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Moving eDNA surveys onto land: Strategies for active eDNA aggregation to detect invasive forest insects

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Abstract

The use of environmental DNA (eDNA) surveys to monitor terrestrial species has been relatively limited, with successful implementations still confined to sampling DNA from natural or artificial water bodies and soil. Sampling water for eDNA depends on proximity to or availability of water, whereas eDNA from soil is limited in its spatial scale due to the large quantities necessary for processing and difficulty in doing so. These challenges limit the widespread use of eDNA in several systems, such as surveying forests for invasive insects. We developed two new eDNA aggregation approaches that overcome the challenges of above-ground terrestrial sampling and eliminate the dependency on creating or utilizing pre-existing water bodies to conduct eDNA sampling. The first, "spray aggregation," uses spray action to remove eDNA from surface substrates and was developed for shrubs and other understorey vegetation, while the second, "tree rolling," uses physical transfer via a roller to remove eDNA from the surface of tree trunks and large branches. We tested these approaches by surveying for spotted lanternfly, *Lycorma delicatula*, a recent invasive pest of northeastern USA that is considered a significant ecological and economic threat to forests and agriculture. We found that our terrestrial eDNA surveys matched visual surveys, but also detected *L. delicatula* presence ahead of visual surveys, indicating increased sensitivity of terrestrial eDNA surveys over currently used methodology. The terrestrial eDNA approaches we describe can be adapted for use in surveying a variety of forest insects and represent a novel strategy for surveying terrestrial biodiversity.

KEYWORDS
environmental DNA, Lycorma delicatula, spotted lanternfly, surveillance, terrestrial

1 | | INTRODUCTION

While the first known use of environmental DNA (eDNA) was to evaluate soil micro-organismal communities (Ogram, Sayler, & Barkay, 1987), this tool is now primarily used to monitor rare, reclusive, inaccessible or dangerous aquatic species whether they are freshwater (Ficetola, Miaud, Pompanon, & Taberlet, 2008; Lafferty, Benesh, Mahon, Jerde, & Lowe, 2018; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012), marine (Foote et al., 2012; Thomsen, Kielgast, Iversen, Møller, et al., 2012) or brackish (Ardura et al., 2015; Forsström & Vasemägi, 2016). In nearly all uses thus far, eDNA within aquatic systems has enabled efficient detection of species presence when other survey methods could not (e.g., Deiner, Fronhofer, Mächler, Walser, & Altermatt, 2016; Jerde,

Mahon, Chadderton, & Lodge, 2011). The ability of eDNA to detect nascent populations of aquatic invasive species has been especially noteworthy as early detection allows these populations to be managed or eradicated (Simberloff et al., 2013). A similar benefit should accrue toward the detection and management of the growing number of terrestrial invasive species, although only limited and highly specialized examples currently exist (Hunter et al., 2015; Valentin, Fonseca, Nielsen, Leskey, & Lockwood, 2018; Williams, Huyvaert, Vercauteren, Davis, & Piaggio, 2018). In particular, the number of non-native insects has grown exponentially since the 1950s (Seebens et al., 2017), making them the most numerous and costly group of invasive animals worldwide (Bradshaw et al., 2016; Fei, Morin, Oswalt, & Liebhold, 2019).

Environmental DNA is biological material, such as shed cells, excrement, exuvia and reproductive secretions among other sources, that is continuously released into the environment by all living macro-organisms (Rees, Maddison, Middleditch, Patmore, & Gough, 2014). This DNA can be collected within samples of water, soil or sediment, and then assigned to species using single-species quantitative PCR (qPCR) or droplet digital (ddPCR) assays or metabarcoding techniques (Comtet, Sandionigi, Viard, & Casiraghi, 2015; Jerde et al., 2011). For eDNA surveys to be efficiently executed within terrestrial settings, any shed eDNA must be collected and aggregated from a source that can be sampled (Figure 1). While this step is also necessary for aquatic eDNA surveys, the task is especially difficult within terrestrial settings. For example, in lentic environments, eDNA deposited by fish diffuses outward away from the source and through the water column, resulting in detectable levels of eDNA within water collected 10 m or more out from the fish themselves (Eichmiller, Bajer, & Sorensen, 2014). Within lotic and marine waters, shed eDNA can travel across much longer distances due to consistent currents, making it possible to collect water samples from a variety of locations well away from the target individuals and still detect their presence (Deiner et al., 2016; Thomsen, Kielgast, Iversen, Møller, et al., 2012). However, within terrestrial environments there are far fewer mechanisms that facilitate dispersion of shed eDNA across a landscape, and most efforts to collect terrestrial eDNA have come from collecting soil samples (e.g., Buxton, Groombridge, & Griffiths, 2018; Kucherenko, Herman, III, & Urakawa, 2018; Leempoel, Hebert, & Hadly, 2019; Sales et al., 2019; Walker et al., 2017) or carrion flies and leaches (Calvignac-Spencer et al., 2013; Lee, Gan, Clements, & Wilson, 2016; Schnell et al., 2012; Schubert et al., 2015). While these methods have proven successful in detecting terrestrial species, collecting eDNA from soil has limitations due to the small amount of material that can be collected and processed with expensive and specialized kits necessary to extract DNA and remove inhibitors, and it may not accurately represent eDNA from organisms above the soil (Taberlet, Bonin, Coissac, & Zinger, 2018). This can be circumvented by using flocculation techniques to adsorb eDNA from soil (Taberlet et al., 2012; Zinger et al., 2016). However, these limit the accessible states of eDNA by targeting only extracellular eDNA, which may not be applicable for the question being addressed given that soil can preserve DNA for extensive periods of time (Andersen et al., 2012). Carrion flies and leeches present a creative alternative that has thus far been implemented for detection of mammalian eDNA (Calvignac-Spencer et al., 2013; Lee et al., 2016; Schnell et al., 2012; Schubert et al., 2015), but they may not be applicable for a wide range of taxa. Aside from these methods, no known techniques to collect eDNA from above-ground substrates (i.e., bark, leaves, rocks, etc.) *en masse* are currently available (but see Nichols, Köenigsson, Danell, & Spong, 2012 for analysis of eDNA from individual twig clippings). Thus, the primary challenge to utilizing eDNA surveys for detecting terrestrial species is collecting and aggregating shed eDNA in a way that does not require exhaustive sampling of deposition sites and substrates across a landscape (Figure 1).

Successful alternative approaches using eDNA to survey for terrestrial species published thus far have utilized water bodies that passively aggregate eDNA shed from target species (e.g., Harper et al., 2019; Rodgers & Mock, 2015; Ushio et al., 2017; Valentin et al., 2018; Williams et al., 2018). These range from using lotic and lentic waterbodies (Deiner et al., 2016; Harper et al., 2019; Sales et al., 2019; Ushio et al., 2017, 2018) to artificial containers such as those used in Williams et al. (2018) that terrestrial species use for drinking, bathing or foraging. Valentin et al. (2018) devised a different eDNA approach for surveying brown marmorated stink bug (*Halyomorpha halys*) in agricultural produce, in which eDNA deposited by *H. halys* on fruits and vegetables was aggregated in the water farmers used to wash their produce and then tested to detect the eDNA left by *H. halys*. Although all the above approaches represent substantial forward progress in using eDNA to survey terrestrial species, they are restrictive in that, for them to work, natural or artificial water bodies must be present and the target species' eDNA must consistently reach them. A next step in the evolution of eDNA surveys is to develop active, user-controlled methodologies that collect and aggregate eDNA deposited onto a variety of above-ground substrates (here defined as terrestrial substrates above the soil, such as leaves, bark and rockfaces). Such methods pave the way for eDNA to be used in terrestrial ecosystems that are under heavy threat from invasive species (Aukema et al., 2011; Liebhold et al., 2013; Lovett et al., 2016; Seebens et al., 2017), and allow for its use in agricultural systems before harvest when harvested products are not routinely washed (Valentin et al., 2018).

A recent addition to the list of invasive species that threaten forests and orchards in the USA is the spotted lanternfly [*Lycorma delicatula* (White) (Hemiptera: Fulgoridae)] (Cooperband, Mack, & Spichiger, 2018; Urban, Smyers, Barringer, & Spichiger, 2018). *L. delicatula* is a generalist phloem-feeding insect that damages plants by direct feeding and blocking photosynthetic activity due to sooty mould that grows from its excrement (Cooperband et al., 2018; Park et al., 2009). Its current known distribution has resulted in the establishment of more than 24,000 km in quarantine zones in Pennsylvania, New Jersey, Maryland, Delaware, and Virginia (Urban, 2020). *L. delicatula* is one of the latest additions to the over 450 tree-feeding pest species that have caused ecological and economic damage to forests in the USA (Fei et al., 2019).

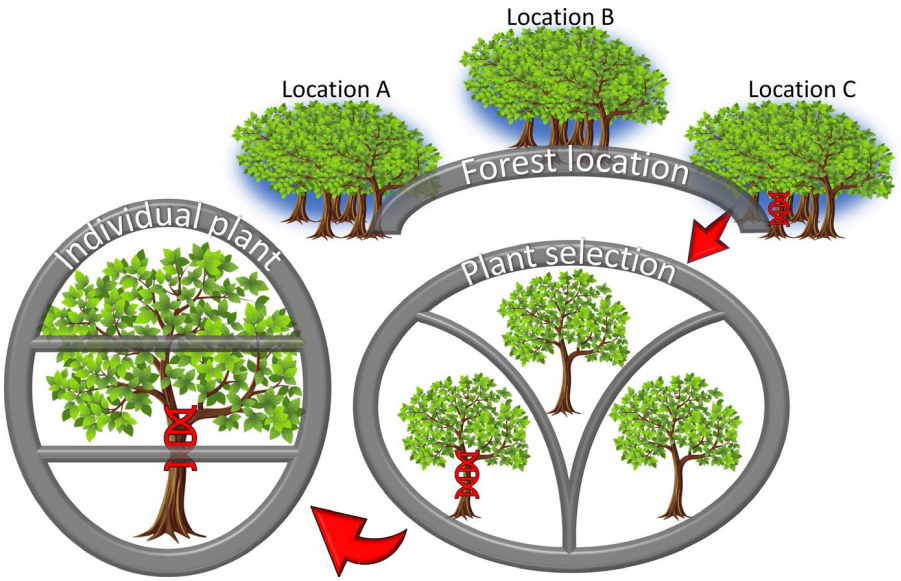

FIGURE 1 Conceptual depiction of the challenges faced when implementing environmental DNA (eDNA) surveys within terrestrial systems. The first challenge comes from identifying which location (e.g., which forest stand) to carry out surveys. Because there is no medium to facilitate eDNA dispersion, the second challenge comes from identifying which unit(s) to survey (e.g., which trees) that contain the target eDNA. The third challenge is two-fold and comes from having the means to collect a sample from a nonaquatic substrate and identifying precisely from where to collect the sample so as not to miss the eDNA entirely

The most valuable strategy for eradication and containment of invasive forest insects is the early detection of newly established local populations (Liebhold & Kean, 2019). For some species, current best-practices to achieve early detection rely on pheromone-baited traps. However, these require substantial research investment before use, which can cause critical delays in their implementation (Tobin et al., 2014). In the case of *L. delicatula*, no commercially available pheromone or species-specific trap exists at present, while for many other species such traps are not effective due to the biology of the species (i.e., no known or low reliance on pheromone signalling). This leaves managers no choice but to rely on visual surveys that are known to detect only the presence of target species when they become too common to efficiently control (Liebhold & Kean, 2019; Tobin et al., 2014). Here, we provide proof-of-concept for two novel approaches for collecting, aggregating and detecting eDNA deposited by a terrestrial insect species in forested ecosystems. Our objective was to develop an active user-controlled eDNA survey protocol for forest landscapes, focusing our efforts on the newly invasive *L. delicatula*. Such an approach paves the way for utilizing eDNA surveys to detect nascent populations of a variety of invasive insects, providing a transformative tool for increasing successful eradication and control of forest insect pests (Liebhold & Kean, 2019; Tobin et al., 2014). Our goal is to transform management of invasive insect species and allow biologists to detect the presence of individuals when they are present in very low numbers, ensuring a higher eradication success rate.

2 | | METHODS

2.1 | | *Lycorma delicatula* species-specific assay development

To detect *Lycorma delicatula* using eDNA, we used a species-specific approach by designing a specific TaqMan genetic assay (hereafter, Lydel assay). To maximize the likelihood that this assay worked across the established and spreading populations of *L. delicatula* in the USA, we obtained nine individuals from Berks County, Pennsylvania, considered the epicentre of the invasion in the USA (Urban et al., 2018). Each individual *L. delicatula* was externally washed with double deionized water to remove surface contaminants. We then carefully cleaned forceps and used 100% ethanol to flame-sterilize them to pull a single leg with the underlying muscle attached, as described by Valentin, Maslo, Lockwood, Pote, and Fonseca (2016), and placed each in a sterile 0.2-ml microcentrifuge tube for extraction of total genomic DNA using the HotSHOT method (Truett et al., 2000). Following Valentin et al. (2016), we elected to design the Lydel assay within the first internal transcribed spacer (ITS1) of the rDNA, a multicopy locus with low intraspecific sequence variability yet high interspecific variability (Gerbi, 1986). We amplified ITS1 using primers BD1 and 4S (Table 1) (von der Schulenburg et al., 2001) and an optimized PCR protocol (Appendix S1).

We ran all PCRs on Veriti 96-Well Thermal Cyclers (Applied Biosystems) and visualized reactions in 1% agarose gel with ethidium bromide. Successful reactions yielded amplicons of ~300 bp that were cleaned using ExoSAP-IT (Affymetrix), then ~ 20 ng of cleaned template DNA was mixed with 25 pmol of each primer and Sanger sequenced (Genscript). We sequenced individual *L. delicatula* PCR amplicons bidirectionally to attain a consensus of the full ITS1 sequence. Sequences (accession nos. MN453253–MN453260) were then assessed, cleaned, and aligned against an rDNA sequence for *L. delicatula* from GenBank (accession no. JF719822.1) in SEQUENCER 5.1 (GeneCodes). We selected a 182-bp conserved region and imported the sequence into PRIMER EXPRESS version 3 (Applied Biosystems, Life Technologies) to design the primers and TaqMan probe following stringent criteria regarding melting temperatures and avoiding hairpins and dimers.

We evaluated the assay's lower limits of detection by creating a dilution series using genomic DNA (gDNA) extractions from *L. delicatula* tissues. We began with an initial concentration of 3.85 ng of

L. delicatula gDNA, verified using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies) for DNA quantification, and then created a series of 1:10 serial dilutions from 0.385 ng down to 0.0385 fg. We carried out qPCR analysis in 20-µl reactions in triplicate and counted dilution levels as an effective positive result if at least two of the three reactions amplified. All qPCRs consisted of 500 nm of each primer, 250 nm of probe, 1 × TaqMan Environmental Mastermix II with no UNG, and 1 µl of each dilution. The optimized reaction protocol was conducted at an initial denaturing step of 96°C for 10 min, followed by 45 cycles of denaturing for 15 s and annealing and extension at 60°C for 1 min. All reactions were run on an Applied Biosystems 7,500 Real-Time PCR System.

Next, we assessed the specificity of the Lydel assay by first carrying out an in silico PCR using NCBI's PRIMER-BLAST. We required the use of our primers in the analysis and used an automatic search mode across the nonredundant (nr) database against all prokaryotes and eukaryotes. In addition, we tested a suite of species (Table 2) we collected from the field. Specimens were collected from sticky band traps deployed in sites where *L. delicatula* visual surveys were conducted in Berks County, Pennsylvania. All insects, regardless of whether *L. delicatula* was detected in the vicinity, were placed individually in 1.5-ml microcentrifuge tubes and submerged in molecular-grade 100% ethanol. Once in the laboratory, all field-collected specimens were thoroughly rinsed with distilled H_2O to ensure any external contamination was removed, then using 100% ethanol flame-sterilized forceps we pulled a leg with connected muscle and placed it in 0.2-ml microcentrifuge tubes for HotSHOT extraction.

We identified field-collected specimens to species by amplifying and sequencing the barcode fragment of the cytochrome oxidase subunit 1 (CO1) mitochondrial DNA (mtDNA) locus. We used CO1 universal primers LCO1490 and HCO2198 (Folmer, Black, W, Lutz, & Vrijenhoek, 1994) in 20-µl reactions using an optimized PCR protocol (Appendix S2). We visualized amplifications in a 1% agarose gel with ethidium bromide, which displayed DNA fragments ~700 bp in size, and prepared them for DNA sequencing. We cleaned successful amplifications using ExoSAP-IT (Affymetrix) and submitted samples in duplicate with 25 pmol of either the LCO1490 or HCO2198 primer mixed and 20 ng of template DNA for cycle sequencing (Genscript). Chromatograms were cleaned and aligned in SEQUENCER 5.1 (GeneCodes), and evaluated for insertions and deletions and translated to amino acids to check for stop codons. Once we had catalogued the different species present in our data set, we proceeded to test them all against the

TABLE 1 Universal primers used throughout the paper to amplify the internal transcribed spacer subunit 1 (ITS1) and the barcode region of cytochrome oxidase subunit 1 (CO1)

Primer	Sequence	Reference
ITS1		
BD1	5′–GTCGTAACAAGGTTTCCGTA–3′	Schulenburg et al. (2001)
4S	5′–TCTAGATGCGTTCGAAGTGTCGATG–3′	Schulenburg et al. (2001)
CO1		
LCO1490	5′–GGTCAACAAATCATAAAGATATTGG–3′	Folmer et al.. (1994)
HCO2198	5′–TAAACTTCAGGGTGACCAAAAAATCA–3′	Folmer et al. (1994)

TABLE 2 Species identification list of specimens collected from the field (*N*), and tested with the *Lycorma delicatula* assay to ensure specificity

	Order	Family	Species	*n*
Field collected	Hemiptera	Cicadellidae	*Draeculacephala savannahae*	2
	Hemiptera	Cicadellidae	*Empoasca papayae*	5
	Hemiptera	Cicadellidae	*Graphocephala coccinea*	26
	Hemiptera	Cicadellidae	*Graphocephala fennahi*	20
	Hemiptera	Cicadellidae	*Graphocephala versuta*	13
	Hemiptera	Cicadellidae	*Gyponana aculeata*	2
	Hemiptera	Cicadellidae	*Gyponana octolineata*	2
	Hemiptera	Cicadellidae	*Jikradia olitoria*	3
	Hemiptera	Cicadellidae	*Osbornellus auronitens*	10
	Hemiptera	Cicadellidae	*Paraphlepsius irroratus*	2
	Hemiptera	Cicadellidae	*Paraphlepsius irroratus/apertus*	22
	Hemiptera	Cicadellidae	*Scaphoideus forceps*	2
	Hemiptera	Cicadellidae	*Tylozygus bifidus*	2
	Hemiptera	Fulgoridae	*Acanalonia conica*	1
	Hemiptera	Fulgoridae	*Fulgoridae sp.*	1
	Diptera	Drosophilidae	*Drosophila tripunctata*	1

Lydel assay using the optimized reaction protocol with a series of *L. delicatula* standards (acting also as positive controls) plus extraction and PCR-negative controls.

2.2 | | Design of new eDNA field collecting and aggregation methods

Lycorma delicatula is a phloem-feeding insect, and while extracting phloem from host plants it produces copious amounts of sugary excrement (honeydew) that it jettisons, falling on leaves, branches, tree trunks and soil surfaces below (Cooperband et al., 2018; Urban et al., 2018). We tested the honeydew from a single *L. delicatula* and found it contains easily detectable DNA (Data S1). This observation led us to devise two methods to collect eDNA from a forest. The first, spray aggregation, collects eDNA from the surfaces of leaves, shrubs and other understorey foliage that may hold traces of *L. delicatula* honeydew. The second method, tree rolling, was developed for collection of eDNA from tree bark surfaces and primary branches.

2.3 | | Spray aggregation

In previous work, we established that insect eDNA can be removed from the surface of crops and put into suspension when submerged in water (Valentin et al., 2016, 2018). We elaborated on this approach by using spray action to suspend any eDNA deposited onto a leaf into solution. Here, water is sprayed onto the leaves of shrubs and other understorey vegetation and collected into a container. The ability of eDNA to suspend into solution in combination with the force created from the spray action removes any eDNA from the substrate being sprayed and carries it into a clean container for collection and aggregation across a landscape. We validated our spray aggregation approach via a series of experiments (Data S2), then field-trialled the method by surveying sites identified as infested with *L. delicatula* individuals as determined by United States Department of Agriculture and the New Jersey Agriculture Extension Service personnel. Before field sample collection, we cleaned all equipment (i.e., buckets, silicon tubing and filter housings) with a 10% bleach solution and rinsed three times in deionized water before use. We collected all spray runoff within cleaned buckets placed directly below the direction of spray, using a 5-L manually pressurized spray canister filled with deionized water (Figure 2a). The sprayer had a maximum working pressure of 36 psi (2.48 bar), and while spraying across vegetation surfaces we ensured consistent pressure throughout operation by regularly pressurizing the canister to the point where the pressure release valve regulated internal pressure. The nozzle of the sprayer was adjusted not to exceed the opening of the buckets being used, which was typically set to a spray surface area of no more than 410 cm^2 from ~30 cm away from the substrate being sprayed. Using a Pegasus Alexis field peristaltic pump (Proactive Environmental Products), we filtered all water collected in buckets through a 10-μm PCTE filter membrane to isolate intracellular eDNA from our aggregates (Turner et al., 2014; Valentin et al., 2018). We then placed filters into sterile 1.5-ml microcentrifuge tubes prefilled with molecular-grade ethanol for storage and transport. Before extraction, we opened all microcentrifuge tubes to allow all ethanol to evaporate, ensuring any eDNA that may have moved into the ethanol was retained (Spens et al., 2017). Filters were then extracted using the HotSHOT extraction method.

We trialled spray aggregation in six locations, ranging in *L. delicatula* population density from high (>10 individuals on a single tree), medium (5–10 individuals per tree) or low (<5 individuals per tree; as designated by state and federal biologists), and two locations where *L. delicatula* was not known to occur. For unknown status sites, we carried out surveys in two teams, one adept at visual surveys for *L. delicatula* and the other deploying eDNA surveys. Before eDNA surveys took place at each of the confirmed locations, trained biologists conducted visual surveys. Visual surveys lasted 3 min, where biologists examined hosts for the presence of *L. delicatula* individuals and examined the understorey plants beneath host trees for the presence of honeydew. If *L. delicatula* individuals were found, the number of nymphs and adults was recorded. eDNA surveys were carried out along the same transect, and visited the same plants as the visual surveys. No information from the visual survey was relayed to the eDNA survey team. After every eDNA survey was concluded, we collected field-negative controls by spraying directly into a clean bucket reserved for control sampling and pumping the water through a 10-μm PCTE filter to ensure equipment had not become contaminated during use, potentially providing false positive data. Buckets were randomly assigned as negative controls before carrying out field collections to prevent consecutive reuse of a control bucket to also control for decontamination of equipment.

2.4 | | Tree rolling

Our tree rolling protocol borrows from existing forensic practices where sterile swabs are used to collect material from crime scenes for genetic analysis by physically transferring material from one substrate to the next (Verdon, Mitchell, & van Oorschot, 2014). To apply this method, we chemically sterilized 10 × 4-cm cotton roller covers by first submerging them in 10% bleach solution for 1 min, followed by a thorough rinsing with deionized water to remove any bleach residue. We then UV-sterilized them for 10 min in a Labconco 3′ Purifier Filtered Enclosure equipped with UV-light (Labconco) to remove any remaining DNA from the surface. We applied a light mist of deionized water to the roller to facilitate the transfer of eDNA from dry surfaces onto the rollers (see Van Oorschot et al., 2003). We then placed each roller within a 2-L bucket, with a lid that had been cleaned with 10% bleach solution, as outlined above, for transport to the field.

Once in the field, we opened the buckets (taking care not to contact the inside portion of the lid) and, using clean gloves, placed rollers on extendable poles ranging from 1.8 to 6.1 m long. Using

FIGURE 2 Demonstration of the two new environmental DNA (eDNA) aggregation methods introduced within this paper: (a) spray aggregation and (b) tree rolling. Spray aggregation uses pressurized water to remove eDNA from the surface of leaves and other materials and become suspended in solution within a bucket held below the direction of spray. Tree rolling uses a dampened cotton roller to transfer eDNA from tree bark, branches or other materials to the roller itself. The roller is then sprayed off in a clean bucket following the same logic as for spray aggregation

firm pressure and steady motions we moved the rollers along the bark surface of lower branches (Figure 2b), followed by a progression down the base of the trunk to ~0.3 m from the ground. Only the tops of branches were sampled because our experience indicated honeydew accumulates on the upper surfaces of objects as it falls once ejected. We focused on avoiding contact of the extendable pole to any parts of the tree to avoid contamination from any residue on the pole's surface. After sampling, we used a new pair of gloves to remove the roller and place it into the clean 2-L bucket from which it was retrieved. In the bucket, the roller was sprayed with deionized water using a pressure sprayer set to 36 psi (2.48 bar) until the roller was saturated with water and all surface debris had been removed. All water remaining in the buckets after the rollers were removed was filtered following the methods outlined above for spray aggregation. Negative controls were also created by spraying into clean buckets reserved for control sampling, following the methods outlined above for spray aggregation to ensure contamination events did not influence our results. As was the case for spray aggregation, we trialled tree rolling in five of the six locations, ranging from high to low population density of *L. delicatula*.

3 | | RESULTS

3.1 | | *L. delicatula* assay design and performance (sensitivity and specificity)

Using the eight *Lycorma delicatula* sequences we generated (accession nos. MN453253–MN453260), and the reference sequence from GenBank (accession no. JF719822.1), we designed primers LydelITS1F (5′-AGCGTTTGACAGCTGACTCTTG-3′) and LydelITS1R (5′-CGCCGAAGCGCAAAAA-3′) and TaqMan MGB quenched probe LydelITS1Tm (5′-CCGCGGGACCGGTA-3′) with an FAM reporter dye. The assay targets a 63-bp fragment entirely within the ITS1 region of *L. delicatula's* rDNA (Figure 3). Of our *L. delicatula* gDNA serial dilutions ranging from the 0.385 ng to 0.0385 fg, all but 0.0385 fg returned positive results, with 0.385 fg returning two of the three replicates as positive and the remaining dilutions returning all three as positive. All positive replicates of the serial dilutions yielded nearly identical C_T values, varying by < 0.05 fractions of a cycle, with a standard curve of − 3.04 (efficiency = 113.3%, r^2 = .99).

During our in-silico PCR analysis, our forward primer amplified one nontarget sequence from *Gluconobacter oxydans* (accession no. LT900338.1) in two locations with a product length of 3 kb, and the reverse primer amplified one nontarget sequence from *Cenarchaem symbiosis* A (accession no. DQ397629.1) in two locations with a product of 3.3 kb (Figure S1). Neither contained the LydelITS1Tm probe sequence, indicating the assay is indeed specific to *L. delicatula*. Of the 131 field-collected specimens from which we attempted to obtain CO1 sequence data, 125 successfully amplified and were sequenced to identify species. These 125 field-collected individuals represented 19 + species from six different families (Table 2; Valentin, 2019). Of these, none cross-amplified with the Lydel assay we designed, indicating it is specific to *L. delicatula* DNA.

3.2 | | New eDNA field collection and aggregation methods

In the six field sites where we implemented spray aggregation, we were able to detect *L. delicatula* DNA across four of them (Table 3; Valentin, 2019). At locations that returned negative results for *L. delicatula* DNA (Warren County, NJ – Farm 3; and Mercer County, NJ – Residence), visual surveys recorded no *L. delicatula*

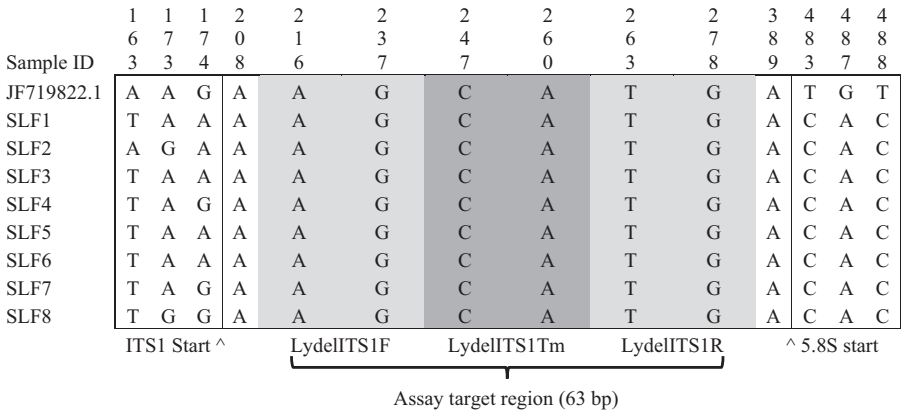

FIGURE 3 Polymorphic sites and SLFITS1 primer/probe locations within the internal transcribed spacer 1. Position numbers refer to the base pair position along the *Lycorma delicatula* reference rDNA sequence from GenBank (accession no. JF719822.1). Sample ID refers either to the reference sequence or the eight SLF ITS1 sequences. qPCR primers and probe are represented by light and dark grey shading, respectively. The thin vertical line within the box indicates the start of the ITS1 intron region and 5.8S exon, respectively

TABLE 3 Survey sites of SLF at different population densities as designated by state and federal biologists from the US Department of Agriculture, Pennsylvania Department of Agriculture, and New Jersey Department of Agriculture

Site	Site density	Number of visits	Number of locations	Visual inspection		eDNA spray aggregation		eDNA tree rolling	
Harrisburg, PA	High	1	2	Y/Y		Y			Y
Warren co., NJ – Farm 1	Medium	2	4	Y/N/Y/Y	Y/N/Y/Y	Y/N/Y/Y	Y/N/Y/Y	Y/N/Y/Y	Y/N/Y/Y
Warren co., NJ – Farm 2	Low	2	2	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y
Warren co., NJ – Farm 3	Low	1	1	N		N		N	
Mercer co., NJ – Residence	Unknown	1	2	N/N		N/N		N/N	
Hunterdon co., NJ – Park	Unknown	1	1	*N*		**Y**		–	

Note: Site densities were determined by identifying the number of *Lycorma delicatula* individuals on a single tree, with high having > 10 individuals, medium having 5–10 individuals and low < 5 individuals. Survey methods are marked as (Y) if it detected *L. delicatula* in a given site, and (N) if it did not. Survey results are grouped by locations per site, with multiple groups displayed representative of the number of visits. Farm 1 was marked as medium overall, although one location within the farm never showed evidence of being infested, while Farm 3 was found to be negative at the time of sampling but was labelled as a low-density site from a visual inspection weeks previously. The abbreviation "co." means county. The bold (Y) for Hunterdon county emphasizes an instance where eDNA detected *L. delicatula* when a visual survey did not. Due to reporting restrictions at the state and federal level, specific site names and locations are not provided.

individuals at the time of sampling. Note that these locations were considered occupied by *L. delicatula* in visual surveys 2–3 weeks before eDNA surveys, or were locations where a positive (Warren County – Farm 3) or suspected *L. delicatula* "hitchhiker" was caught and reported to New Jersey state biologists (Mercer County – Residence). At the Warren county, NJ – Farm 1, Site 2, there was never evidence of *L. delicatula* before eDNA sampling, and eDNA was not detected. However, at a Hunterdon county park location where visual surveys did not find *L. delicatula*, the eDNA survey produced a positive detection. In the five sites where we trialled tree rolling, we found three sites to be positive for the presence of *L. delicatula* using our eDNA methods. The sites that were negative with spray aggregation and visual surveys were also negative for tree rolling aggregation (Table 3; Valentin, 2019).

All negative controls (i.e., field controls, extraction controls and qPCR controls) generated throughout the study were negative, indicating an absence of contamination and successful decontamination of equipment before and after each field collection. Combined with the results of the specificity testing, this indicates positive results were not due to false positive detection.

4 | | DISCUSSION

We provide proof-of-concept evidence that our user-controlled and active collection and aggregation techniques can be utilized to survey for a fully terrestrial invasive insect species, *Lycorma delicatula*, within forested ecosystems. Furthermore, we provide preliminary evidence that our eDNA survey protocol can detect the presence of *L. delicatula* individuals when visual surveys cannot, thus suggesting that our approach represents a transformative tool to detect and eradicate early infestations of this species. The success of the aggregation approaches we developed almost certainly benefited from *L. delicatula* excreting copious amounts of honeydew, which we showed contains easily detectable amounts of DNA. However, given the sensitivity of the Lydel assay we developed, very little

DNA-laden material needs to be captured to obtain a positive qPCR result. This level of assay sensitivity has two implications for general use of our eDNA surveys for *L. delicatula* and other invasive insects.

First, the fact that individual *L. delicatula* do excrete large amounts of eDNA that we can easily collect and aggregate suggests that our approach can detect the presence of even a few individuals within a single survey site. Furthermore, given that *L. delicatula* individuals are highly mobile, it is also likely that even a small number of individuals will disperse their eDNA over a sufficiently large enough area that the likelihood of our methods encountering it is quite high. The combination of assay sensitivity, tools for actively collecting and aggregating shed eDNA, and a species whose biology results in constant and high deposition of eDNA describes the perfect situation where an eDNA survey will provide marked increases in eradication and control success. To the extent that these attributes are shared across species (e.g., other phloem-feeding insects), our eDNA survey approach should be equally powerful at detecting low-abundance populations in the ecosystems they inhabit. For example, spray and roller aggregation can be used to collect and aggregate DNA deposited by a wide variety of insects that feed on fruits in orchards or vineyards, and these surveys can be conducted before harvest when fruits are most vulnerable.

Second, the advantage to using active user-controlled collection and aggregation methods instead of more passive approaches is that detection rates can be increased by stepping up survey effort. For example, if water bodies (ponds) are used to passively collect eDNA shed by terrestrial mammals or birds (e.g., Harper et al., 2019; Ushio et al., 2017; Ushio et al., 2018; Williams et al., 2018), then the power of the survey to detect the presence of target species is limited by the rate at which these species naturally use ponds, the rate at which they shed eDNA into these ponds, and the number of ponds in the landscape. Only a few of these rates are available for active manipulation by a surveyor, and thus even a highly sensitive eDNA assay will be limited in its detection power. In contrast, and beyond the inherent limitations imposed by the rate of eDNA deposition by target species, spray and roller aggregation techniques supported by a sensitive assay are limited in their detection power only by the logistical and financial resources available for deployment. Therefore, acquiring a positive detection with our terrestrial eDNA approaches becomes a sampling limitation rather than one of eDNA concentration in any discrete location (e.g., one tree or shrub).

While sampling effort can also be increased with more traditional survey methods, eDNA has demonstrated its potential to detect populations more efficiently (e.g., Jerde et al., 2011). Here we had a similar finding, where using spray aggregation we detected *L. delicatula* in a site that was previously unknown to have a population (i.e., site 6, Hunterdon county – Park). We chose to sample in this location because prior to our efforts a single dead *L. delicatula* adult was found and reported through the New Jersey Agricultural Experiment Station website. Several weeks later when we surveyed the site we found no indication of an active population via visual surveys, but we obtained positive eDNA results. Given the window of detectability for terrestrial eDNA is less than 1 week (Valentin, Kyle, Allen, Welbourne, & Lockwood, unpublished data) it is highly unlikely the *L. delicatula* DNA we detected came from the reported dead individual, indicating our result was attributed to live individuals present within the last week of our survey. This further demonstrates the power of eDNA surveys compared to visual ones. That said, eDNA carries with it an added cost (e.g., laboratory materials) in carrying out surveys. A comparison of the cost-effectiveness of eDNA compared to traditional methods would be highly beneficial to land managers in determining under which invasion population densities does eDNA become the survey method of choice.

Our approaches are broadly applicable to a suite of ecosystems and species as they are designed to circumvent the common challenge of collecting deposited eDNA across a variety of aboveground substrates (Figure 1). Because the spatial grain of the aggregated sample (i.e., stratified sampling) can be manipulated to best serve the goals of the survey design (Valentin et al., 2018), when surveying for an invasive insect within a forest, for example, the aggregate can be as fine grained as a single tree or as coarse as a forest patch. This ability to manipulate the scale of the eDNA survey is a unique product of our collection and aggregation approaches (spray and roller aggregation) and is particularly useful in conducting delimiting surveys that seek to identify the range edge of an invading population (Tobin et al., 2013). Additionally, given the ability of eDNA to detect populations at very low abundance, our methods can be used after eradication efforts to gauge the success of these efforts and monitor the site for resurgence of the population. Finally, our approach to collecting and aggregating eDNA is applicable to a variety of species that shed eDNA onto any terrestrial surface, thereby opening its use for the conservation monitoring of threatened, rare or cryptic terrestrial species.

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AUTHOR CONTRIBUTIONS

R.E.V. and J.L.L. designed the research; R.E.V., J.L.L. and D.M.F. secured the funding; R.E.V., S.G. and K.E.K. performed the laboratory tests; J.L.L. and D.M.F. contributed reagents and analytical tools; R.E.V. analysed the data; A.L.N. and G.C.H. obtained field samples for specificity testing; R.E.V., A.L.N. and K.E.K. assisted with fieldwork; all parties wrote the manuscript.

DATA AVAILABILITY STATEMENT

Sequence data generated for *L. delicatula* during this study were submitted to GenBank at https://www.ncbi.nlm.nih.gov/genbank/ under accession numbers (MN453253–MN453260). Additional data files for barcodes and *L. delicatula* site detections were submitted to Open Science Framework (https://osf.io/dashboard) under https://doi.org/10.17605/OSF.IO/FEJRN Valentin (2019).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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