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A qPCR assay for the rapid and specific detection of Shining ram's-horn snail (Segmentina nitida) eDNA from Stodmarsh National Nature Reserve, UK

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Abstract

Segmentina nitida Müller 1774 is a freshwater snail which was formerly widespread throughout England and south Wales. Since the 1840s it has seen a rapid decline in its range which has been attributed to deteriorating water quality due to nutrient enrichment, lowering of water tables and over-management of the ditches in which it resides. Segmentina nitida has therefore been identified as a UK Biodiversity Action Plan (UKBAP) priority species which recommends further research for its conservation. Here we have developed a Taqman based qPCR eDNA assay for the detection of S. nitida at the Stodmarsh National Nature Reserve and compared the results with a manual survey of the ditches at this location. Our eDNA analysis exhibited an observed percentage agreement of 84% with a kappa coefficient of agreement between manual and eDNA surveys of 0.56. Three ditches determined to be negative for Segmentina nitida by eDNA analysis were manual survey positive, and a further two ditches that were negative by manual survey were positive by eDNA analysis revealing the potential for improved overall detection rates using a combination of manual and eDNA methodologies. eDNA analysis could therefore augment manual survey techniques for Segmentina nitida as a relatively quick and inexpensive tool for collecting presence and distribution data that could be used to inform manual surveys and management of ditches.

A qPCR assay for the rapid and specific detection of Shining ram's-horn snail (*Segmentina nitida*) eDNA from Stodmarsh National Nature Reserve, UK

Short Title: eDNA detection of Shining ram's-horn snail

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Abstract

Segmentina nitida Müller 1774 is a freshwater snail which was formerly widespread throughout England and south Wales. Since the 1840s it has seen a rapid decline in its range which has been attributed to deteriorating water quality due to nutrient enrichment, lowering of water tables and over-management of the ditches in which it resides.*Segmentina nitida* has therefore been identified as a UK Biodiversity Action Plan (UKBAP) priority species which recommends further research for its conservation. Here we have developed

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a Taqman based qPCR eDNA assay for the detection of *S. nitida* at the Stodmarsh National Nature Reserve and compared the results with a manual survey of the ditches at this location. Our eDNA analysis exhibited an observed percentage agreement of 84% with a kappa coefficient of agreement between manual and eDNA surveys of 0.56. Three ditches determined to be negative for *Segmentina nitida* by eDNA analysis were manual survey positive, and a further two ditches that were negative by manual survey were positive by eDNA analysis revealing the potential for improved overall detection rates using a combination of manual and eDNA methodologies. eDNA analysis could therefore augment manual survey techniques for *Segmentina nitida* as a relatively quick and inexpensive tool for collecting presence and distribution data that could be used to inform manual surveys and management of ditches.

Key Words

Ditch, environmental DNA, qPCR, *Segmentina nitida* , Shining ram's-horn snail, water samples.

Introduction

Management and conservation of rare or at-risk species requires knowledge of species distribution via the detection of populations which could be at low densities. Traditionally, population monitoring is carried out by visual detection, identification, and counting. However, for the last decade environmental DNA (eDNA) has been used as a non-invasive sampling technique as it is reliable, cost effective, less harmful to the ecosystem and correlates well with conventional survey results (Biggs et al., 2015; Darling 2019).

eDNA describes the DNA that is present within an environmental sample for example water, soil, sediment, or air. The eDNA present in an environment includes DNA that originates from sloughed cellular material (e.g. skin cells) or that is excreted (e.g. faeces and urine) or secreted (e.g. saliva) by organisms occupying the environment in question (Rees et al., 2014). Similarly, the DNA of organisms that visit the environment can also be present, for example birds or mammals drinking from a water body. The presence of an organism's DNA within a water body is short lived as DNA has been shown to be degraded to undetectable levels within days to weeks and is affected by UV light, pH and microbial activity (Shogren et al., 2017; Seymour et al., 2018; Zulkefi et al, 2019). This suggests that the detection of an organism's DNA is a demonstration of its presence or very recent presence and is a suitable surrogate for the detection/capture of the organism itself (Pilliod et al., 2013; Strickler et al., 2015;). The analysis of eDNA by sensitive PCR-based approaches, therefore, has become an important tool for measuring species presence/absence and has been successfully used to detect many aquatic species including the highly invasive*Potamopyrgus antipodarum* New Zealand mud snail within rivers (Goldberg et al, 2013; Clusa et al. 2016).

The Shining ram's-horn snail *Segmentina nitida* (Muller, 1774) is a small (4-6 mm diameter) rare European freshwater snail (Figure 1) which is highly sensitive to changes in management of ditches and is found only in good quality, well vegetated ditches (Rowson et al, 2021). Although once common in UK, and widespread across lowland England and south Wales, the range of this species has substantially declined since the 1840s (Kerney, 1999). Its presence has been verified at only a few locations in southeast England where it is mainly confined to marshes and shallow drainage ditches with dense emergent vegetation (Watson and Ormerod, 2003). *S. nitida* is listed as a rare and declining Section 41 species and also a Ramsar criteria feature of Stodmarsh National Nature Reserve (NNR). In order to provide sensitive management of ditches at this site, there is a requirement to know where the*S. nitida* occurs. Here, we describe a qPCR assay for the detection of *S. nitida* and demonstrate the detection of this species within ditch water samples from Stodmarsh NNR with known presence/absence status.

Materials and methods

Sample collection

22 ditch water samples were collected from ditch surface water by Natural England staff at Stodmarsh NNR between the 16th and 27th November 2020 (Figure 2). 10 ditch water samples outside the known range of S. nitida were collected by ADAS staff between the 10th January and the 4th February (sample information can be found in supplemental file 1, table S1 and S2). 20x 30 mL water samples were collected along the

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length of each of the ditch and pooled into sterile sampling bag. Up to 500 mLs of this water was filtered through a 0.22 µM PES sterivex filter (Millipore) using a 50 mL luer-lock syringe (Table 1). After filtration of water, 95% ethanol was added as a preservative.

Snail specimens were collected during an invertebrate survey carried out at the same 22 ditches at Stodmarsh NNR by Dan Bennett of The Ecology Co-op, contracted by Natural England between December and January 2020/2021 (specimen information and detailed sampling methodology can be found in supplemental file 1, table S3). Manual surveys of the 22 ditches were performed using an adapted invertebrate sampling protocol from Drake (2007). The emphasis was on the free-style netting of suitable looking micro-habitats (e.g. emergent vegetation stands) that are likely to be most productive for this assemblage. Effort was deliberately not divided in proportion to the extent of features nor length of ditch, since species are not distributed in this fashion. Snail specimens from *S.nitida* and nine other similar ram's-horn shaped species, from the 16 species of snail) found during invertebrate survey were identified and preserved in 95% ethanol prior to shipment to the laboratory: *Anisus vortex, Bathyomphalus contortus, Gyraulus crista, Gyraulus albus, Hippeutis complanatus, Planorbarius corneus, Planorbis carinatus, Planorbis planorbis, Segmentina nitida, Valvata cristata.*

DNA Extraction

Upon return to the laboratory, the preservative solution was removed from the sterivex filters and any DNA containing material captured on the filter membrane was recovered by addition of 720 µL of ATL lysis buffer and 40 µl proteinase K to the filter, followed by incubation at 56°C in a water bath with regular mixing by vortexing. The supernatant was then extracted using the DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions with final resuspension in 200µl of elution buffer. All DNA samples were quantified using a Qubit 3.0 Fluorometer (Invitrogen) following the manufacturer's instructions then stored at -20 0C before use.

Each snail specimen was individually transferred to a clean, sterile mortar and ground into a fine paste using a pestle and liquid nitrogen. For some snail species, the individual specimens were pooled prior to grinding (see Table 1). After use mortar and pestles were immediately immersed in 10 % bleach for a minimum of 10 minutes and then cleaned in between samples with 10 % Distel (Tristel), rinsed with dH_2O and then autoclaved at 121 0C for 15-20 minutes. DNA was extracted by the DNeasy blood and tissue kit (Qiagen) -with a final elution volume of 50 or 200µl - in a separate laboratory remote from water sample extraction and qPCR set up, using dedicated tissue extraction equipment. Disposable laboratory coats were worn, and benches and equipment were wiped down with a 10% bleach solution before and after use. Extracted DNA was quantified using a Qubit 3.0 Fluorometer (Invitrogen) following the manufacturer's instructions then stored at -20 °C before use.

Snail specimen identification

All PCR set up was performed in a clean 'PCR room' within a UV sterilisable cabinet and in a separate laboratory to DNA extraction using dedicated equipment and PPE. To ensure the unidirectional workflow DNA extracts are collected from the DNA extraction laboratory and transferred to the PCR set-up laboratory. Laboratory personnel do not return to the DNA extraction laboratory during that same day thus maintaining the unidirectional workflow.

PCR was performed to confirm the identity of the provided snail specimens using the mICOIintF/jgHCO2198 primer combination (Leray et al. 2013). These primers amplify a fragment of the Cytochrome c Oxidase subunit I gene (COI) and have been shown to perform well in invertebrate metabarcoding studies (Leray et al. 2013; Geller et al. 2013). PCRs were set up in a total volume of 25 µL consisting of: 2 µL of extracted template DNA, 2.5 µL of each primer (0.4 µmol/L), 12.5 µL of Itaq (BioRad) Sybr Green mastermix, and 5.5 µL ddH2O. Each sample was run in duplicate on a Bio-Rad CFX Connect real-time PCR machine as follows: an initial incubation for 1 minute at 950C; followed by 35 cycles with a melting temperature of 95°C for 1 minute; an annealing temperature of 400C and a final extension step at 720C for 90 seconds before holding at 40C until collection of PCR products for analysis. After PCR and amplicon clean-up, PCR products were

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Sanger sequenced using mICOIintF and returned sequences identified using BLAST.

Species-specific assay development

In order to design primers specific to the *S. nitida* the DNA sequences for the cytochrome oxidase 1 (COI) gene for *S. nitida*and the nine other co-occurring similar ram's-horn shaped species commonly found at the Stodmarsh NNR were downloaded from Genbank and their sequences aligned using BioEdit version 7.2.5. Primers and probes were designed using PrimerBLAST with default settings except for targeting a 70-300 bp fragment and including only base pairs between 70 and 600 in the *S. nitida* consensus sequence as this corresponded to the most variable region on the multi-species alignment. Ten potential primer/probe combinations were generated and reduced to four using PrimerBLAST and looking for cross-species amplification (Supplemental file 2).

The four potential primer/probe combinations were tested firstly on DNA extracted from *S. nitida* followed by the other nine co-occurring snail species to test for cross-species reactivity. PCRs were set up in a total volume of 25 µL consisting of: 3 µL of extracted template DNA, 1 µL of each primer/probe (0.2 µmol/L forward primer; 0.4 µmol/L reverse primer; 0.1 µmol/L probe), 12.5 µL of TaqMan® Environmental Master Mix 2.0 (containing AmpliTaq GOLD DNA polymerase), and 6.5 µL ddH2O. Each sample was run as 12 replicates on a Bio-Rad CFX Connect real-time PCR machine as follows: an initial incubation for 5 minutes at 56.30C then 10 minutes at 95°C; followed by 55 cycles with a melting temperature of 95°C for 30 seconds and an annealing temperature of 59.60C for 1 minute.

Once specificity of primer/probe combinations was confirmed, the primer concentrations of two primer/probe combinations (primer/probe combinations 2 and 9) were optimised by independently varying final primer concentrations (the probe was held at a final concentration of 0.1 µmol/L) (Wilcox *et al* , 2015). The sensitivity of the assay was tested by creating a six-level standard curve dilution series ($3x10^{-1}$ to $3x10^{-7}$ µg/µl). The standard curve was created by quantifying the DNA extracted from*S. nitida* sample 7a on a Qubit Fluorometer (Thermo Fisher Scientific) and diluting the DNA to the desired concentrations using the elution buffer provided in the DNeasy Blood and Tissue kit (Qiagen). 12 replicates of each dilution were run using the optimised primer/probe concentrations to determine the standard curve slope, the limit of detection (LOD) and limit of quantification (LOQ).

The optimised assay using primer/probe combination 9 (Snit9F: 5'- CCACTTTTAATTGGGGCTCCG-3'; Snit9R: 5'- CCATGTGCAATAGGACCGCT-3'; Snit9P: 5'- TGAAGGAGGTGTTGGTACTGGGTG-3', FAM/BHQ-1) was used to determine the presence/absence of *S. nitida* within the 22 ditch samples from Stodmarsh NNR and the 10 ditch samples from outside of the known range of *S. nitida* .

Inhibition testing

All DNA extracts were tested for inhibition by adding 3µL of extract into a qPCR for the amplification of an internal positive control (DNA derived from the plasmid pSD3). PCRs were set up in a total volume of 25 µL consisting of: 3 µL of extracted template DNA, 3 µL of internal positive control DNA (0.08 ng/µl),1 µL of each primer/probe (0.2 µmol/L forward primer; 0.4 µmol/L reverse primer; 0.1 µmol/L probe; Supplemental file 2), 12.5 µL of TaqMan® Environmental Master Mix 2.0 (containing AmpliTaq GOLD DNA polymerase), and 3.5 µL ddH2O. PCR cycling was as above.

Statistical analysis

To measure the agreement between the two survey methods, that is, manual survey and eDNA analysis, Cohen's kappa coefficient (Cohen 1960) was calculated as follows:

$$k = \frac{\Pr(a) - Pr(e)}{1 - Pr(e)}$$

where Pr(a) is the relative agreement among rates, and Pr(e) is the hypothetical probability of chance agreement, using the observed data to calculate the probabilities of each method randomly giving a positive

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detection. If the methods are in complete agreement, then j = 1. If there is no agreement other than what would be expected by chance, j = 0.

Once PCR analyses had been performed, the program PRESENCE version 13.12 [available from http://www.mbr-pwrc.usgs.gov/software/presence.html (Mackenzie et al. 2002)] was used for occupancy modelling of the data. A single-season model was used which assumes that species are never falsely detected at a site when absent, but that may or may not be detected when present; the detection of a species at an individual site is independent of the detection of the species at all other sites; and the probability of detecting the species across all sites is constant.

Results

Detection

The limit of detection (LOD) and limit of quantification (LOQ) of primer/probe combination 9 were found to be $3x10^{-4}$ng/µL and $3x10^{-5}$ ng/µL respectively. The LOD and LOQ have various definitions in the eDNA literature, here LOD is defined as the lowest standard concentration at which 95% of technical replicates amplify and LOQ is the lowest standard concentration for which the coefficient of variation (CV; equal to the standard deviation quantity divided by the mean quantity of a group of replicates) value is <35% (Klymus *et al* . 2019). The LOD corresponded to a Ct value of 37.47 which encompassed 100% of the Ct values in this study i.e. all positive amplifications were above the limit of detection. Detection in a water sample was indicated by at least 1 of 12 positive qPCR replicates (Biggs et al. 2015).

All Stodmarsh NNR ditch water samples and ditch samples from outside the known range of *S. nitida* were subjected to inhibition testing, all samples except two from Stodmarsh NNR (136 and 146) did not cause inhibition of qPCR. Samples 136 and 146 caused complete inhibition of the qPCR inhibition assay.

All Stodmarsh NNR ditch water samples and ditch samples from outside the known range of *S. nitida* were then subjected to the optimised assay for *S. nitida* detection, with seven ditches from Stodmarsh NNR being positive for *S. nitida* DNA (Table 1). One ditch (ditch 34) with very low positivity (1/12), was re-tested to confirm positivity (3/12 on repeat). All remaining ditches were negative for *S. nitida* DNA, therefore other than those ditches where *S. nitida*was found by manual survey - ditches 62, 70 and 108 (Table 1) - *S. nitida* is likely to be absent.

Occupancy Analysis

Using the observed percentage agreement of the two methods of 0.84 (1 = 100%) and the probability of random agreement of 0.64, Cohen's kappa coefficient was calculated as 0.56 for manual survey-positive ponds versus their qPCR analysis results i.e. moderate agreement.

Site occupancy modelling was used to calculate the occupancy estimate and the probability of detection of *S. nitida.* Using the combined manual survey and qPCR assay results the occupancy estimate was calculated to be 0.35 (95% CRI 0.19, 0.56) with a probability of detection of 0.67 (Table 2).

Discussion

This study was carried out to design and evaluate the use of an eDNA assay for the detection of *S. nitida* in ditches at Stodmarsh NNR and to compare to manual survey data taken shortly after water samples were collected. *S. nitida* DNA was detected at 62.5% (5 of 8) of the ditches where *S. nitida* presence was detected by manual survey and in two additional ditches where *S. nitida* was not detected by manual survey (7 ditches in total). A further 22 ditches sampled within Stodmarsh NNR and outside the known distribution of*S. nitida* were negative for *S. nitida.* This resulted in an observed percentage agreement of 84% and a kappa coefficient of 0.56 which shows a moderate agreement between the results. When taken together, manual survey in combination with eDNA analysis has led to improved *S. nitida* detection rates, that is, 10 of 32 ditches rather than the 8 reported by field survey. However, it still should be noted that where eDNA was detected but no *S. nitida* found by manual survey (ditches 98 and 115) it is unknown whether the animals were present but not detected by manual survey (present in very low numbers), or whether they

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are absent and the eDNA drifted into the ditch e.g. through hydrological connections. Ditches 98 and 115 are not directly adjacent to occupied ditches and the direction of flow would not 'normally' facilitate drift between the ditches concerned in normal flow conditions, but ultimately they are connected nevertheless. Site occupancy models can be used to account for imperfect detection and were used by Schmidt et al. (Schmidt et al. 2013) to demonstrate their applicability to eDNA surveys. When applied to the data within this study, site occupancy estimates were greater than the actual observed proportion though not significantly when combining field survey with eDNA assay. This matches the observed increase in positive detections from 8/32 to 10/32 ditches when both techniques were combined.

Where rare or threatened species are concerned, it is likely that their detection by either manual survey or eDNA will be imperfect leading to an underestimation of its distribution.

During particular time periods or developmental stages, some species can be difficult to detect potentially biasing survey outcomes (Gotelli and Colwell 2001; Mackenzie

et al. 2006). This may have been the case here for the two ditches where*S. nitida* was found by manual survey but not via qPCR of eDNA. A study in Poland showed that *S. nitida* breed during April-May (Ksiażkiewicz and Goldyn, 2008) and although conditions may be slightly different in the UK as these ditch water samples were taken outside the likely breeding season their detection will be more difficult as there is likely to be less *S. nitida* DNA in the water. The sampling strategy of taking 20x 30 mL samples along the length of each ditch should have overcome the fact that eDNA can be highly localised in space and time (Li et al, 2019). To achieve a higher level of coverage (especially for longer ditches), more samples may need to be taken. This could allow for targeting more locations within the ditches where*S. nitida* would most likely be present, that is, where the ditch is thickly vegetated, thus improving the probability of detection.

A further cause of false negative results can be PCR inhibition (Jane et al. 2015, McKee et al. 2015). Upon testing of the DNA extracts only two samples, from ditches 136 and 146, were found to cause complete inhibition of the qPCR inhibition assay. These were not from ditches that were positive for *S. nitida* on manual survey so are unlikely to be false negatives. Therefore, it is unlikely that inhibition is the reason for the qPCR of the samples from ditches 62, 70 and 108 being negative for *S. nitida* .

It is also likely that the volume of water filtered will play an important role in the detection of *S. nitida* . The volume of water filtered was between 200 mL and 500 mL for all samples - the volume determined by how soon the filter clogged. A larger pore sized filter eg. 0.45µM or 0.8µM may have allowed a larger volume of water to be filtered which could enable more *S. nitida* eDNA to be recovered at sites where it was found by manual survey. It is common for volumes of water between 500 mL and 5L to be filtered although there is little consensus on the minimum volume (Bruce et al., 2021). Small volumes (0.25 L) have been shown to contain detectable eDNA from macroinvertebrate species when a range of volumes up to 2 L were sampled and analysed (Machler et al., 2016) and increasing the volume of filtered water has been shown to have a positive effect on eDNA capture and PCR amplification efficiency (Muha et al., 2019).

Finally, the density of snails in the ditch system will play an important role in its detectability. The relative abundance of *S. nitida* was recorded during the manual survey (Supplemental file 1), and at sites where the eDNA assay was negative for *S. nitida* but it was found during manual survey, the abundance was recorded as occasional or rare. Studies on the New Zealand mud snail have shown that this snail's eDNA can be detected when snails are present at low densities (Goldberg et al, 2013), this species is similar in size to *S. nitida* , however, far larger volumes of water (4 L) were filtered (using multiple filters if necessary) prior to analysis which could account for its detectability at low density although eDNA transport would need to be considered as these samples were taken from a river system.

Since this study was carried out a study (Hobbs et al., 2021) investigating the population structure of *S. nitida* individuals from Poland, Germany, Sweden and the UK to identify differences both within and between populations has been published. The study found that there are two distinct genetic lineages (also distinct in shape), one in western Europe (UK, Germany – Lineage 1) and one in eastern Europe (Poland, Sweden – Lineage 2). Although only a UK population was tested during the present study it likely that the assay

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designed herein would also detect *S. nitida* populations in eastern Europe as during the primer design phase the three *S. nitida* sequences retrieved from Genbank were from specimens collected in Poland, Denmark and Germany. Future work could involve testing our primer/probe combination on individuals from these other populations.

To confirm the results of the eDNA assay designed herein further manual survey is required to corroborate the two additional eDNA assay positive ditches found. For those manual survey samples where *S. nitida*specimens were found but the eDNA assay did not detect *S. nitida*DNA a larger pore sized filter/filtration of more water may be required to enable eDNA assay corroboration. This study has shown that eDNA assays can be used for the detection of *S. nitida* and if used in the future could have time and cost savings and could inform manual survey and therefore management of the ditches.

List of Abbreviations

Cytochrome oxidase I (COI), Environmental DNA (eDNA), quantitative polymerase chain reaction (qPCR), National Nature Reserve (NNR).

Ethics approval and consent to participate

Water samples and snail specimens were collected from several ditches at Stodmarsh NNR and no institutional animal care and use committee (IACUC) or animal welfare protocol was required for their collection as generally invertebrates do not require approval for use (except cephalopods). No endangered or protected species were collected during field survey and all land was accessed with the permission of the landowners facilitated by the Stodmarsh National Nature Reserve Manager.

Competing interests

None

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Data Accessibility Statement

All data can be found within the main manuscript or supporting files.

Authors contributions

HR and MC conceived and designed the experiments; CB performed the experiments; HR and BM analysed the data and prepared the figures; MC facilitated provision of the samples from the surveyor; HR wrote the manuscript; all authors reviewed the manuscript.

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Table 1. Summary of *S. nitida* survey and PCR status of the 32 ponds studies

Sample ID	DNA (ng/µL)	*S. nitida* species-specific qPCR result	*S. nitida* manual survey
1	<1 ng/µL	0/12; negative	N/A
2	<1 ng/µL	0/12; negative	N/A
3	<1 ng/µL	0/12; negative	N/A
4	<1 ng/µL	0/12; negative	N/A
5	<1 ng/µL	0/12; negative	N/A
D1	<1 ng/µL	0/12; negative	N/A
D2	<1 ng/µL	0/12; negative	N/A
Top	1.7	0/12; negative	N/A
LHS	1.34	0/12; negative	N/A
RHS	<1 ng/µL	0/12; negative	N/A
34	2.14	1*/12; positive	positive
42	2.95	0/12; negative	negative
44	1.71	0/12; negative	negative
56	6.56	12/12; positive	positive
58	11.1	0/12; negative	negative
60	3.96	0/12; negative	negative
62	2.11	0/12; negative	positive
65	3.78	9/12; positive	positive
70	0.84	0/12; negative	positive
87	18.1	12/12; positive	positive

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Sample ID	DNA (ng/μL)	*S. nitida* species-specific qPCR result	*S. nitida* manual survey
92	4.88	6/12; positive	positive
98	4.36	3/12; positive	negative
106	1.66	0/12; negative	negative
108	2.32	0/12; negative	positive
115	2.06	4/12; positive	negative
131	1.08	0/12; negative	negative
135	1.46	0/12; negative	negative
136	<1 ng/μL	0/12; negative	negative
146	<1 ng/μL	0/12; negative	negative
153	1.89	0/12; negative	negative
155	<1 ng/μL	0/12; negative	negative
161	1.35	0/12; negative	negative
Positive control	5 ng/μl	N/A	N/A
Negative control	n/a	N/A	N/A

Σαμπλες μαρκεδ ας '<1 νγ/μΛ ωερε σαμπλες ωηερε τηε ΔΝΑ ςονςεντρατιον ωας τοο λοω το μεασυρε υσινγ τηε Χυβιτ βροαδ ρανγε κιτ. Γρεεν ηιγηλιγητεδ σαμπλες ωερε φουνδ το βε ποσιτιε φορ Σ. νιτιδα βψ βοτη χΠΤΡ ανδ μανυαλ συρεψ· ορανγε ηιγηλιγητεδ συρεψς ωερε φουνδ το βε ποσιτιε φορ Σ. νιτιδα βψ χΠΤΡ βυτ νοτ μανυαλ συρεψ· ρεδ ηιγηλιγητεδ σαμπλες ωερε φουνδ το βε ποσιτιε φορ Σ. νιτιδα βψ μανυαλ συρεψ βυτ νοτ βψ χΠΤΡ.

**qPCR was repeated for this sample to confirm positivity and 3/12 replicates were found to be positive for S. nitida.*

Table 2. Parameter estimates for a combination of manual survey and qPCR methods using a single-season model Ψ(-), p(-),that is, assuming constant occupancy and detection.

Model	N	-2 log likelihood	Ψ (95% CRI)	Est. P (95% CRI)	SE (P)
Manual survey plus qPCR	2	60.54	0.35 (0.19, 0.56)	0.67 (0.37, 0.87)	0.14

Where N = number of parameters, Ψ = occupancy estimate, P = estimated detection rate. Sample size = 32 sites

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