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A duplex ddPCR assay for simultaneously detecting *Ips sexdentatus* and *I. typographus* (Coleoptera: Curculionidae) in bulk trap samples

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ABSTRACT

Bark beetles in the family Curculionidae present a growing hazard to forests worldwide. In addition to native bark beetles, introduction of exotic species can pose a serious threat to North American forests. Ips typographus (Boerner) and I. sexdentatus (Linnaeus), both native to Europe, are two such pests that have caused widespread forest loss in their native ranges. International trade has led to increased interceptions of Scolytine beetles at U.S. ports. Most intercepted individuals are not identified to species due to lack of expert identifiers, poor specimen quality, or incomplete taxonomy. These same problems affect identification for domestic surveys. Therefore, development of molecular methods for identification of potentially invasive Ips species is essential. Due to the need to scrutinize large numbers of beetles in an efficient manner, we describe a duplex droplet digital PCR (ddPCR) assay to identify I. typographus and I. sexdentatus simultaneously in bulk trap samples containing 500 Scolytinae specimens using a scalable, two step DNA extraction. This ddPCR method is highly effective for processing the entire contents of beetle traps and identifying these potentially invasive species in a timely and definitive manner. We also describe a non-destructive DNA extraction technique that preserves specimens for morphological identification.

Keywords: ddPCR, bark beetles, invasive species, bulk sample
1. INTRODUCTION

Several bark beetles in the family Curculionidae are known for their ability to cause the widespread destruction of forests. Two such species, *Ips typographus* (Boerner) and *Ips sexdentatus* (Linnaeus), have caused significant dieback of spruce (*Picea* Mill. Pinaceae) and pine (*Pinus* L. Pinaceae) during outbreak events in their native range. *Ips typographus* has caused widespread destruction of spruce forests, leading to the loss of 30 million m$^3$ of forest in Germany in the late 1940s and a further 9.5 million m$^3$ in Norway and Sweden in the 1970s (Schwerdtfeger 1957; Eidmann 1983; Risberg 1985; Bakke 1989). Despite the implementation of more rigorous forest management practices in recent years, outbreaks of *I. typographus* continue to occur as noted by the loss of 30 million m$^3$ of spruce in Switzerland and Austria from 2000 to 2010 (Krehan et al. 2010; Steyrer and Krehan 2011). *Ips sexdentatus* has caused significant losses in pine forests and plantations in Southern Europe, although not to the same extent as *I. typographus* (e.g., Fernández Fernández 2006; Rossi et al. 2009). Storms that down large swaths of timber provide increased habitat for broods which, combined with increased temperatures and drought conditions, are thought to be a major driver in bark beetle outbreaks (e.g., Facolli and Stergulc 2004; Wermelinger 2004; Facolli 2009). These beetles are also vectors for numerous species of pathogenic microbes, including blue stain fungus (*Ophiostoma* spp.), which can be fatal to host trees and exacerbate outbreak damage (Krokene and Solheim 1997; Wegensteiner and Weiser 2004).

Both *I. typographus* and *I. sexdentatus* are broadly distributed from Western Europe across South and Central Asia, and are found as far east as China and the Korean Peninsula (Cognato 2015). *Ips typographus* primarily feeds on *Picea*, while *I. sexdentatus* prefers *Pinus*. Several species of Nearctic spruce-feeding bark beetles are morphologically similar to *I. typographus* (Cognato 2015), and the North American six-spined *I. apache* and *I. calligraphus* are superficially similar to *I. sexdentatus*. In 1995 and 2002, *I. typographus* individuals were trapped in Indiana and Maryland, respectively, as part of the Cooperative Agricultural Pest
Survey (CAPS) program in those areas (Waltz 1996; USDA 2016). Since these initial
discoveries, no additional detections have been made, but such incidents underscore the
potential for *I. typographus* to establish and spread in North America.

Expanding worldwide international trade facilitates dispersal of potentially invasive
species from their historical ranges. For example, from 1985 to 2000, 6,825 interceptions of
Scolytinae beetles were made at U.S. ports of entry (Haack 2003). Only 40% of these
interceptions were identified to species, with an additional 34% identified to genus (Haack
2003). A similarly low rate of specific identification exists within *Ips*, with only 24% of intercepts
made between 2007 and 2017 identified to species (USDA PestID Database).

Such high rates of non-specific identification for intercepted insects is often due to the
specimens themselves (either they are damaged or at immature stages, which are difficult to
identify), or the group in question is taxonomically unresolved (e.g., Gilligan and Passoa 2014;
Johnson et al. 2017). Taxonomic issues have historically plagued *Ips* despite recent
phylogenetic studies aimed at improving identification (e.g., Cognato and Sperling 2000; Jordal
and Cognato 2012). Additionally, identification of bark beetles to the species level is time
consuming given their small size and overall similarity, the need for genitalic dissection, and a
lack of resources for larval identification (Carvey et al. 1994; Cognato 2015). The use of
pheromone traps for detection and population monitoring introduces further complications.

Pheromone-baited trapping to survey for the presence of a target insect species has been a
common practice used by entomologists for more than 50 years. Despite the benefit that
pheromone trapping has provided to entomologists, some pheromones lack specificity, including
those developed for attracting beetles (Witzgall et al. 2010). Bark beetle traps often contain a
mixture of species from numerous Scolytinae genera that are attracted to the pheromones cis-
verbenol, ipsdienol, and 2-methyl-3-buten-2-ol (the *Ips* lure recommended by CAPS), further
inhibiting rapid identifications (Birgersson et al. 2012). We have recently attempted to address
the problems associated with identifying target species in pheromone trap samples by
developing methods that allow for the processing of entire trap samples using a common mass
extraction of DNA and species-specific probes to identify the target (Gilligan et al. 2015; Zink et al. 2017; Tembrock et al. 2017; Zink et al. 2018). With the Scolytinae-specific primer sets
developed recently as part of phylogenetic studies within that group (Jordal et al. 2011),
methods such as real-time PCR and droplet digital PCR (ddPCR) are well suited for rapid
identification of potentially invasive Ips species in bulk trap samples. Herein, we describe a
duplex ddPCR method for the simultaneous detection of I. sexdentatus and I. typographus from
bulk samples containing numerous Scolytinae or other non-target species in a single reaction.

2. MATERIALS AND METHODS

2.1 Sample collection and identification

Ips sexdentatus and I. typographus were obtained from routine trapping efforts by
colleagues in Spain (University of Valladolid) and Sweden (Swedish University of Agricultural
Sciences), respectively. Identifications of I. sexdentatus and I. typographus from all trapped
individuals were made by experts in these taxa. Specimens from Europe were preserved in 70%
ethanol and stored at −20°C.

North American specimens were obtained by hanging panel or funnel traps baited with
cis-verbenol, ipsdienol, and 2-methyl-3-buten-2-ol in diverse locations across Colorado’s
northern Front Range during the summer months of 2017 and 2018. Locations included
municipal and private tree-limb diversion centers and montane coniferous forests historically
impacted by bark beetle outbreaks. Identifications of trapped species were made using
morphological characters by experts at the C.P. Gillette Museum of Arthropod Diversity at
Colorado State University in Fort Collins, CO, USA. Specimens were stored in 70% ethanol at
−20°C.

Additional specimens of assorted North American Ips species were obtained from the
C.P. Gillette Museum of Arthropod Diversity. These specimens were used for non-destructive
DNA extraction. Because the CO1 barcode data is minimal and the current taxonomy for Ips
and related groups is inconsistent and incomplete, we did not employ the dataset from
107 specimens in BOLD (Barcode of Life Data system; Ratnashingham and Hebert 2007) for
108 identification. Rather, we generated a customized set of CO1 barcodes based on Jordal et al.
109 (2011) to confirm morphological identifications to species. This data was also used for
110 development of the primers and probes used for this assay. All DNA sequences used in this
111 study were uploaded to GenBank under accession numbers MK643351-MK643403.

112

2.2 DNA extraction

Extraction of DNA to be used for initial sequencing of *Ips* specimens and local trap
115 catches was carried out using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia,
116 California, USA). Whole adult specimens were pulverized using a pestle in a 1.5mL
117 microcentrifuge tube and incubated in a solution of 180µL Buffer ATL and 20µL Proteinase K
118 overnight at 56°C in a GeneMate Digital Dry Bath (VWR International, Radnor, Pennsylvania,
119 USA). Cells were then lysed with Buffer ATL, and DNA was column purified using Buffers AW1
120 and AW2 and eluted in 50µL Buffer AE. Cross-contamination was prevented by using
121 disposable pestles, and filter pipette tips were used for all DNA-handling steps. Tissue-free
122 extraction controls were carried out along with each extraction to ensure no contamination
123 occurred. DNA concentration and purity of Qiagen extracts was estimated by a NanoDrop 2000
124 Ver. 1.6 spectrophotometer (Thermo Scientific/NanoDrop, Wilmington, Delaware, USA) from
125 2µL of DNA extract. Readings were taken in duplicate to ensure consistency and averaged for
126 reporting.

DNA was extracted from whole museum specimens using the Qiagen DNeasy Blood
128 and Tissue Kit (Qiagen) with a modified protocol to avoid destruction of the specimens. Each
129 specimen was catalogued, removed from its pin or point, and placed in 180µL Buffer ATL with
130 20µL Proteinase K followed by overnight soaking at 56°C in a GeneMate Digital Dry Bath (VWR
131 International, Radnor, Pennsylvania, USA). Following overnight incubation, each individual was
132 removed from the buffer solution, dried completely, and returned to its pin or point. The
remaining buffer solution was used to carry out the DNeasy extraction protocol as outlined above. The DNA concentrations were determined using a NanoDrop 2000 as above.

To simulate a trap catch, bulk DNA extractions were carried out by pooling ratios of target and non-target beetles in 7mL Falcon tubes with 2.3mm zirconia/silica beads. The samples were pulverized for one minute at high speed in a mini beadbeater (Biospec Products, Bartlesville, Oklahoma, USA). After grinding, 50µL of “squish buffer” (10mM Tris HCl, 0.5mM EDTA, and 12.5mM NaCl; Gloor et al. 1993) was added for each beetle (e.g., 5 beetles = 250µL squish buffer; at this point the 1:500 sample was moved to a 50mL tube), and the tubes were then incubated at 80°C (56°C in tube)/500rpm in a Thermomixer FP rotating dry bath (Eppendorf AG, Hamburg, Germany) for four hours. Total contents of the tubes were transferred to 1.5mL microcentrifuge tubes and spun down at top speed for 10min in a Centrifuge 5424 (Eppendorf AG). The supernatant from each extraction was saved and stored at −20°C until use.

To reduce impurities, squish buffer extractions of bulk trap samples were bead purified using Agencourt AMPure XP solid-phase paramagnetic bead purification to remove excess salts and biomolecules including pigments (Agencourt Bioscience Corporation, Beverly, Massachusetts, USA). Briefly, an equal volume of supernatant from a squish buffer extraction and AMPure XP beads were combined in a 1.5mL microcentrifuge tube and incubated for 5min at room temperature. The tube was then placed on a 6-Tube Magnetic Separation Rack (New England BioLabs Inc., Ipswich, Massachusetts, USA) to pelletize the beads. The supernatant was removed and discarded. The pellet was washed twice with an equal volume of freshly prepared 70% ethanol. After the final wash, the pellet was dried, and the DNA was eluted by removing the tube from the magnetic rack and resuspending the pellet in 50µL of reagent-grade water. The tube was incubated at room temperature for 5min, after which it was returned to the magnetic rack to pelletize the beads, and the DNA eluate was removed to a clean tube and stored at −20°C until use.
Squish buffer extractions contain high levels of impurities given the simplicity of the method, which leads to difficulties estimating the DNA concentration. Quantification of DNA in squish buffer extractions was therefore performed using three independent methods, with DNA concentration measured using each method both before and after bead purification. First, DNA concentration was measured by NanoDrop, then by Qubit and lastly, by EvaGreen-based ddPCR using universal mtDNA primers designed to amplify an approximately 200 base pair region of the Scolytinae mitochondrial genome (Table 1).

2.3 PCR, sequencing, and primer design

All PCRs for this study were performed on a Bio-Rad C1000 Touch thermocycler with a deep-well reaction module (Bio-Rad Laboratories, Inc., Hercules, California, USA). To generate CO1 sequence data, conventional PCR was carried out using TaKaRa Ex Taq HS polymerase (Takara Bio, Shiga, Japan) in a 50µL total reaction volume using manufacturer’s recommendations for 10x Ex Taq Buffer, dNTP mixture, and water with 0.2mM each of forward and reverse primers. Primer S1718 and Primer A2237 from Jordal et al. (2011; Table 1) were used to amplify a segment of CO1 from both targets and a variety of non-targets using an annealing temperature of 60°C for 1 min and an extension temperature of 72°C for 30 sec over 40 cycles of PCR. Amplification was confirmed on a 1% agarose gel and the PCR product was purified using a Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA). Sanger sequencing was conducted using the S1718 and A2237 primers at the University of Chicago Cancer Research Center DNA Sequencing Facility using an Applied Biosystem 3730XL DNA sequencer (Applied Biosystems, Foster City, California, USA). Electropherograms were inspected to determine the quality of each sequencing reaction, with high quality sequences then assembled, trimmed, and aligned using Geneious Pro 8.1.8 (Biomatters, Auckland, New Zealand; Kearse et al. 2012).

Edited sequences generated from this study were combined with CO1 sequences from Jordal et al. (2011) and aligned in Geneious Pro 8.1.8 using the MUSCLE (Multiple Sequence
Comparison by Log-Expectation; Edgar 2004) algorithm. The alignments were manually examined for fixed (within each individual target *Ips* species) and variable (differing from the target *Ips* species in all non-targets) SNPs from which distinct probe and primer sets could be designed. We employed the general guidelines for ddPCR probe and primer design in which as many species-specific SNPs as possible were integrated into the probe and primer regions. Both amplicons were designed to be less than 200bp in total length. We employed the following Primer 3 0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012) settings for determining primer and probe Tm: divalent cations = 3.8; monovalent cations = 50; dNTPs = 0.8mM; and the Santa Lucia (1998) formula for thermodynamic parameters and salt correction. The alignment was further analyzed using TCS statistical parsimony algorithm (Clement et al. 2000) as implemented in PopArt 1.7 (Leigh & Bryant 2015) to examine CO1 sequences for similarities and differences among and between species sampled. Primers and probes for each species were designed to target different regions of the sequenced segment of CO1.

An additional set of primers was designed to amplify a homologous region, outside of CO1, of the mitochondrial genome of Scolytinae for the purpose of quantifying the amount of mtDNA recovered during bulk extractions. The complete mitochondrial sequences from *Ips sexdentatus* and five non-target species (*Gnathotrichus materiarius*, *Hylastes attenuates*, *Pityophthus pubescens*, *Pityogenes bidentatus*, and *Orthotomicus laricis*; Table 2) were downloaded from the NCBI database. The genomes were aligned in Geneious using the MUSCLE algorithm as above as well as the MAFFT (multiple alignment using fast Fourier transform; Kotah et al. 2002) algorithm to confirm the alignments. The same Primer 3 0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012) settings specified above were used to design one set of forward and reverse primers (Table 1), and the amplicon was designed to be less than 200bp in length to work with ddPCR.

2.4 Droplet digital PCR (ddPCR)
For ddPCR testing, DNA was first applied to a QIAshredder column (Qiagen, Valencia, California, USA) and spun at 15,000rpm for 2min in a Centrifuge 5424 (Eppendorf AG, Hamburg, Germany) to reduce fragment size. The ddPCR mix consisted of 2x Bio-Rad ddPCR Supermix for Probes (no dUTP), 500nM of each primer, 250nM of each probe, 1µL of DNA, and water to complete the dilution of the supermix to 20µL. The ddPCR protocol was carried out as described in Zink et al. (2017). After droplet preparation and transfer using the Bio-Rad system (Bio-Rad Laboratories Inc.), thermocycling was performed in a Bio-Rad C1000 Touch thermocycler with a deep-well reaction module (Bio-Rad Laboratories Inc.) using the following program: 95°C for 10min, (94°C for 30s, 58°C for 1min, 72°C for 30 s) x 39, 98°C for 10min, with a final hold at 4°C. A ramp rate of 2°C/s was used between all steps and the lid temperature was maintained at 105°C throughout. Droplets were then read on the Bio-Rad QX200 Droplet Reader (Bio-Rad Laboratories Inc.) and data was recorded with QuantaSoft Software version 1.7.4.0917 using default settings.

A duplex ddPCR assay was designed to detect either or both targets using hydrolysis probes. The *I. sexdentatus* probe was 5’ tagged with FAM and the *I. typographus* probe was 5’ tagged with HEX (Biosearch Technologies, Petaluma, California, USA). These fluorophores can be detected simultaneously on different fluorescence channels by the Bio-Rad QX200 droplet reader (Bio-Rad Laboratories Inc., Hercules, California, USA). Each probe was tested individually and then simultaneously to ensure the duplex assay gave consistent signal for both species with no interference.

Universal bark beetle mtDNA primers were used to confirm the presence of beetle DNA in samples with non-targets only using ddPCR with EvaGreen dye chemistry. As described above, a ddPCR mix was made using 2x Bio-Rad ddPCR EvaGreen Supermix (Bio-Rad Laboratories Inc.), 750nM of each forward and reverse primer, 1µL of shredded DNA, and water to bring the total volume of the reaction to 20µL. Droplets were generated in the same manner as above using EvaGreen Droplet Generation Oil (Bio-Rad Laboratories Inc.). The droplets were then transferred to an Eppendorf semi-skirted 96-well plate which was sealed using the
239 PX100 Plate Sealer (Bio-Rad Laboratories Inc.). Thermocycling was carried out on a Bio-Rad
240 C1000 Touch thermocycler with a deep well reaction module using the following program: 95°C
241 for 10min, (94°C for 30s, 58°C for 1min, 72°C for 30s) x 39, 4°C for 5min, 90°C for 5min, with a
242 final hold at 4°C. A ramp rate of 2°C/s was used between all steps. After thermocycling was
243 complete, the droplets were read on a Bio-Rad QX200 Droplet Reader (Bio-Rad Laboratories
244 Inc.).

245

2.5 Assay performance at low template concentrations
246 Serial dilutions of target DNA were run for each species, first separately then in duplex.
247 DNA from multiple targets was used to control for sample purity. Target DNA concentration
248 varied from about 10ng/µl to 10⁻⁸ng/µl. Assay preparation and ddPCR conditions were the same
249 as outlined above.

250

2.6 Bulk trap sampling by ddPCR
251 To simulate field samples, increasing numbers of non-target beetles were combined with
252 a single individual of each target, with up to 500 non-targets to a single target. Extractions were
253 completed as described above using a mini beadbeater (Biospec Products, Bartlesville, OK)
254 and squish buffer. Crude extract was shredded using a QIAshredder (Qiagen, Valencia,
255 California, USA) column and then used for ddPCR in a duplex assay to detect both *I.
256 sexdentatus* and *I. typographus* simultaneously, as described above.

257

2.7 False Positive Testing
258 To determine the False Positive Rate (FPR) for the duplex reaction, 52 wells of non-
259 target were run using the ddPCR assay described above. The FPR and Limits of Detection
260 (LoD) for the probe-based assays were determined as described in Zink et al. (2017) based on
261 look-up tables provided by Bio-Rad based on published tables by Armbruster and Pry (2008).
2.8 Data Analysis

To determine the threshold cutoff for positive droplets and exclude rain (droplets of an intermediate amplitude between positive and negative) from further analysis, the program 'definetherain' (definetherain.co.uk; Jones et al. 2014) was used to determine the independent cutoffs for the FAM and HEX channels. This was done by exporting amplitude and cluster data from QuantaSoft. The amplitude data from each channel of the positive control run with each experiment was then placed in individual Excel .csv files and analyzed using definetherain. The resulting thresholds for each channel were input into QuantaSoft to redefine the threshold for all wells in each experiment.

Extrapolating the template DNA concentration from EvaGreen ddPCR using general primers was carried out by taking the mean of the estimated sizes of available Scolytinae genomes using the base pair count from the fully sequenced *Dendroctonus ponderosae* genome (Keeling et al. 2013), and the estimated size of a *Xyleborus* sp. using flow cytometry (Hanrahan & Johnston 2011) and averaging them; this resulted in an average genome size of 219.4Mbp. By calculating the molar mass of the genome, we concluded that there are approximately 4,223 copies of the genome per nanogram of DNA in an extraction. Based on the number of copies detected by ddPCR (copies/µL of template) we were able to estimate the ng/µL of bark beetle DNA in the original sample.

3. RESULTS

3.1 DNA Extraction

The non-destructive DNA extraction technique used in this paper resulted in DNA concentrations ranging from 0.85–70.25 ng/µL, as measured by NanoDrop (Fig. 1A). DNA concentration and quality were not directly correlated with sample age (Fig. 1B). These extractions were used to confirm the specificity of our primer and probe design for the target species. Results from the non-destructive DNA extraction technique were comparable to results from destructive DNA extraction techniques using column-based extraction with regards to final
DNA concentration (Fig. 1A; Table 3). The museum samples were not damaged, and DNA was successfully isolated from species collected up to 88 years ago.

### 3.2 Conventional PCR and ddPCR Primer and Probe Design

Each primer and probe set were used for ddPCR on purified DNA from target and non-target species to ensure there was no off-target amplification undetected by conventional PCR and agarose gel imaging (Fig. 2). Neither probe reacted with any non-target curculionid species captured locally in Colorado, which consisted mainly of *Scolytus schevyrewi, Xyloborinus saxeseni, Orthotomicus latidens, Dendroctonus rufipennis,* and *D. valens* (Figs. 2A–B).

To determine if there was any cross reaction in a wider sampling of *Ips* species from North America, we sampled 10 *Ips* species as well as several specimens of *D. ponderosae* due to its relative abundance in the sampling area from museum collections. These samples were also used to investigate whether there was any cross reaction between the primers and probes we developed for our target *Ips* species. Using ddPCR we found there was no cross reaction using the newly designed primers and probes between North American species and any of the European *Ips* (Figs. 2E–F).

### 3.3 Duplex *Ips* assays

The duplex assay was run with purified DNA from both targets and a range of non-targets to ensure there was no cross-reactivity when the primers and probes were confined to the same droplets (Figs. 3A–B). There was one positive droplet in the reaction with *I. sexdentatus* and three positive droplets in the reaction with *X. saxeseni* in the HEX channel of the duplex assay but these do not indicate cross reaction of the probes with non-target DNA because they are below the FPR for the duplex assay when non-target DNA is present (Fig. 3B).

To account for occasions when the two target species co-occur in a trap, the DNA from *I. sexdentatus* and *I. typographus* was combined and serially diluted until the concentration of
DNA from each species was approximately $10^{-8}$ ng/µL (Figs. 3C–D). Each of the serial dilutions were run on ddPCR in the duplex assay to determine the lower limit of detection for each species when using purified DNA. Both were detected at concentrations around $10^{-7}$ ng/µL but not at $10^{-8}$ ng/µL, which corresponds to around 0.08 copies of the target region from each species in each µL of reaction or 1.6 copies/20µL well of reaction.

### 3.4 Bulk Sampling

Field trapped samples with targets present were simulated by combining increasing numbers of locally caught bark beetles with one of each target prior to pulverization. DNA concentration for these samples was measured by Qubit and ddPCR to determine the total DNA concentration as well as the number of copies of a region of Scolytinae mtDNA (non-specific) in each sample (Table 4).

Target and non-target specimens in ratios of 1:5, 1:10, 1:50, 1:100, and 1:500 were extracted and analyzed using ddPCR. Both targets were easily detectable in all ratios, with, at times, all droplets containing copies of at least one, if not both targets. However, in the 1:100 and 1:500 ratio extractions, we noticed multiple clusters of droplets in the FAM channel (I. sexdentatus) and a decreasing amplitude for positive droplets in the HEX channel (I. typographus) (Figs. 4A, 4C). Both effects are particularly apparent in the histogram for each of the ratios 1:100 and 1:500 and likely represent artifacts related to sample impurity (Figs. 4B, 4D). Each of the bulk samples also produced heavy rain and wide peaks on the histograms, further indicating impure input DNA (Figs. 4B, 4D). Due to these anomalies, we ran all the squish buffer extractions through a standard bead purification protocol using magnetic Ampure beads. When the duplex assay was performed on the bead purified samples, the indeterminate clusters of droplets in the FAM channel disappeared and the amplitude of positive droplets in the HEX channel returned to the amplitude seen for the positive control (Figs. 5A, 5C). Additionally, each bulk sample exhibited only one peak for positive droplets in the FAM channel histograms (Fig. 5B). The tailing effects and wide peaks seen in the histograms for both
channels were also resolved, resulting in narrower overall peaks with reduced rain (Figs. 5B, 5D).

Because the *Ips* lures used in this study are relatively non-specific, we also conducted testing with a broad sampling of non-target coleopterans. Trap catches routinely included specimens from other insect orders as well as arachnids. We separated Coleoptera specimens from each trap catch and combined them in ratios of 1 target: 25 non-targets. In addition, a squish buffer extraction was carried out on 25 non-targets from the same group. Initially, the targets were not detectable in the ratio extraction and both samples showed an elevated amplitude for negative droplets (Figs. 6A–B). After bead purification both targets were detected at a low level in the ratio extraction and the amplitude of the negative droplets for both was reduced to a normal level for the assay (Figs. 6C–D).

### 3.5 False positive rate

The false positive rate for the probe assay using bulk samples was based on 52 negative control wells run through the standard assay with a positive control well to determine the cutoff for rain. The negative control DNA was extracted from 25 locally collected bark beetles. In 52 wells, there were two FAM (*I. sexdentatus*) positive droplets equal to a FPR of 0.04 false positives/well. At this rate, the threshold to call a well positive is 1 positive droplet, and the sample would be wrongly called positive 0.47% of the time with a 99% confidence interval. The limit of detection for the FAM primer and probe set is 7 copies/20 µL reaction. The same 52 wells were run with the HEX (*I. typographus*) primers and probe resulting in 25 positive droplets after elimination of rain droplets or a FPR of 0.48 false positives/well. The threshold for the HEX assay is 4 droplets, which will only be incorrectly called 0.18% of the time. The limit of detection for the HEX assay is 11 copies/20 µL reaction (Supplemental Figure S1).

### 3.6 Estimation of DNA concentration in bulk samples
DNA extractions in squish buffer are notably impure because they are not filtered through a column, resulting in difficulties estimating the concentration of DNA in the samples. For these experiments, DNA concentration was estimated in three ways: by NanoDrop, by Qubit, and by ddPCR using EvaGreen chemistry. DNA concentration measured by NanoDrop was likely overestimated in most cases before bead purification based on the clarity and color of each sample, especially for samples containing non-bark beetle Coleoptera (samples labeled ‘Trap’), which had higher concentrations of pigment (Table 4). Of the quantification methods tested, Qubit was most consistent before and after bead purification of the samples (Table 4). DNA concentration has not previously been estimated using ddPCR beyond copy number (Plotka et al. 2017). Samples run before and after bead purification were used to estimate the amount of bark beetle DNA extracted, which was extrapolated from detected copy number based on the two estimated genome sizes and the resulting average mass of bark beetle genomes (Table 4). As expected, lower concentrations of bark beetle DNA were measured in the two samples that contained non-bark beetle Coleoptera, as compared to samples that contained only bark beetle species in the presence or absence of either target (Table 4).

4. DISCUSSION

Droplet digital PCR has been shown to be effective for identifying rare DNA sequences in complex samples, including for the identification of single individuals in large trap samples made up of many specimens and several different species. While other authors have used ddPCR to analyze environmental samples and detect a specific target (e.g., Cao et al. 2014; Nathan et al. 2014), these studies simulate positive samples by spiking the field sample with purified target DNA. These methods are informative as to the limits of ddPCR technology but fail to replicate real-world detection scenarios. When extracting DNA from environmental samples, the total amount of bulk sample as well as the volume of extraction buffer can be determinant to the outcome. These samples also contain the DNA of multiple individuals (potentially both target
and non-target), as well as many different species. Thus, developing an efficient and scalable DNA extraction technique for bulk samples is essential for producing a robust assay.

4.1 DNA extraction techniques are integral to developing a ddPCR assay

Use of ddPCR for identifying DNA in environmental samples has proven effective due to the resiliency of the reaction to PCR inhibitors (Dingle et al. 2013). This allows for the use of “dirty” DNA extractions, like those obtained with squish buffer, to produce reliable results. We have employed this method previously when using ddPCR to identify a single target species in a high background of non-target species without any interference from other biomolecules (Zink et al. 2017; Zink et al. 2018). When this method was used for bark beetles however, the DNA extractions were too impure to provide reliable results. While the total contents of the samples were never chemically analyzed to determine what caused the artifacts shown in Fig. 4 and Figs. 6A–B, the results indicate that a compound, possibly at a very low concentration, was interfering with signal amplitude even in the absence of primers and probes (Supplemental Fig. S2). The exact mechanism by which signal detection was affected is less important than the fact that it occurred, and did so in a cumulative manner, with the interference increasing with the increased mass of non-target tissue. Fortunately, bead purification proved to be an effective method to resolve the issues observed with bulk extractions, allowing for the use of ddPCR to identify the targets in all samples tested (Fig. 5, Figs. 6C–D). Furthermore, because bead purification resolved the issues with target detection, it was determined that the signal interference was not due to the binding of the primers or probe with non-target DNA, but was due to impurities in the squish buffer extraction. Since the number of identified copies of both targets remained low in the bulk sample using non-bark beetles, bead purification may not be sufficient if the sample contains a very high concentration of fluorophore-obscuring biomolecules (Fig 6). Incidentally, these samples were a deep red color after squish buffer extraction and remained cloudy even after bead purification. These results indicate that
continued research is needed for the improvement of DNA extraction methods related to complex arthropod environmental samples for use with ddPCR.

4.2 The total tissue mass used for DNA extractions affects detection more than the number of non-target individuals

The bulk extraction results are promising and represent a reliable, reproducible way to identify invasive *I. sexdentatus* and *I. typographus* simultaneously in North American trap samples. While our testing consisted of bulk samples of up to 500 specimens, based on the reported concentrations for the highest number of non-targets, larger samples should also give positive results. As can be seen in the difference in concentration between the 1:100 ratio and the 1:500 ratio (Figs. 5A and C), the relative mass of the specimens has a larger effect on the ability to detect either of the targets than the number of specimens. This explains the variation in concentration between the 1:100 ratio, the 1:500 ratio, and the 1:25 ratio consisting of non-bark beetle non-targets (Figs. 5A, 5C, 6C-D). *Ips sexdentatus* and *I. typographus* were among the largest of the Curculionidae used in this study, with the majority of the North American trap samples used here consisting of *S. schevyrewi* and other small bark beetles, often only half the size of the target *Ips* species. The non-bark beetle specimens included were all approximately twice as large as the targets which, along with the decreased purity of these samples due to squish buffer extraction, likely contributed to the low calls for each ddPCR run performed with these samples. For practical application, it may be beneficial to run a predetermined weight of an overall trap catch to reduce the possibility of not identifying the target among the larger proportion of DNA that may occur if high numbers of larger specimens are included in sampling.

4.3 Methods for estimating DNA concentration from bulk samples

We used three different methods to measure the concentration of template DNA in bulk extractions but since an average bark beetle genome size was assumed, the resulting calculation of DNA concentration in the original sample is an estimate. In addition, none of the
three methods is exact, as all are light-based and subject to distortion by the presence of molecules that absorb or emit the same wavelengths as DNA or intercalating dyes. While all three methods have their shortfalls, the most important factor for measuring DNA content using these methods is sample purity, with highly pigmented or cloudy samples giving a lower or more skewed reading. Traditional estimation by NanoDrop is ineffective as the squish buffer extraction only lyses cells with no further purification, allowing all soluble macromolecules to enter the solution, any number of which absorb light wavelengths between 230nm and 280nm, leading to biased NanoDrop readings. Even after bead purification, the NanoDrop readings were often still contaminated, as evident in the shape of the absorbance curve. Qubit readings were more consistent between bead purified and non-bead purified bulk squish buffer extractions but give a reading for total DNA content in the sample and not Scolytinae specific. Using a simple ddPCR assay based on EvaGreen dye chemistry with primers specific to bark beetle mtDNA, we were able to estimate the total content of bark beetle DNA in a sample (Table 4). EvaGreen ddPCR is the only method of the three evaluated methods that distinguishes the presence of specific types of DNA, as the primers are designed to amplify only Scolytinae DNA, therefore allowing us to determine the total proportion of bark beetle DNA in a sample containing all manner of insect specimens that are attracted to the general *Ips* lure. The two samples that contained only Coleoptera from trap samples (labeled “Trap” on Table 4) had lower bark beetle DNA concentrations when extrapolated from copy number by ddPCR, but not when concentrations were measured by NanoDrop or Qubit. Using the ddPCR method, we can roughly determine the presence of Scolytinae bark beetles in an area from bulk trap samples. A broad scale detection method such as this could be a useful tool in assessing the general effectiveness of different treatments and forest management practices on reducing the bark beetle number.

5. CONCLUSION
The introduction and establishment of an invasive bark beetle population in North American forests could prove devastating, especially when environmental conditions are favorable for an outbreak. Herein we present an assay for the detection of two species of routinely intercepted European Ips bark beetles from survey traps. Using ddPCR we were able to identify both I. sexdentatus and I. typographus simultaneously when they were included in a sample of up to 500 native North American bark beetles. The probe-based duplex assay presented here for simultaneous identification of both species is the first of its kind developed for use in routine surveys and drastically reduces the processing time per beetle compared to morphological identification of each trapped specimen. Our completed assay uses a two-step DNA extraction and purification protocol to optimize trap samples for identification by ddPCR. We also developed a method for quantifying the amount of total bark beetle DNA in trap samples using ddPCR with EvaGreen dye chemistry, which could have broad scale applications for forest monitoring and management practices. In addition, the primers described in this paper could be adapted for real-time or conventional PCR assays when specimens are intercepted to verify I. sexdentatus or I. typographus identifications when ddPCR is unavailable. The non-destructive DNA extraction technique for molecular testing described here is useful, in these situations, as it preserves beetle specimens for further morphological identification.

ACKNOWLEDGEMENTS

We thank our European collaborators Dr. Juan Alberto Pajares Alonso from the University of Valladolid in Spain for collecting I. sexdentatus and Dr. Martin Schröder of the Swedish University of Agricultural Science for collecting I. typographus. We thank Dr. Boris Kondratieff and Dr. Donald Bright for identifying the beetles collected for this project and Janet Hardin for her assistance with trapping beetles. We thank the three anonymous reviewers for their helpful comments that improved this manuscript. The findings and conclusions in this
preliminary publication have not been formally disseminated by the U.S. Department of Agriculture and should not be construed to represent any Agency determination or policy.

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA; USDA is an equal opportunity provider and employer.
REFERENCES


Handy, K. J. 2009. NAPIS data for EWB/BB target species. Personal communication (email) to L. Jackson on 23 March 2009, from KJ Handy (USDA-APHIS-PPQ-EDP).


### Table 1. Primers and probes used in this study (Tm=melting temperature in °C)

<table>
<thead>
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<th>Description</th>
<th>Sequence</th>
<th>Tm</th>
<th>Source</th>
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<td>S1718</td>
<td>Common CO1 forward primer</td>
<td>5'-GGAGGATTTGGAATTGATTAGTTCC</td>
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<td>Jordal et al. 2011</td>
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<tr>
<td>A2237</td>
<td>Common CO1 reverse primer</td>
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<td>Is-306F</td>
<td><em>Ips sexdentatus</em> forward primer for ddPCR</td>
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<tr>
<td>Is-448R</td>
<td><em>Ips sexdentatus</em> reverse primer for ddPCR</td>
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<td>This study</td>
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<td>Is-351P</td>
<td><em>Ips sexdentatus</em> probe for ddPCR</td>
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<td>It-111F</td>
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<td>It-203R</td>
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<td>It-141P</td>
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<td>Col_MtF</td>
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<td>55.4</td>
<td>This study</td>
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### Table 2. Sequences used to find common mtDNA primers for Scolytinae

<table>
<thead>
<tr>
<th>Species Name</th>
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<tr>
<td><em>Hylastes attenuatus</em></td>
<td>KX035212.1</td>
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<td><em>Gnathotrichus materiarius</em></td>
<td>KX035218.1</td>
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<tr>
<td><em>Pityogenes bidentatus</em></td>
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<tr>
<td><em>Orthotomicus laricis</em></td>
<td>NC_036291.1</td>
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Table 3. DNA concentration from extractions of whole, pulverized beetles measured by NanoDrop

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Collection year</th>
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<tr>
<td>Anthribidae</td>
<td>2017</td>
<td>98.25</td>
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<td>Ciidae</td>
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<td><em>Hylesinus</em> sp.</td>
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<tr>
<td><em>Ips calligrapha</em></td>
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<td>70.45</td>
</tr>
<tr>
<td><em>Ips confusus</em></td>
<td>2017</td>
<td>86.3</td>
</tr>
<tr>
<td><em>Ips pilifrons</em></td>
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<td>76.1</td>
</tr>
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<td><em>Ips pini</em></td>
<td>2017</td>
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<td><em>Pityophthorus juglandis</em></td>
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<td>2017</td>
<td>9.4</td>
</tr>
<tr>
<td><em>Scolytus rugulosus</em></td>
<td>2017</td>
<td>35.45</td>
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Table 4. DNA concentration measured in three ways for each bulk sample. All values are in ng/µl

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<thead>
<tr>
<th>Sample</th>
<th>Nanodrop Squish Buffer</th>
<th>Nanodrop Bead Purified</th>
<th>Qubit Squish Buffer</th>
<th>Qubit Bead Purified</th>
<th>EvaGreen Squish Buffer</th>
<th>EvaGreen Bead Purified</th>
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<th>ddPCR Bead Purified</th>
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<td>3.56</td>
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<td>347.5</td>
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<td>14.7</td>
<td>3.9</td>
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<td>1:500</td>
<td>3566.3</td>
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<td>73.9</td>
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<td>No Target</td>
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<td>56.4</td>
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<td>167</td>
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<td>171</td>
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<td>Target (Trap)</td>
<td>3566.3</td>
<td>58.65</td>
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<td>3.1</td>
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<td>No Target (Trap)</td>
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<td>158</td>
<td>11.5</td>
<td>0.3</td>
<td>0.13</td>
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Figure Legends

**Fig. 1** Non-destructive DNA extraction techniques are effective for preserved museum specimens. Museum specimens were soaked in lysis buffer which was then used for a column-based DNA extraction. A) The DNA concentration from non-destructive DNA extractions is shown along with the species name and collection year for each specimen as measured by NanoDrop. B) The DNA concentration from each extraction is plotted with relation to the collection year.

**Fig. 2** *Ips sexdentatus* and *I. typographus* are detectable by ddPCR. Probe-based ddPCR assays were developed to detect each target species without reacting with North American species. *Ips sexdentatus* (A; blue) and *I. typographus* (B; green) and primers and probes were tested with a selection of often-sampled bark beetle species in the Western United States. Positive droplets are shown in color, negative droplets are grey. *Ips sexdentatus* (C) and *I. typographus* (D) primers and probes were tested for reaction with more rarely-trapped bark-beetle species in the Western region. The *I. sexdentatus* (E) and *I. typographus* (F) assays were tested with non-destructive DNA extracts of museum specimens of *Ips* species from across the United States.

**Fig. 3** *Ips sexdentatus* and *I. typographus* are detectable in a duplex ddPCR assay. The two assays function together as a duplex with no cross-reaction when tested with non-target species commonly trapped in the Western United States with droplets positive for *I. sexdentatus* shown in blue in A), and droplets positive for *I. typographus* shown in green in B). Droplets negative for both targets are shown in grey. The assay was also used to detect *I. sexdentatus* and *I. typographus* simultaneously in a serial dilution of DNA from both species with droplets positive for *I. sexdentatus* shown in blue in C), and droplets positive for *I. typographus* shown in green in D). Droplets negative for both samples are shown in grey.
Fig. 4 The duplex assay can be used to detect both targets in bulk trap samples. Target Ips species were combined with increasing numbers of non-target bark beetles prior to DNA extraction and then used in the duplex assay. A) FAM channel (I. sexdentatus) results are shown for all bulk extractions, positive control, and no tissue control (NTC) with positive droplets in blue and negative droplets in grey. B) The histograms showing the amplitude and frequency of positive and negative droplets in the FAM channel displays positive droplets represented by the peaks on the right and negative droplets represented by the peaks on the left. The four histograms represent the positive control, 1:100, 1:500, and NTC reactions. C) HEX channel (I. typographus) results are shown for the same samples with positive droplets shown in green and negative droplets shown in grey. D) The histograms showing amplitude and frequency for droplets in the HEX channel displays positive droplets in the peaks on the right and negative droplets in the peaks on the left. The histograms represent the positive control, 1:100, 1:500, and NTC reactions.

Fig. 5 Bead purification eliminates clustering and tailing effects on the duplex assay. The bulk extractions were bead purified to remove impurities that distort the droplet reader output. The duplex assay was run with bead purified bulk extraction template DNA with FAM positive droplets (I. sexdentatus) shown in blue and negative droplets shown in grey(A), and the resolved histograms (B) with positive droplets in the peaks on the right and negative droplets in the peaks on the left; and HEX positive droplets (I. typographus) shown in green and negative droplets shown in grey (C), and the resolved histograms for the HEX channel with positive droplets represented by the peaks on the right and negative droplets represented by the peaks on the left. The histograms in B and D represent the positive control, 1:100, 1:500, and NTC reactions.

Fig. 6 The duplex assay can be used on bulk samples that include non-target species. Bulk extractions were performed on samples that included non-target species to determine whether full trap catches could be extracted without sorting. Prior to bead purification (A, B) no positive droplets (blue for I. sexdentatus and green for I. typographus) were detected for bulk samples with targets present using squish buffer extractions, excluding positive controls. Negative droplets are shown in grey after bead
purification, FAM positive (*I. sexdentatus*) droplets are shown in blue and negative droplets are shown in grey (C), and HEX positive (*I. typographus*) droplets are shown in green with negative droplets shown in grey (D).
### A

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection Year</th>
<th>DNA concentration (ng/µL)</th>
</tr>
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<tbody>
<tr>
<td>Ips paraconfusus</td>
<td>1941</td>
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<tr>
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<td>2014</td>
<td>27.05</td>
</tr>
</tbody>
</table>

### B

![Graph showing DNA concentration over time](image)

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