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ORIGINAL ARTICLE

Detection of a cryptic terrestrial insect using novel eDNA collection techniques

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Abstract

Sensitive detection techniques are key to inform management practices for invasive and pest species by identifying the pest species' distribution or identifying the presence of biological control agents that may negate the need for chemical control. Environmental DNA (eDNA) can be detected to infer the presence of rare, cryptic, and invasive species. This study provides a method that expands the application of eDNA techniques within terrestrial systems. Our study is focused on a cryptic biological control agent of the pointed snail *Cochlicella acuta*, the fly parasitoid *Sarcophaga villeneuveana*, which remains within the snail shell for most of its lifecycle, making it difficult to detect. Three sites were sampled using traditional surveys and three types of eDNA sampling 1/ crushing live snails, 2/ water washing live snails, old snail shells or vegetation, and 3/ vacuuming live snails or vegetation. An assay was developed to amplify a 129 bp fragment of *S. villeneuveana*, with positive detections recorded in crushed snail samples, water washed live snail, water washed vegetation, and laboratory vacuumed vegetation. Presence of the endoparasitoid was validated by traditional survey techniques conducted at the same time, indicating that all techniques tested are comparable for detection of the parasitoid. The new technique which only requires a small vegetation sample from the field post-harvest and a portable vacuum has great potential to be applied to other insects and environments, particularly cryptic species, pests, and biological control agents in crops.

KEYWORDS
biological control, *Cochlicella acuta*, crop, cryptic species, detection, eDNA sampling, parasitoid, *Sarcophaga villeneuveana*

1 | INTRODUCTION

Invasive insects cost a minimum of US$ 70 billion per year globally (Bradshaw et al., 2016). Early detection and surveillance have been shown to be key to successful control (Epanchin-Niell & Liebhold, 2015; Rout et al., 2011, 2014). Similarly, there is a need for detection of cryptic species in the landscape. Some pests (introduced or native) can be cryptic and hard to find. Sensitive detection techniques are therefore needed to facilitate early detection and control measures.

Sensitive detection techniques can inform management practices for invasive pest species by determining the distribution of a pest species or identifying the presence of beneficial control insects

that may negate the need for chemical control methods. For example, parasitoids are insect species that can effectively regulate host populations (Bellows, 2001; Boivin et al., 2012) but endoparasitoids are difficult to detect as they live within their host during their immature stage. Determining the presence of a parasitoid usually involves collection of multiple host individuals to either rear the host until the adult parasitoid emerges or dissect the host to look for immature endoparasites. Traditional molecular techniques can be useful to identify the parasitoid within the host (Derocles et al., 2012; Gariepy et al., 2008), but require careful collection and individualized analysis of a host to identify the parasitoid and in the case of low parasitism rates, these could be missed. New detection techniques that can effectively survey multiple individuals simultaneously would be useful in order to determine the distribution of cryptic beneficial species, especially in the context of biological control programs and integrated pest management to assess the establishment and impact of an introduced parasitoid or predator. Rapid detection methods would also be advantageous to estimate the diversity of parasitoids in ecological studies on parasitoid-host interactions and trophic webs (Sow et al., 2019; Zalucki et al., 2015).

Environmental DNA (eDNA) refers to DNA present in the environment and can be isolated directly from samples such as water, soil, or air. eDNA surveys were first applied to the field of microbiology (Ogram et al., 1987). Since 2008, the isolation and analysis of eDNA has expanded to eukaryotes (Ficetola et al., 2008) to identify species from their DNA which has been shed directly into the environment either as free-floating DNA or tissue fragments such as skin, or cells from feces, urine, and saliva (Bohmann et al., 2014; Taberlet et al., 2012).

Analysis of eDNA has expanded rapidly in the last decade with studies spanning systems such as water (Deiner et al., 2017; Doi et al., 2017; Goldberg et al., 2015), nivean and subnivean environments (Franklin et al., 2019) and terrestrial environments (Fahner et al., 2016; Prosser & Hedgpeth, 2018; Valentin et al., 2018). The detection of eDNA is beneficial for use in remote locations or sites difficult to survey using traditional methods, and for the detection of rare or cryptic species, in particular invasive species (Bylemans et al., 2016; Furlan & Gleeson, 2016; Jerde et al., 2013; Ramsey et al., 2017; Valentin et al., 2018) and threatened species (Cowart et al., 2018; Weltz et al., 2017). Environmental DNA can also be isolated from a collection of biological material such as macroinvertebrate samples (such as kick net samples) and gastrointestinal contents (Deagle et al., 2005; Nichols et al., 2019; Pompanon et al., 2012). The detection of eDNA from biological material usually requires a strategic search for the material required. Provided here is the application of eDNA barcoding within terrestrial systems, using a novel approach of vacuuming.

Our study is focused on a cryptic biological control agent of the pointed snail *Cochlicella acuta*, the fly parasitoid *Sarcophaga villeneuveana*. *Cochlicella acuta* is one of four pest species of introduced Mediterranean terrestrial snail in southern Australia that cause major damage to grain crops. All four species have the peculiar behavior of climbing to higher ground to escape hot soil temperatures and aestivate. Aestivating snails get harvested with the grain, resulting in grain contamination (Baker, 2008; Baker et al., 1991). Due to the difficulty in controlling snails, a biological control program was instigated by CSIRO and South Australian Research and Development Institute (SARDI) (Coupland & Baker, 2007). Classical biological control aims at reunifying an invasive organism with its co-evolved natural enemies by introducing these in the invaded range (van Driesche et al., 2009; Hajek & Eilenberg, 2018). Only highly specific natural enemies are considered for introduction as they need to pass strict host specificity testing and risk assessments (van Lenteren et al., 2006). Biological control can be a highly effective and sustainable control method, as once established, the natural enemy populations are self-sustaining and keep the invasive organism below damaging levels (Bale et al., 2008; Clewley et al., 2012). After extensive host specificity testing on native species in quarantine the parasitic fly *S. villeneuveana* (then known as *S. penicillata*) was introduced from Europe to South Australia in the early 2000s (Leyson et al., 2003). Despite having been established successfully, it has not kept *C. acuta* populations below damage levels and the current distribution of *S. villeneuveana* in South Australia is unknown. Detection through traditional surveillance is difficult because *S. villeneuveana* develops within the snail shell, making it an ideal candidate for eDNA sampling. Traditionally, the only way to detect the presence of the parasitoid is to find a pupa at the entrance of the snail's shell, wait for the fly to emerge, or dissect snails to look for the presence of larvae. This is time-consuming and not always feasible. As the snails are harvested with the grain, it is expected that if *S. villeneuveana* is present at the site, their DNA will accumulate in the contaminated grain and chaff enabling eDNA detection of the parasitoid with minimal effort.

The objectives of this study were to: (i) develop a novel technique for eDNA collection using a handheld vacuum with collection filter in a terrestrial environment and (ii) determine whether this method can successfully detect *S. villeneuveana* in South Australia.

2 | METHODS

2.1 | Study system

Cochlicella acuta Müller (Gastropoda: Geomitridae) originates from the Mediterranean region. It was first detected in Australia in 1953 in South Australia (Baker, 1986), and has since spread to Western Australia, South Australia, and parts of Victoria (Atlas of Living Australia). *Cochlicella acuta* is a small conical snail <18 mm long that has become a major pest of grain crops. While it mostly feeds on organic matter, it can cause grain contamination issues by being harvested with the grain (Baker, 2008; Baker et al., 1991). *Cochlicella acuta* are active from autumn to spring, which is also when they breed, laying several clutches of eggs during the season. They primarily have a biennial lifecycle, with snails breeding after their second summer of aestivation (Baker et al., 1991). When temperatures start rising in early summer, they climb on vegetation or structures and can form large aggregations. Some will also cluster at the base

of plants or under rocks and logs when present (Baker et al., 1991). During summer, they may become active for short period of time after rain or heavy dew (Leonard, 2003; Perry et al., 2020).

Existing management practices are often costly (e.g., molluscicide baits), labor-intensive (e.g., cabling fields prior to harvest to dislodge snails, rolling, slashing and grazing and post-harvest cleaning with snail-crushing rollers) (Leonard, 2003) and provide insufficient control of snails to enable growers to consistently deliver grain that meets delivery standards for snail contamination. Conical snails are particularly challenging because they are less susceptible to molluscicides, frequently evade cultural control by aestivating under refuges (e.g., stones, rotting wood, roots of plants (Baker et al., 1991)), and avoid detection in post-harvest cleaning methods due to their similarity in size and shape to grain (SARDI unpublished results).

A classical biological control program for *C. acuta* was instigated in the 1990s and surveys of natural enemies were conducted in southern France, Italy, Portugal, Spain, and Morocco (Coupland & Baker, 2007). Only one parasitoid fly species passed the strict host specificity tests (Leyson et al., 2003). *Sarcophaga villeneuveana* (Diptera: Sarcophagideae), then known as *S. penicillata*, was introduced to the Yorke Peninsula in South Australia in 2000–2004 (Leyson et al., 2003), yet after 20 years, its range is still restricted to that region (SARDI unpublished results). In its native range, *S. villeneuveana* is a primary parasitoid of *C. acuta*, but can also parasitize *Cochlicella conoidea* and *Cochlicella barbara* (Coupland & Baker, 1994; Fendane et al., 2018; Thomann et al., 2020). Female *S. villeneuveana* lays a larva on the shell of an aestivating snail (Figure 1a). The larva enters the shell through the operculum. It will remain inside, feeding on the host tissue until it completes its development and emerges as an adult, leaving the pupal case inside (Figure 1b) (Coupland & Baker, 1994). Several generations can occur over the season (Coupland & Baker, 2004). While *S. villeneuveana* established successfully in Australia, parasitism remains low (Thomann et al., 2020). A molecular study of *C. acuta* in its native range showed that there are three main mitochondrial lineages and that the *C. acuta* that was introduced in Australia is part of the same lineage encompassing Morocco and the Iberian Peninsula (Jourdan et al., 2020). However, *S. villeneuveana* in Australia was sourced from southern France, where *C. acuta* is in a different lineage, making a potential mismatch of the parasitoid and its host (Thomann et al., 2019). For this reason, other strains of *S. villeneuveana* are being tested for their efficacy at controlling Australian *C. acuta*, and the most efficient one will be introduced, providing host specificity testing showed it poses no risk to the Australian environment.

2.2 | Study design and sample collection

Three sites were sampled on the Yorke Peninsula, Southern Australia (Figure 2). Sites were chosen based on previous knowledge of *S. villeneuveana* presence: site 1: not detected for more than 5 years; site 2: first detected in 2018; site 3: fly established for more than 10 years.

To assess *S. villeneuveana* parasitism rates, *C. acuta* were sampled at three sites using a belt transect sampling method. Four 25-m transects were selected at each site running parallel to a paddock boundary. Snails were sampled by directly searching a two-meter-wide continuous strip for 5 min along each transect. *Cochlicella acuta* specimens collected for parasitism assessment were ≥5 mm with the shell exhibiting color texture. Empty snail shells can remain for several years in the field. To avoid sampling snails that have died in previous seasons, old, bleached shells devoid of color and texture were excluded. Snail specimens were brought back to SARDI laboratories to quantify parasitism rates.

Parasitism rates were determined by dissecting snails to search for evidence of fly parasitism, including fly larvae and pupae, empty fly puparia, and dead or live flies. Parasitized snails were usually easy to detect due to the lack of flesh at the apex or a fly pupa near the shell aperture (Figure 1b). The parasitism rate was calculated by dividing the number of parasitized snails by the total number of snails collected.

2.3 | eDNA sampling

To detect the flies using DNA-based molecular techniques, three types of sampling were used: vacuuming live snails ($n = 5$) and vegetation ($n = 15$); water washing live snails (minimum 1000 snails per sample) ($n = 6$), old snail shells ($n = 2$) and vegetation ($n = 8$); and crushing live snails (minimum 1000 snails per sample) ($n = 6$) for a total of 42 samples.

For the vacuum treatment, live snails and plants around snails were sampled under field conditions at all three sites using a handheld vacuum (Dirt Devil) with the mouth of the vacuum fitted with a folded 9 cm diameter 1.2 μm glass fiber filter (MicroScience). Five negative field controls were produced prior to sampling by removing clean filters from the packaging at each site and placing in collection tubes. Live snails were aestivating at the time of sampling and were found attached on vegetation and fence posts in large aggregations. Vegetation and fenceposts with high densities of snails were vacuumed for 30 s, allowing sampling of several thousand snails, to

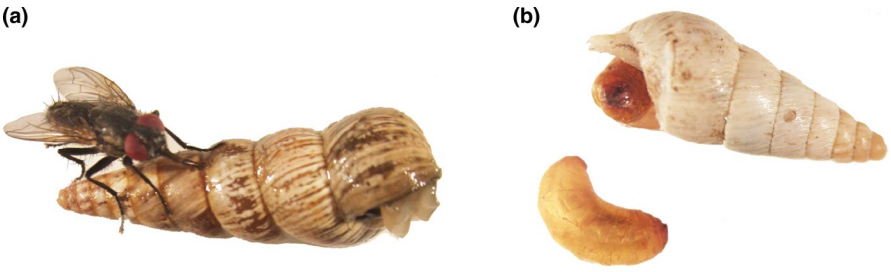

FIGURE 1 (a) *Sarcophaga villeneuveana* on live *Cochlicella acuta*. (b) Late instar larva *Sarcophaga villeneuveana* dissected from a shell and pupa inside *Cochlicella acuta* shell

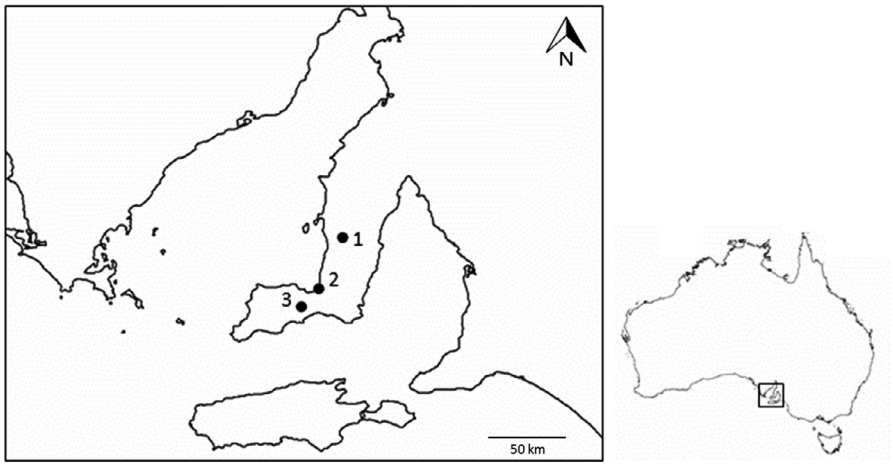

FIGURE 2 Sites sampled on the Yorke Peninsula, South Australia. Site 1: parasitoid not detected in >5 years, site 2: parasitoid first detected in 2018, site 3: parasitoid established for >10 years

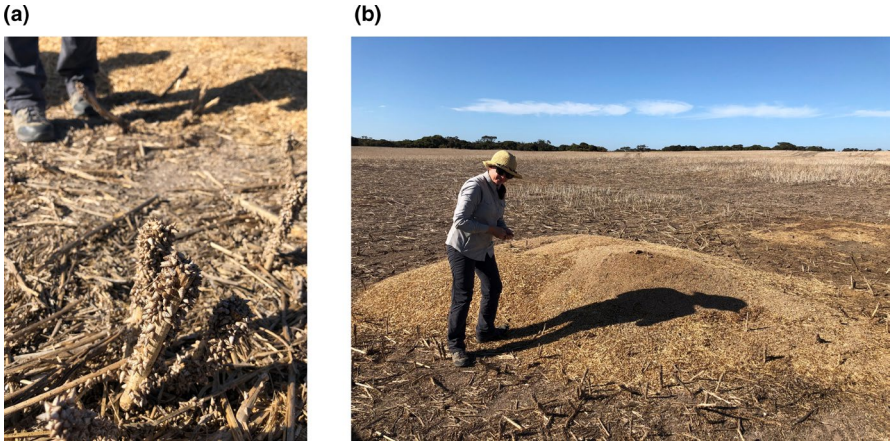

FIGURE 3 (a) High density of aestivating *Cochlicella acuta*. (b) Discarded snails and chaff following harvest

capture DNA of *S. villeneuveana* directly from potentially parasitized snails (Figure 3a) as parasitized snails remain attached to their support, even after death (Coupland & Baker, 1994). Filters were carefully removed using sterilized forceps and stored in 100% ethanol until processing. Tools were cleaned in 20% bleach and flamed in ethanol after each sample. All equipment was soaked in a 20% bleach solution between sites for a minimum of 20 min and rinsed with running UV sterilized tap water for several seconds, air dried and stored separately in new Ziploc bags.

At each site, a minimum of 1000 aestivating *C. acuta* were collected from fence posts and vegetation. As parasitization rates are usually low (<5%), having at least 1000 snails increased the chance of having several parasitized snails in the sample. *Sarcophaga villeneuveana* does not parasitize snails <5 mm, therefore small snails were excluded. Old bleached shells were also collected from the ground at sites 2 and 3 as they could contain DNA from *S. villeneuveana* that have already emerged. At site 2, three vegetation samples (0.5–1 kg) free of snails were taken (i) mowed dry vegetation on road reserves under high snail density, (ii) chaff from harvested fields, (iii) chaff where wheat was sieved post-harvest). At site 3, two samples were taken (i) chaff where canola was sieved post-harvest, (ii) canola contaminated with snail shells (Figure 3b). All samples were processed 24–72 h after being collected in a dedicated environmental DNA laboratory, which is spatially separated from DNA extraction and PCR rooms. *Sarcophaga villeneuveana* specimens or tissues had never been brought to this laboratory. Vegetation samples were first vacuumed in the laboratory as described above, before proceeding with the water wash treatment.

All samples were placed in Ziploc bags and soaked with UV filtered water. Snails were soaked for at least 90 min and vegetation samples for a minimum of 4 h. The wash water was filtered through 4.7 cm diameter 1.2 μm glass fiber paper (Filtech, Wollongong, NSW, Australia), filter funnel manifold (Pall Australia Pty Ltd), and a peristaltic pump (Geopump®, Geotech, Colorado, USA). Snails were further processed by crushing them in water with the remaining liquid filtered as above. Five laboratory negatives were generated by filtering UV sterilized water with clean filters at random stages of the filtering process. In total, 54 samples were prepared for assay testing and real-time PCR, including five site negatives, five laboratory negatives, and two extraction negatives, one for each extraction group. Vacuum samples collected in the field were stored in 100% ethanol and moved to a −20 freezer in the trace DNA laboratory until extraction was completed while all samples filtered in the laboratory were stored in a −20 freezer in the trace DNA laboratory until extraction was completed. All samples were archived in a −80 freezer in the trace DNA laboratory at the University of Canberra.

TABLE 1 Mean (±SE) number of *C. acuta* (per 5 min search) dissected and mean (±SE) parasitism rate of *S. villeneuveana* on *C. acuta* at three sites on the Yorke Peninsula, South Australia

Site	Mean (±SE) number of snails	Mean (±SE) number of parasitized snails	Mean (±SE) parasitism rate (%)
1	16.75 (7.26)	0	0
2	405.25 (146.07)	6.00 (3.39)	2.64 (1.32)
3	301.00 (93.65)	2.00 (1.08)	1.54 (0.93)

TABLE 2 Details of primers and hydrolysis probe designed to amplify a short fragment of the COI mitochondrial DNA region for *Sarcophaga villeneuveana*

Primer name	sequence (5′-3′)
Svill_211F	GCCCCCGATATAGCCTTTCC
Svill_379-1R	AATCAACAGAAGCTCCGCCA
Svill_probe	(FAM) ACCTCCTGCACTTACACTTCTC (MGB)

Abbreviations: FAM, fluorescent dye label; MGB, minor groove binder.

2.4 | Positive control DNA extraction and PCR amplification

Two pupae and three adult *S. villeneuveana* that emerged from field-collected *C. acuta* were collected in the Warooka area near site 3 and used as positive controls during primer testing and real-time PCR. One pupa was crushed using two ball bearings and the other was added whole to the lysis buffer. All adult samples were sliced in half and macerated before continuing with the extraction protocol. DNA was extracted from tissue samples using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

DNA was extracted from field samples plus two extraction negatives using the Qiagen DNeasy Blood and Tissue Kit. Prior to extraction, filter papers were dried in individual sterilized petri dishes for 10 min in a sterilized fume hood to remove excess ethanol. The smaller filters (4.7 cm) used to filter water samples were extracted following the Renshaw et al. (2015) modification of the Qiagen manufacturer's protocol while the larger (9 cm) filters used for the vacuumed samples were extracted using a modification of the Renshaw et al. (2015) protocol as follows: 2x ATL buffer and proteinase K added before initial lyses and 2x AL buffer and 70% ethanol to ensure filter was saturated in liquid, extraction continued following the manufacturer's protocol.

A 710 bp fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified in the two pupae and three adult *S. villeneuveana* samples collected for use as positive controls using universal primers LCO1490: 5'-ggtcaacaaatcataaagatattgg-3' and HC02198: 5′-taaacttcagggtgaccaaaaaatca-3′ (Folmer et al., 1994). PCR amplifications contained 50 ng of DNA, 1x MyTaq HS Red (Meridian Bioscience, Memphis, Tennessee, USA), 0.4 μM each forward and reverse primer and ddH20 to a total volume of 25 μl. PCR conditions consisted of 95°C for 5 min followed by 15 cycles of 95°C for 20 s, a touchdown cycle of 65–50°C for 20 s and 72°C for 60 s, followed by 30 cycles of 95°C for 20 s, 50°C for 20 s and 72°C for 60 s followed by a final extension period of 72°C for 4 min. PCR products stained with SYBR safe (ThermoFisher Scientific) were visualized on a 2% agarose gel, by electrophoresis, and visualized under UV light. PCR products were purified using Difinity RapidTip® (Sigma) following the manufacturer's protocol.

Amplicons were sequenced in both forward and reverse, and reactions consisted of 1 μl of purified PCR product, 3.5 μl of BigDye™ Terminator (Applied Biosystems), 1x sequencing buffer, 2 μM primer, and ddH_2O to a total volume of 20 μl. Cycling conditions were 94°C for 5 min, 30 cycles of 96°C for 10 s, 50°C for 5 s followed by 60°C for 4 min. Sequencing reactions were purified using the ethanol/EDTA precipitation method (Applied Biosystems, 2009). Sequencing was performed on an AB 3730xl DNA Analyser (ThermoFisher Scientific) at the ACRF Biomolecular Resource Facility within the John Curtin School of Medical Research, Australian National University.

2.5 | Assay design and real-time PCR amplification

To ensure specificity to the target species, a reference DNA database was developed for all *Sarcophaga* species found in South Australia. Of the 21 species of *Sarcophaga* known from South Australia (Meiklejohn et al., 2013), COI genes were available from GenBank (accessed in February 2019) for 20 species including 13 sequences from *S. villeneuveana* individuals previously collected in Warooka, South Australia (Thomann et al., 2019) (Table S1). A *S. villeneuveana* species-specific primer set and a hydrolysis probe (Table 2) were designed using the primer3 plugin (Koressaar & Remm, 2007; Untergasser et al., 2012) in the software package Geneious v10.2 (Biomatters). Reference DNA sequences were aligned using MUSCLE (Edgar, 2004) in the software package Geneious v10.2 (Biomatters). Attempts were made to maximize base-pair mismatches in the primer and probe region with non-target taxa. The efficiency of the assay was tested on a dilution series of *S. villeneuveana* DNA with a starting concentration of 10 ng/μl. Eight dilutions were run with 11 replicates each and used to establish a standard curve.

Real-time PCR was carried out on the 54 field-collected samples and negative controls in triplicate 20 μl reactions, consisting of 10 μl TaqMan® Environmental Master Mix 2.0 (Applied Biosystems®), 0.75 μM Svill TaqMan Assay, 2 μl DNA and made up to a final volume of 20 μl with Ultrapure DEPC water. PCR cycling was conducted on a Viia™ 7 Real-Time PCR System (Applied Biosystems®) with thermal cycling conditions set at 95°C (5 min) followed by 55 cycles of 95°C (30 s), 50°C (30 s) and 72°C (30 s), 95°C (15 s) and followed by a melt curve of 1 min increasing 0.2°C every 15 s (60–95°C) with a final extension at 72°C for ten mins. One extraction negative, three

PCR negatives, and two positive controls were run on each plate. A negative result was considered if there was no exponential phase at any point during the 55 reaction cycles. Every sample that amplified was considered a positive amplification and was subsequently sequenced to confirm it was *S. villeneuveana* using the same conditions outlined in the above section for the positive control samples.

2.6 | Data analysis

Real-time PCR replicates were considered positive if there was an exponential phase at any point during the 55 cycles. If two of the three replicates were positive, then the replicate with the lowest CT-value was sequenced following the method described above. Forward and reverse sequences were aligned in Geneious v10.2 (Biomatters) and subsequently checked and edited manually.

3 | RESULTS

3.1 | Parasitism rates

Visual searches for *C. acuta* identified the snail's presence at each site; however, snail abundance varied with site 2 having the highest abundance (i.e., mean number of snails collected per 5 min). *Sarcophaga villeneuveana* parasitism was only detected at sites 2 and 3, where snail numbers were much higher (Table 1). Parasitism rates were low at both sites ranging from 0.3%–6.1% at site 2, and 0.3%–4.3% at site 3.

3.2 | Assay design and real-time PCR amplification

Primers and a hydrolysis probe were designed initially on a mitochondrial COI alignment of *S. villeneuveana* from South Australia to maximize the number of base pair mismatches with all other South Australian species of *Sarcophaga* available on GenBank (Tables 2 and 3). The assay was developed to amplify a 129 bp fragment and tested in-silico on the entire reference DNA database. All COI sequences of *S. villeneuveana* from South Australia were identical, as would be expected from a single introductory event. In silico analyses of 19 out of 20 non-target *Sarcophaga* species found within South Australia identified that all contained ≥8 mismatches in the primer-probe region (Table 3). Little is known of the remaining species: *S. emuensis*. The dilution series showed reliable detection (>95%) of DNA to 0.00002ng per reaction (slope: −3.374; Y-intercept: 21.617; R^2: 0.998; efficiency: 97.892%; error: 0.016).

All positive and negative controls performed as expected. Thirteen of the 54 samples showed positive DNA detections in either two ($n = 3$) or three ($n = 10$) replicates and were detected in filtered snail samples ($n = 3$), crushed snail samples ($n = 4$), filtered vegetation samples ($n = 3$) and lab vacuumed samples ($n = 3$). All positive detections in the vegetation samples were from post-harvest discarded vegetation piles. All positive amplicons ($n = 13$) were subsequently prepared and sent for sequencing and showed a 100% identity to *S. villeneuveana* (Accession number KU746570.1). All positive detections were from site 2 and 3 where *S. villeneuveana* was known to occur (Table S1).

TABLE 3 The number of nucleotide mismatches present in the primer/probe region of the assay for 20 *Sarcophaga* species present in South Australia

	F primer	Probe	R primer	Total
Sarcophaga villeneuveana (target)	-	-	-	-
Sarcophaga africa	3	5	1*	9
Sarcophaga aurifrons	4	7	1–2*	12–13
Sarcophaga australis	3	4–5	1*	8–9
Sarcophaga bidentata	3	4	2–3	9–10
Sarcophaga bifrons	3	4	2	9
Sarcophaga crassipalpis	2*	7	1*	10
Sarcophaga dux	4	6	1–2*	11–12
Sarcophaga froggatti	4	4–5	2	10–11
Sarcophaga furcata	3	4	5	12
Sarcophaga impatiens	3	4–5	3	10–11
Sarcophaga kohla	3	5	2*	10
Sarcophaga longifilia	2*	4–5	2	8–9
Sarcophaga omikron	5	4–5	2	11–12
Sarcophaga peregrina	2–4	5–6	2	9–12
Sarcophaga praedatrix	4	6	1*	11
Sarcophaga ruficornis	3	6	1*	10
Sarcophaga sigma	4	7	1*	12
Sarcophaga spinigera	3	5	1*	9
Sarcophaga torvida	4	7–8	4	15–16

Note: Asterisks indicate where mismatches are present in the 3′ region of the primer.

4 | DISCUSSION

This research has demonstrated the successful application of a species-specific eDNA assay in the detection of an endoparasitoid in a terrestrial environment and some promising results for a novel eDNA collection method. Results show successful detection of *S. villeneuveana* in an area where flies are well established (site 3) as well as an emerging area (site 2). *Sarcophaga villeneuveana* was successfully detected from filtered crushed snails, filtered snails, filtered vegetation and laboratory vacuumed vegetation. Presence of the endoparasitoid at these two sites was validated by traditional survey techniques conducted at the same time, indicating that new techniques tested here are comparable for detection of the parasitoid. It was assumed that filtered crushed snails would detect *S. villeneuveana* as any parasitoid present would also have also been

crushed in the process, but it is interesting that washing the snails and filtering the wash provided enough DNA of the endoparasitoid for positive detections. It would therefore be possible to collect large number of snails from the field and process these in the field or laboratory, without the need for dissection to assess the presence of the parasitoid. More interestingly, the washed and laboratory vacuumed vegetation did provide detections, for vegetation samples discarded after harvest (grain and chaff). The harvesting process may have contributed to the accumulation of DNA onto the vegetation. Other studies have used a wash technique and filtering method to detect an invasive insect species on vegetation (Valentin et al., 2018). However, the vacuuming technique is new and is by far the most promising as it only requires a small sample of discarded vegetation post-harvest and does not require either snail collection or time-consuming filtration.

While UV-treated water was used in the laboratory to soak and filter several different sample types, the ability to collect samples dry in the field, or use a portable vacuum cleaner to filter air around samples, increases the utility of this method. And while the field vacuumed samples did not successfully amplify, the success of the laboratory vacuumed samples shows promise for the application in the field, which if perfected could help avoid the difficulties associated with carrying bulky samples through remote field locations, or spending hours filtering water samples on-site. Instead, vegetation samples can be collected from the field and vacuumed in the laboratory to allow detection of an endoparasitoid. This would enable researchers, agriculturalists, and biosecurity officers to collect samples in the field and transport to the laboratory with little effort and few restrictions.

The reasons behind the lack of detection in the field vacuum samples are unclear. The effect of suspending filters in ethanol in this study is unknown and could have impacted the recovery of DNA. Where possible, alternate preservation methods such as freezing may increase eDNA detection rates (Hinlo et al., 2017). As the field sample was done remotely, suspension in ethanol was the most practical method of preservation available. The field vacuum samples had to travel extensively before being processed in the laboratory while the laboratory vacuumed samples didn't. This could explain the detection of *S. villeneuveana* DNA in the laboratory vacuumed samples in the laboratory, but not in the field vacuumed samples. Further research into the vacuuming techniques described in this study is warranted in the field. It is possible that using a more powerful vacuum, increasing sampling time or intensity, and not suspending filters in ethanol could contribute to the detection of the target species.

A limitation to this study was the lack of negative controls for the equipment. While the sterilization technique used here is similar to other studies (Bylemans et al., 2016; Furlan & Gleeson, 2016), it would still have been useful to have included equipment controls in order to assess the potential for false negatives due to bleach residue or false positives due to contamination.

The assay tested here is specific to the detection of *S. villeneuveana* in South Australia against 19 non-target *Sarcophaga* species. One remaining *Sarcophaga* species – *S. emuensis* – is poorly studied and little is known of its distribution (Meiklejohn et al., 2012). While it is possible that this species co-exists with *S. villeneuveana*, it seems unlikely that it would provide non-target amplification with the assay given the large variation in genetic sequence at the primer/probe sites for all species of *Sarcophaga* found in South Australia. To account for the possibility of non-target amplification with this or other unknown species, it is recommended that all positive amplicons be sequenced to confirm a match with the target *S. villeneuveana*.

Monitoring the establishment and spread of a newly introduced organism is essential in order to predict its potential distribution (Marsico et al., 2010) and it is a key component of any biological control program (Lavandero et al., 2004). In the case of *S. villeneuveana*, the information generated from this study will be useful to understand the species distribution, which is currently unknown in Australia. Traditionally, to assess the presence of the snail parasitoid, snails are collected and cultured until parasitoids emerge or snails are dissected to look for the presence of larvae or pupae. This can be very time-consuming, making large-scale sampling difficult and expensive. The DNA sampling technique described here using discarded vegetation from the harvest could replace the need for traditional sampling to assess presence of *S. villeneuveana* in the field. Also, a new strain of *S. villeneuveana* is currently being tested for introduction into Australia. This strain also provides a 100% match to the assay designed here (GenBank accession number MN534874). Laboratory assays indicate that this new strain may provide a better control of *C. acuta* populations than the one already established (Caron, unpublished results). If it is shown to be more efficient and if it passes the strict biosecurity requirements, it may be released in southern Australia. This DNA sampling technique will provide an efficient way of establishing where *S. villeneuveana* is currently present and will help with deciding where to release the new strain as well as tracking its spread.

This new DNA sampling technique has great potential to be applied to other insects and environments, particularly cryptic species such as parasites, pests, and biological control agents in crops; making eDNA barcoding techniques even easier to use without the need for UV sterilized water and complex filtering systems in the field and the laboratory. Further research to compliment this pilot study is required to reach the full potential of this technique in both grain crops and other systems.

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interest regarding the publication of this article.

AUTHOR CONTRIBUTIONS

The author contributions are as follows: conception of the study: CDC, DMG, EMF, VC; acquisition, analysis, or interpretation of the data: CDC, EMF, KAM, VC; writing of the manuscript: CDC, DMG, EMF, KAM, VC.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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