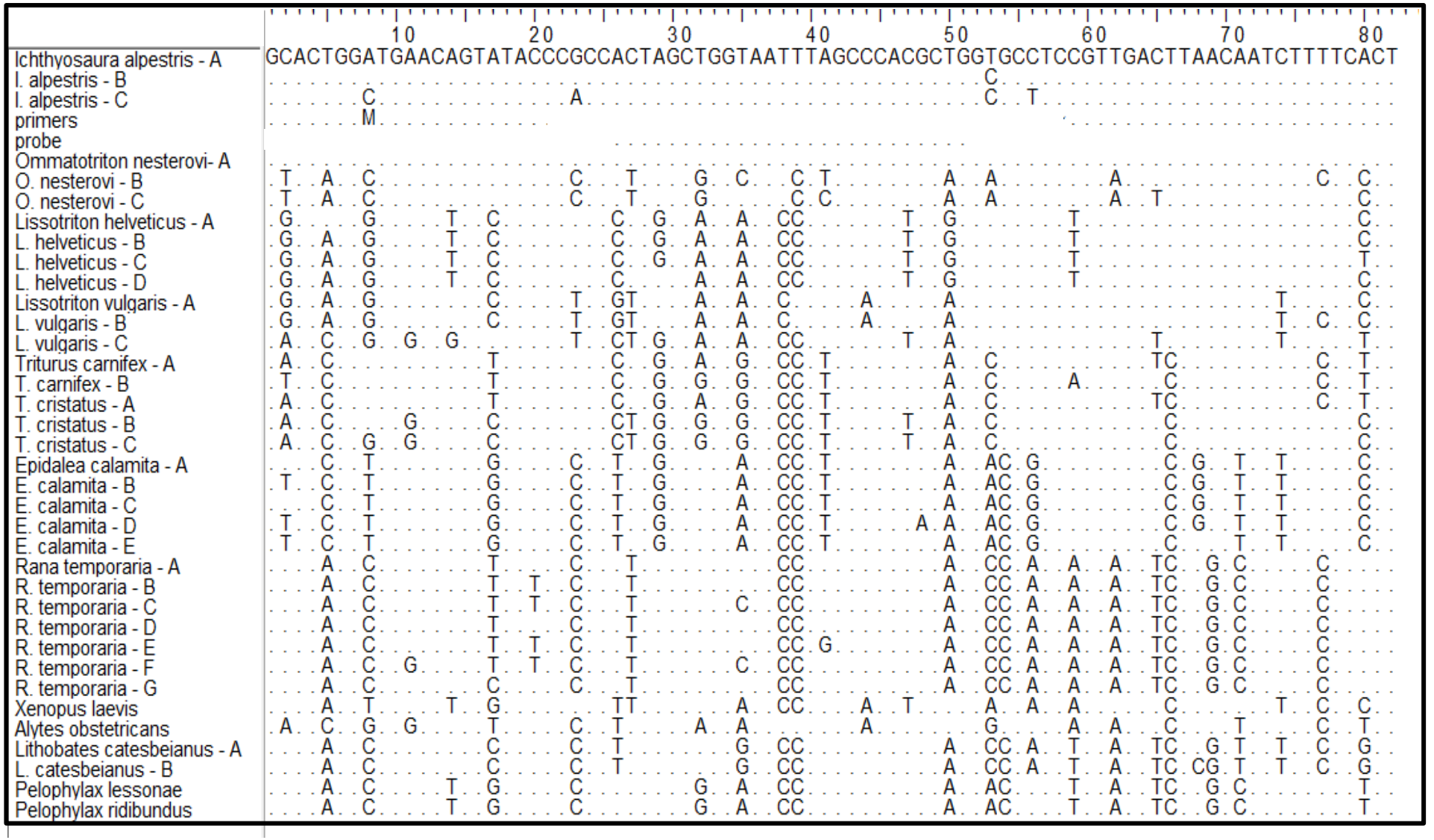

Figure 1. Sequence alignment of the region of the CO1 gene targeted in the alpine newt assay. Different haplotypes for each species are labelled A-G. See text for discussion of *Ommatotriton nesterovi* haplotype A.

The PCRs were set up in a reaction volume of 25 µl as follows:

- 12.5 µl Taqman Environmental Mastermix 2.0 (Applied Biosystems 4396838)
- 1 µl alpine newt primer/probe mix
- 8.5 µl nuclease-free water (Qiagen 129114)
- 3 µl DNA extract

The degradation control primers and probe (labelled with Yakima Yellow) that are used for Cellmark's sampling kits were included in the primer/probe mix. The alpine newt primer/probe mix consists of:

- 10 µM alpine newt forward primer
- 10 µM alpine newt reverse primer
- 10 µM degradation control forward primer
- 10 µM degradation control reverse
- 2.5 µM alpine newt probe
- 2.5 µM degradation control probe

The thermal cycling conditions for the assay using a 7500 real-time PCR system (Applied Biosystems) are:

- 56.3°C - 5 min
- 95°C - 10 min
- 55 cycles of: 95°C - 30 sec
  52°C - 1 min

2.1 Samples

2.1.1 Swabs

DNA was obtained by swabbing the skin of the animals using Copan cotton tipped swabs. The swab samples were dried, shipped to Cellmark at ambient temperature and then frozen on arrival.

Swabs were obtained from a collection of captive animals that had been obtained from a nature reserve in the UK. Skin swabs of smooth newt (*Lissotriton vulgaris*), common frog (*Rana temporaria*), common toad (*Bufo bufo*), and great crested newt (*Triturus cristatus*) were collected at Pendeford Mill LNR in Wolverhampton and Fibbersley LNR in Willenhall by the Birmingham and Black Country Amphibian and Reptile Group committee and volunteers. Five palmate newt (*Lissotriton helveticus*) skin swab samples were collected in

Macclesfield, Cheshire by ACIEEM. These skin swabs were obtained from 3 male palmate newts. It was unknown which swab came from which palmate newt.

2.1.2 Water

Water samples were collected using Cellmark great crested newt kits, shipped at ambient temperature and then stored at 4°C prior to DNA extraction.

Three samples (private garden pond in Oxfordshire, River Ock in Abingdon, pond in Abingdon Business Park) were collected by Cellmark staff. Alpine newts were thought to be absent from these locations. The sample from the garden pond was known to contain frogs/frog spawn at the time of sampling.

Ten pond water samples were collected according to the great crested newt protocol (Biggs and others. 2014). All samples were collected by the Birmingham and Black Country Amphibian and Reptile Group committee and volunteers at the locations listed in Table 1.

Table 1. Ponds sampled for alpine newt eDNA.

DNA extract #	sampling date	location	pond ID	newts present (pos/neg) field data
1	21/06/2022	Spennells Valley NR	S084307505	alpine & great crested newt neg
2	21/06/2022	Spennells Valley NR	S084307505	alpine & great crested newt neg
3	21/06/2022	Spennells Valley NR	S084307505	alpine & great crested newt neg
4	12/06/2022	Fens Pools SAC	S092298877	alpine newt pos
5	12/06/2022	Fens Pools SAC	S092298877	alpine newt pos
6	12/06/2022	Fens Pools SAC	S091988882	alpine & great crested newt pos
7	12/06/2022	Fens Pools SAC	S091168981	alpine newt pos
8	12/06/2022	Fens Pools SAC	S091988882	alpine & great crested newt pos
9	12/06/2022	Fens Pools SAC	S091988882	alpine & great crested newt pos
10	12/06/2022	Fens Pools SAC	S092298877	alpine newt pos

2.1.3 DNA extraction

The DNA from swab samples was extracted with the EZ1 DNA Investigator Kit (Qiagen) on the EZ1 and EZ1 Advanced XL (Qiagen) robots according to the manufacturer's instructions. The DNA was eluted in 50 µl of TE.

The water samples were extracted with the DNeasy Blood & Tissue Extraction Kit (QIAGEN) using the methodology outlined in Biggs *and others*. 2014. The DNA was eluted in a volume of 100 µl.

2.1.4 DNA quantitation

DNA quantification when using mixed source samples and targeting mitochondrial DNA can be problematic. Skin swabs are very likely to contain a percentage of DNA that is not derived from the target species making total DNA quantitation of the sample inaccurate. DNA quantitation when using a mitochondrial target for qPCR can be problematic due to the variability in the number of mitochondria per cell type. Two methods of DNA quantitation were used for this study to get an indication of both the amount of total DNA and the amount of vertebrate mitochondrial DNA in the samples.

The DNA extracted from the swabs was quantitated using the Quant-iT PicoGreen ds DNA Assay (Invitrogen), the QuantiNova SYBR Green PCR Kit (QIAGEN) and/or the QuantiFast SYBR Green PCR Kit (QIAGEN). Fluorescence readings (PicoGreen) and real-time PCR (qPCR) were performed on a 7500 Real-Time PCR system (Applied Biosystems).

The PicoGreen assay was set up according to the manufacturer's instructions except that 45 µl of diluted PicoGreen reagent and 2 µl of sample were used per reaction. For the qPCR assays (QuantiFast and QuantiNova), a fragment of the 12S rRNA gene was targeted. The 12S primers were designed to amplify the mitochondrial DNA target of most vertebrate and some invertebrate species. qPCR reactions were set up and amplified according to the manufacturer's instructions with the exception that the QuantiFast assays were scaled down to a total of 15 µl with an input of 2 µl of DNA. Dilutions of human genomic DNA (Roche 11691112001) were used for the DNA standards.

2.1.5 PCR Inhibition testing

The 10 pond water DNA extracts were tested for PCR inhibition using the Universal Exogenous qPCR Positive Control for TaqMan Assays (Yakima Yellow-TAMRA Probe) Kit (Eurogentec). The reactions were set up according to the manufacturer's instructions except that the total reaction volume was scaled down to 25 µl and 3 µl of DNA extract was added to each reaction. qPCR was carried out on a 7500 Real-Time PCR system.

2.1.6 Alpine Newt Assay Sensitivity

The sensitivity of the alpine newt assay was tested using the DNA extracted from 3 alpine newt swab samples. The DNA concentrations of the undiluted extracts are listed in Table 2. The samples were diluted with nuclease-free water (Qiagen 129114) to the following concentrations: 10,1,0.1 and 0.01 pg/µl of DNA (PicoGreen quantitation values) and 0.1, 0.01, 0.001, 0.0001 pg/µl of DNA (12S quantitation values). For each of the three samples, alpine newt detection reactions were set up in duplicate at every DNA concentration listed including the neat DNA extracts. The degradation marker DNA was not added to these reactions.

Table 2. DNA concentrations of the alpine newt samples used for sensitivity studies.

Sample	PicoGreen ng/µl	12S ng/µl
Alpine newt A (AlpN A)	4.435	0.102
Alpine newt B (AlpN B)	5.801	0.112
Alpine newt C (AlpN C)	0.335	0.008

2.1.7 Species Specificity

DNA obtained from smooth, great crested and palmate newts, common frog, common toad and human DNA (Roche 11691112001) was tested for amplification in the alpine newt assay. Three samples from each species were tested except for great crested newt (n=2) and palmate newt (n=5; samples were obtained from 3 animals). The DNA concentrations of the samples ranged from 6.016/1.092 ng/µl (smooth newt 3) to <0.004/0.000 pg/ul (common toad 2) for the PicoGreen/12S assays respectively.

The samples were assayed in duplicate as described in the “Alpine newt assay design and amplification conditions” section above. The degradation marker DNA was not added to these reactions. Undiluted DNA extracts from 4 alpine newt samples were used as positive controls.

DNA extracted from water collected from a private garden pond in Oxfordshire, the River Ock, and a pond in the Abingdon Business Park were also tested. The DNA extracts were quantitated and tested for inhibition. The PicoGreen DNA quantitation values were 5.73 and 2.40 ng/µl for the ponds and 0.71 ng/µl for the river. No inhibition was detected in the extracts. The alpine newt assay was carried out as described in the “Alpine newt assay design and amplification conditions” section above. Alpine newt DNA was used as a positive control.

2.1.8 Pond Samples

The DNA extracted from the 10 pond water samples listed in Table 1 was tested for the presence of PCR inhibitors. No inhibition was detected in the samples. The samples were then tested for the presence of alpine newt DNA. Alpine newt DNA obtained from swab samples was used for standards/control DNA in the assays. Twelve replicate PCRs were set up for each sample and the DNA extraction negative controls.

DNA extract 7 was tested a second time (12 replicates) and the DNA was quantitated using the QuantiFast SYBR Green assay.

3. Results & Discussion

3.1 Assay Sensitivity

Three alpine newt samples (AlpN A, AlpN B, AlpN C) were used to test the sensitivity of the alpine newt assay. The C_t values of the alpine newt amplicon when 3 µl of each DNA extract was tested in duplicate are shown in Table 3.

Table 3. C_t values of the alpine newt amplicon.

Sample	C_t reaction 1	C_t reaction 2
AlpN A (alpine newt A)	26.92	26.93
AlpN B (alpine newt B)	26.85	26.73
AlpN C (alpine newt C)	30.34	30.25

The C_t values obtained when 3 µl of diluted DNA were tested are shown in Table 4. Due to the variation in DNA concentrations obtained with the PicoGreen and 12S qPCR DNA quantitation methods (Table 2.), dilutions were set up for each sample using both sets of concentrations. The sensitivity of the assay was 3 pg (PicoGreen) and 0.03 pg (12S qPCR). One replicate of the AlpN A sample had a C_t value at 0.3 pg of DNA (PicoGreen).

Table 4. Ct values of the alpine newt amplicon for DNA concentrations based on PicoGreen quantitation of the neat DNA extract and DNA concentrations based on 12S DNA quantitation of the neat DNA extract.

Sample	PicoGreen DNA concentration (pg/µl)	C_t	12S DNA concentration (pg/µl)	C_t
AlpN A	10	34.93	0.1	37.71
AlpN A	10	36.56	0.1	36.59
AlpN B	10	34.76	0.1	37.19
AlpN B	10	36.22	0.1	38.29
AlpN C	10	34.94	0.1	33.15
AlpN C	10	35.28	0.1	32.84
AlpN A	1	U	0.01	U
AlpN A	1	38.24	0.01	U
AlpN B	1	U	0.01	U
AlpN B	1	38.29	0.01	38.64
AlpN C	1	U	0.01	36.27
AlpN C	1	38.85	0.01	35.73
AlpN A	0.1	38.34	0.001	U
AlpN A	0.1	U	0.001	U
AlpN B	0.1	U	0.001	U
AlpN B	0.1	U	0.001	U
AlpN C	0.1	U	0.001	U
AlpN C	0.1	U	0.001	U
AlpN A	0.01	U	0.0001	U
AlpN A	0.01	U	0.0001	U
AlpN B	0.01	U	0.0001	U
AlpN B	0.01	U	0.0001	U
AlpN C	0.01	U	0.0001	U
AlpN C	0.01	U	0.0001	U

3.1.1Species Specificity

Eight alpine newt skin swabs were extracted, the DNA was quantitated and then tested with the alpine newt assay. Alpine newt DNA was detected in all but 1 of these DNA extracts. The DNA concentration in this extract was too low to be measurable with either the PicoGreen or 12S DNA assays. In the 3 attempts at PicoGreen quantitation, this sample had fluorescence values that were lower than the negative control. The C_t value was undetermined when 12S quantitation of this sample was attempted. There was carryover of the magnetic beads used in the DNA extraction process and the eluate was discolored, therefore, the lack of DNA was most likely due to failure during the extraction process but lack of DNA on the swab cannot be ruled out.

DNA obtained from smooth, great crested and palmate newts, common frog, common toad, and human was tested for amplification in the alpine newt assay. The C_t values of the 4 alpine newt samples used as positive controls ranged from 26.9 (AlpN A) to 30.34 (AlpN C). No C_t values (undetermined) were obtained for the other species tested.

Water samples from two ponds (private garden pond in Oxfordshire, pond in the Abingdon Business Park) and the River Ock were tested for amplification using the alpine newt assay. These samples were taken from locations where alpine newts were thought to be absent. Neither amplification of the alpine newt marker nor evidence of DNA degradation was detected in the water samples (Table 5).

Table 5. Alpine newt assay results for water samples collected from areas where newts were thought to be absent. AlpN B is an alpine newt sample that was used as a positive control.

Sample /collection location	target name	C_t
AlpN B 10 pg/µl	alpine newt	35.27945
AlpN B 10 pg/µl	degradation	29.96351
Oxfordshire garden pond	alpine newt	Undetermined
Oxfordshire garden pond	degradation	27.83609
River Ock	alpine newt	Undetermined
River Ock	degradation	27.25349
Abingdon Business Park pond	alpine newt	Undetermined
Abingdon Business Park pond	degradation	27.39985
negative	alpine newt	Undetermined
negative	degradation	29.93671

Pond Samples

Twelve alpine newt qPCRs were set up for each of the 10 pond samples and 3 DNA extraction negative controls. All replicates of the 3 extraction negatives were negative for the alpine newt marker. The results for the pond samples are shown in Table 6 and Appendix 2. All ponds where alpine newts were observed, except for extract 7, had positive replicates.

Table 6. The number of alpine newt positive replicates obtained from 10 pond water samples. *great crested newt

DNA extract #	location	pond ID	newts present (pos/neg) field data	# of positive replicates in alpine newt qPCR assay
1	Spennells Valley NR	S084307505	Alpine & GCN* neg	0/12
2	Spennells Valley NR	S084307505	Alpine & GCN neg	0/12
3	Spennells Valley NR	S084307505	Alpine & GCN neg	0/12
4	Fens Pools SAC	S092298877	Alpine newt pos	8/12
5	Fens Pools SAC	S092298877	Alpine newt pos	9/12
6	Fens Pools SAC	S091988882	Alpine & GCN pos	5/12
7	Fens Pools SAC	S091168981	Alpine newt pos	0/12
8	Fens Pools SAC	S091988882	Alpine & GCN pos	9/12
9	Fens Pools SAC	S091988882	Alpine & GCN pos	6/12
10	Fens Pools SAC	S092298877	Alpine newt pos	10/12

The negative results for pond ID S091168981 (DNA extract 7) were unexpected as alpine newts have been observed in this location. The C_t values of the degradation control in the replicates of DNA extract 7 was slightly higher ($\bar{x}$=35.37; n=12) than the other samples on the plate (alpine newt negative replicates $\bar{x}$=33.38, n=17; alpine newt positive replicates $\bar{x}$=33.97, n=30). This sample was retested and the 12 replicate qPCRs were again negative for the alpine newt marker. DNA quantitation of this extract using the 12S assay indicated a concentration of 0.058 ng/µl DNA.

The increased C_t values of the degradation control marker could indicate that there was DNA degradation or that some of the sample was lost during the extraction process. Either of these could have resulted in the concentration of amplifiable alpine newt DNA being

below the detection threshold of the assay. Alternatively, the amount of alpine newt DNA collected from this pond could have been below the level of detection for this assay.

4. Conclusions & recommendations

In preliminary testing of the alpine newt assay using DNA extracted from skin swabs of various amphibian species, the only positive results were obtained from alpine newt samples. In addition, negative results were obtained from ponds/stream where alpine newts were thought to be absent.

Seven ponds where alpine newts had been observed were tested using the great crested newt sampling and extraction protocols (Biggs *and others* 2014). Alpine newt DNA was detected in 6 of them. It is unclear why alpine newt DNA was not detected in the 7th pond (see discussion above).

Using the 5-level validation scale proposed in Table 3 of Thalinger *and others*. (2021), this assay has been validated to level 3 with additional testing in non-target species.

It is recommended that further eDNA testing is done on ponds where there is supporting data on whether alpine newts are present or absent. Given that there have been multiple introductions of alpine newts into the UK (Beebee & Griffiths, 2000) and the potential of these introduced animals coming from more than one population, it would be valuable to test skin swabs or eDNA samples from a wider distribution of alpine newt populations in the UK to rule out false negative results due to population specific genetic differences.

Appendices

Appendix 1. **Reference DNA sequences for design of assay**

Appendix 2. **Pond assay qPCR data.**

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