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Research

Using the rDNA Internal Transcribed Spacer 1 to Identify the Invasive Pest *Rhagoletis cerasi* (Diptera: Tephritidae) in North America

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Abstract

The cherry-infesting fruit fly *Rhagoletis cerasi* Loew is a significant commercial pest in Europe that has recently invaded North America. To date, it has been trapped only in Canada and northwestern counties of New York. It has the potential to spread further and threaten production and movement of cherry commodities. Timely diagnosis of the pest will facilitate surveys and quick response to new detections. Adult morphology of the pest is distinct from other flies in North America. However, when flies are significantly damaged on traps or the immature life stages are found in fruits, molecular methods of identification are important to confirm presence and host-use records. Other than DNA sequencing of genes from flies which takes over a day to complete, there are no timely methods of molecular identification for this pest. In this study, we report the first sequence record of the internal transcribed spacer 1 (ITS1) from *R. cerasi* and develop two diagnostic tests for the pest based on ITS1 differences among species in North America. The tests use loop-mediated isothermal amplification (LAMP) and multiplex, conventional polymerase chain reaction (mcPCR) technologies that target the same region of the *R. cerasi* ITS1 sequence. Both tests performed well when tested against collections of *R. cerasi* from North America and Europe, generating Diagnostic Sensitivity estimates of 98.4–99.5%. Likewise, the tests had relatively high estimates of Diagnostic Specificity (97.8–100%) when tested against *Rhagoletis* Loew species present in North America that also use cherry as a developmental host.

Key words: LAMP, multiplex PCR, molecular diagnosis, *Prunus*

The fruit fly genus *Rhagoletis* Loew (Diptera: Tephritidae) includes over 70 species that are distributed in Neotropical, Palearctic, Nearctic, and Oriental regions of the world (Norrbom et al. 1999, Smith and Bush 1999, Hulbert 2018, Korneyev and Korneyev 2019). In North America, there are 24 described species of which 11 are reported as economically significant by White and Elson-Harris (1992). Unlike some of the highly polyphagous pests in the fruit fly genera *Bactrocera* Macquart (Diptera: Tephritidae) and *Ceratitis* MacLeay (Diptera: Tephritidae), *Rhagoletis* pests have relatively narrow host ranges and typically use related species in the same plant family or genus as hosts for larval development. Examples of major commercial crops impacted by *Rhagoletis* in the United States include cherries, blueberries, and apples (Yee et al. 2014, Rodriguez-Sanoa et al. 2015, Wakie et al. 2019).

The three major *Rhagoletis* cherry pests native to North America are *Rhagoletis cingulata* Loew, *Rhagoletis indifferens* Curran, and *Rhagoletis fausta* (Osten Sacken) (Yee et al. 2014). In the past decade, two invasive fly species that damage cherries have been introduced to North America. The first to arrive was *Drosophila suzukii* Matsumura (Diptera: Drosophilidae). Its first confirmed record in the United States was from California in 2008 and the species has since spread throughout much of the country (Hauser 2011). In addition to cherry, this pest can use other fruits such as raspberry, strawberry, and wild plants like honeysuckle as hosts (Bellamy et al. 2013, Leach et al. 2019). The second invasive fly to arrive to North America was the European cherry fruit fly, *Rhagoletis cerasi* (L.). This pest, native to Europe, was first detected in Ontario, Canada in 2016 and then in New York in 2017 (Barringer 2018, Wakie et al. 2018). Like *D. suzukii*, this invasive species can also use honeysuckle as a host, but it has only been reported as a commercial pest of cherry.

The presence of new fruit fly species in the United States that attack cherries will complicate accurate and timely identification of immature life stages when collected from fruits in infested fields or

intercepted during transport. Fruits will not always exhibit signs of damage when eggs or larvae are present; therefore, techniques have been developed to extract larvae from fruits for the purpose of surveillance (Yee 2014, Shaw et al. 2019). The morphology of late instar *Drosophila* and *Rhagoletis* is distinct, but a molecular test was developed to separate *D. suzukii* from other species of *Drosophila* (Murphy et al. 2015). A similar molecular test has not been developed to assist in identification of *R. cerasi*. This species can be distinguished from other cherry-infesting *Rhagoletis* species when trapped as adults by comparing wing-banding patterns. However, characters in third instar larvae such as tubule number on anterior spiracles and total body length as are not regarded as reliable (White and Elson-Harris 1992). No diagnostic characters have been reported for reliable identification of earlier life stages of the fly. Accurate identification of invasive species is important for pest management response by plant protection organizations (Lyal and Miller 2020). Behavioral and developmental differences between *Rhagoletis* species regarding tolerance to climate, host acceptance, and timing of adult emergence are useful to develop effective monitoring, management, and pest exclusion practices (Johannesen et al. 2013, Yee et al. 2014, Wakie et al. 2019). Wakie et al. (2018) suggest that trapping and chemical treatments established for native cherry pests in North America might be effective for management of *R. cerasi*. Identification of these pests to species is needed to document pest distributions and improve surveillance and management practices for the species.

In this study, we investigate the internal transcribed spacer 1 (ITS1) of the nuclear rDNA array as a source of diagnostic characters for *R. cerasi*. The ITS segments of rDNA have been shown to be highly variable between even closely related species and therefore useful for insect diagnostics in general (e.g., Coleman 2009), and for fruit fly diagnostics in particular (e.g., Barr et al. 2006, 2017; Boykin et al. 2014; Sutton et al. 2015; Prezotto et al. 2019). The three native species that use cherry as hosts in North America are not closely related to *R. cerasi* (Smith and Bush 1997, Smith et al. 2005) which suggests that diagnostically informative ITS1 differences should exist among these species. To date, ITS1 sequences have not been reported for all of the cherry-infesting *Rhagoletis* species and it is possible that intragenomic or intraspecific variation (Leo and Barker 2002, Douglas et al. 2004, Coleman 2009, Barr et al. 2011) could prevent reliable use of ITS1 as diagnostic for the pest. Therefore, new sequence data are required to complete a comparison of diagnostic utility.

In addition to documenting differences in ITS1 sequences, the sequence data set will be used to develop and test two diagnostic methods to identify *R. cerasi* that are alternatives to a DNA sequencing-based diagnostic protocol. The first is a loop-mediated isothermal amplification (LAMP) technology and the second is a multiplex, conventional polymerase chain reaction (mcPCR) technology that measures PCR product using agarose gel electrophoresis. Conventional PCR techniques are appropriate for implementation of diagnostic testing at a wide range of laboratories based on the routine use of PCR temperature cycling technology. In contrast, LAMP can be accomplished using instruments that hold a constant isothermal temperature (Wong et al. 2017) and it has been proposed as a readily deployable technology for molecular diagnostics of insects including fruit flies (Huang et al. 2009, Blaser et al. 2018). Since less time is often required to complete an isothermal reaction, examining a LAMP technique could contribute to Single-day Turn-Around Time (STAT) testing of pests.

Materials and Methods

Specimens and DNA Extractions

The specimens used in this study were organized into two data sets. The first included 242 adult specimens that were identified using morphological examination and the second was comprised of 119 larvae and pupae collected from fruits in New York identified using DNA barcoding of the cytochrome oxidase c I gene (COI) or from a lab colony in Greece (N. Papadopoulos Lab). The species and collection information for specimens are provided in Table 1. One of the adult specimens of *R. fausta* included in the study was submitted as a DNA isolate from a phenol–chloroform nucleic extraction performed at the Laboratory of B. A. McPheron at Penn State in 1993 and identified by H. Y. Han. The entire fly was destroyed in the extraction process and not available for reexamination but COI sequence (GenBank MW136107) data matched the initial morphological determination. The larvae collected from snowberries in New York were identified as *R. zephyria* Snow based on host, collection location, and COI data (Supp File S1 [online only]).

All DNA extractions performed at the PPQ Texas facility were completed using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the description of Barr et al. (2012). A leg from each adult fly was used for nucleic acid extraction. The remaining bodies of the adult flies were saved in ethanol as voucher material. The only exception to the use of leg tissue from adult specimens was a single adult fly from the *R. cerasi* Greek lab colony. This fly was used to extract two DNA samples: the first was performed using a leg (PPQ Mission Lab DNA Isolate code BX190304-011) for comparison to other flies in the study but subsequently the entire body (BX190502-001) was extracted to provide a sample of higher DNA concentration as a control in methods testing studies. Small larvae at early instar stage were destroyed in the extraction process by crushing the insect. The larvae from later instars were extracted using an excised section of tissue. Using a razor, the midsections of larvae were excised and used to extract DNA following the DNeasy Blood and Tissue Kit manufacturer protocol including a 180-min incubation step in buffer. Posterior and anterior ends of these larval specimens were then stored in ethanol as vouchers. Twenty *R. cerasi* larvae from the Greek colony were extracted following the aforementioned protocols but were treated at different incubation times of 10 min ($N = 5$), 30 min ($N = 5$), 60 min ($N = 5$), and 180 min ($N = 5$) to compare effect of incubation time of extract on test methods. The final *R. cerasi* larva (BX190524-001) from the Greek colony included in the study was extracted from the entire specimen. DNA quantity was estimated using NanoDrop 8000 spectrophotometer (Thermo Scientific/NanoDrop, Wilmington, Delaware) and 1.5 µl of DNA extract per sample.

PCR and Sequencing of COI to Identify Immature Flies Used in the Study

After excluding the 21 larvae from a rearing colony, 98 of the 119 immature flies in the study (Table 1) were identified using DNA sequencing of the COI gene. *Rhagoletis* DNA samples were amplified for COI using Applied Biosystems GeneAmp PCR system 9700 instruments. Takara Bio *Ex* Taq polymerase (Takara Bio USA Inc., Mountain View, CA) was used for all reactions. Unless indicated otherwise, reactions were performed in 25 µl volumes of sterile water with final concentrations of 1× buffer (including 25 mM $MgCl_2$), 0.2 mM each dNTP, 0.4 µM of each primer, and 0.625 U of Ex Taq HS DNA polymerase. PCR primers are listed in Table 2. All primers were synthesized as salt-free oligos (Operon Eurofins, Louisville, KY and Biosearch Technologies, Novato, CA) and resuspended in 1× TE. All PCR experiments were performed using 1 µl of template DNA or sterile water as a negative control.

Prior to PCR amplifying and sequencing the COI gene from immature flies, the COI gene was amplified using different primer combinations in a pilot study using adult flies as DNA template. Initial COI analysis using the Folmer et al. (1994) DNA barcoding primers

Table 1. Specimens used in the study

Species	Location (collection year)	Adult	Immature
R. cerasi		**136**	**87**
	Belgium (2019)	5	0
	Canada: Ontario (2016)	5	0
	Germany: Dossenheim (2010)	5	0
	Germany: Dossenheim (2018)	5	0
	Greece: Lab Colony (2018)	5	21
	Hungary (unknown)	5	0
	Switzerland: Visperterminen (2004)	4	0
	United States: New York (2017)	46	0
	United States: New York (2018)	56	0
	United States: New York (2019)	0	66
R. cingulata		**49**	**3**
	United States: Florida (2008–2016)	21	0
	United States: Massachusetts (2008)	2	0
	United States: South Carolina (2009)	1	0
	Mexico: Zacatlan, Puebla (2003)	1	0
	Germany: Baden-Württemberg, Dossenheim (2006)	1	0
	Germany: Rhineland-Palatinate (2004–2005)	3	0
	United States: Indiana (2019)	*10*	*0*
	United States: New York (2019)	*10*	*3*
R. indifferens		**16**	**0**
	United States: Washington (1998)	5	0
	United States: Washington (2008)	1	0
	United States: Washington (2019)	10	0
R. fausta		**11**	**3**
	Extraction	1	0
	United States: New York (2019)	10	3
R. meigenii (Loew)		**0**	**1**
	United States: New York (2019)	0	1
R. cornivora Bush		**5**	**0**
	United States: Michigan (2017)	5	0
R. pomonella (Walsh)		**15**	**0**
	Mexico (1996)	5	0
	United States: Michigan (2017)	10	0
R. zephyria		**10**	**25**
	United States: Washington (2019)	10	0
	United States: New York (2019)	0	25

LCO-1490 and HCO-2198 did not generate high-quality sequences for *R. cerasi* specimens. A comparison of three *R. cerasi* extractions (BX160718-002, BX171120-009, and BX171120-10) using various primer sets demonstrated that the TY-J-1460 and C1-N-2191 primers (Simon et al. 1994) generated superior COI sequences to the Folmer et al. primers (data not shown). All immature specimens were sequenced using the Simon et al. (1994) primers.

All COI PCRs were performed using cycling parameters of 4-min denaturation at 94°C followed by 39 cycles of 20 s at 94°C, 20 s at 55°C, 30 s at 72°C, and an extension of 5 min at 72°C. PCR products were visualized using 1.2% TAE agarose gels prestained with ethidium bromide. The size of products was compared to TriDye 100-bp ladder (New England Biolabs, Beverly, MA) to inspect fragment length. PCR products were purified with ExoSAP-IT (USB Corp., Cleveland, OH) prior to DNA sequencing. The amplicons were sequenced using the two PCR primers and ABI BigDye Terminator v.3.1 chemistry at commercial centers Functional Biosciences (Madison, WI). All sequences were edited and assembled into contigs using the program Sequencher v5 (Genecodes, Ann Arbor, MI) and aligned using MEGA7 (Kumar et al. 2016). Sequences were compared to COI data records at National Center for Biotechnology (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Barcode of Life Data System (http://www.boldsystems.org/) to support species determinations. The data were also translated to protein sequences to search for possible pseudo-copies in the data set. Larva identifications were based on >99% matches to existing records for a species. All 98 sequences generated from immature flies in the study are available in Supp File S1 (online only).

PCR and DNA sequencing of 18S gene

The 18S gene was amplified from four fruit fly species (i.e., *R. cerasi*, *R. indifferens*, *R. pomonella* (Walsh), and *Anastrepha ludens* (Loew) (Diptera: Tephritidae)) using highly conserved NS3-F and NS4-R primers (Table 2) under the following cycling conditions: 3 min at 94°C followed by 35 cycles of 20 s at 94°C, 30 s at 60°C, 60 s at 72°C, and a final extension of 5 min at 72°C. All other conditions for PCR, agarose gel electrophoresis, DNA sequencing, and data editing were completed following methods described for COI. Sequences were submitted to GenBank (MW088962–MW088970). These sequences were used to develop primers to be used as controls of DNA quality in the LAMP and PCR diagnostic tests.

PCR and DNA sequencing of ITS1

The ITS1 fragment of rDNA was amplified from *R. cingulata*, *R. indifferens*, and *R. fausta* specimens using two sets of primers. The first set used the primers balITS1f and balITS1r (Table 2) reported by McKenzie et al. (1999). The cycling parameters for this primer pair were 2 min at 94°C followed by 39 cycles of 60 s at 94°C, 60 s at 60°C, 60 s at 72°C, and an extension of 5 min at 72°C. This primer set was not successful at amplification and sequencing of all specimens. Using PRIMER3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/), additional primers were developed for amplification of a 363-bp segment of ITS1 based on comparison of a *R. cingulata* sequence to other species using records downloaded from GenBank for *R. cingulata* (X94554.1), *R. pomonella* (X94555.1), *R. completa* Cresson (AY66111.1 and HQ677028), and *R. zoqui* Bush (HQ677025.1). The new primer set ITS1-RC363F and ITS1-RC363R (Table 2) was amplified using the protocols described for balITS1f and balITS1r. The sequences of the amplified ITS1 363-bp fragment matched the data from ITS1 sequences generated using the McKenzie et al. (1999) primers. ITS1 sequences generated using various primer sets were submitted to GenBank for two *R. cingulata* specimens: MW183266 (primer set baITS1F/balITS1r) and MW183267 (primer set ITS1-RC363F/ITS1-RC363R); for two *R. indifferens* specimens: MW183271–72 (primer set baITS1F/balITS1r); and for three *R. fausta* specimens: MW183268 (primer set baITS1F/balITS1r) and MW183269–70 (primer set baITS1F/ITS1-RC363R).

DNA sequencing of the ITS1 amplicon generated using primers balITS1f and balITS1r was not successful for *R. cerasi* samples. These primers generated multiple bands for the species and gel-excised bands did not sequence well or did not match ITS1 records. The new primers ITS1-RC363F and ITS1-RC363R were successful for bidirectional sequencing of the species but the amplified fragment was relatively short.

PCR was performed again using the *R. cerasi* DNA extracted from the whole adult fly as template (BX190502-001) and the

Table 2. Primers used in the study

Primers	Sequence (5′ to 3′)	Source
TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC	Simon et al. (1994)
C1-N-2191	CCCGGTAAAATTAAAATATAAACTTC	Simon et al. (1994)
baITS1f	GGAAGGATCATTATTGTGTTCC	McKenzie et al. (1999)
baITS1r	ATGAGCCGAGTGATCCACC	McKenzie et al. (1999)
ITS1-RC363F	TTGTAATGCATCAGGGCAAT	This study
ITS1-RC363R	TGATCCACCGCTTAGAGTGA	This study
NS3-18s	GCAAGTCTGGTGCCAGCAGCC	White et al. (1990)
NS4-18s	CTTCCGTCAATTCCTTTAAG	White et al. (1990)
C5F3	TCATTCATTTTGCGACGG	This study
C5B3	AGTACTACCACTCCGGTTA	This study
C5FIP	ACTCGATGCACTAAAGAAGGATTTTTCTGAAGCAATTTTGGATGT	This study
C5BIP	AGGTGTAGGGTTTCATTCCATTTTAATATCTGACCCTGGACGA	This study
LAMP 18sFF2-F3	GGGGCATTAGTATTACGACG	This study
LAMP 18sFF2-B3	AGCTACACCCAATTGCTAG	This study
LAMP 18sFF2-FIP	ATCTTTGGCAAATGCTTTCGCCGAGAGGTGAAATTCTTGGA	This study
LAMP 18sFF2-BIP	ATCAAGAACGAAAGTTAGAGGTTCGCATCGTTTATGGTTAGAACTAGG	This study

aforementioned amplification conditions with various combinations of the four ITS1 primers. These four combinations of forward and reverse primers generated products that were gel-excised and cloned using the TOPO TA Cloning Kit for Sequencing (Thermo Fisher Scientific, Waltham, MA). Colonies were transformed using Electrocomp (Thermo Fisher Scientific) competent cells, grown, and selected on Luria-Bertani agar plates with kanamycin. Transformed colonies were selected for every individual (i.e., each PCR product), plasmid DNA was purified using a miniprep kit (Qiagen), and plasmids were tested for insertion of ITS1 via restriction digestion. Plasmids were sequenced using universal M13 forward and reverse primers by Functional Biosciences. To provide an estimate of variation among species data, the ITS1 sequence from *R. cerasi* was compared to records generated for *R. cingulata*, *R. indifferens*, and *R. fausta* using NCBI's BLAST pairwise comparison in the blastn suite under default settings. The coverage and percent identity of aligned regions was recorded for each pairwise comparison to *R. cerasi*. The ITS1 sequence generated from cloned PCR product was submitted to GenBank: MW183264.

LAMP Design and Conditions

The program PrimerExplorer (https://primerexplorer.jp/e/) was used to design all LAMP primers using a single exemplar sequence of *R. cerasi* as the query sequence in the search. The searches were performed using the Automatic Judgment option and default settings. Alignments of 18S and ITS1 data were used to compare if locations of output primers were in conserved regions of the genes. Regions of high conservation among species for the 18S data (data not shown) were used to select four primers to serve as control reaction of DNA quality. Regions of low conservation between *R. cerasi* (cloned sequence) and other species in our data were used to select four diagnostically informative primers for *R. cerasi*. LAMP reactions require at least four primers to amplify a DNA target, but it is possible to include one or two additional primers called loop primers that increase sensitivity of the reaction. The program searches did not result in acceptable loop primers for inclusion in the tests. Therefore, the LAMP protocols used four primers each: F3, B3, FIP, and BIP. All primers are listed in Table 2.

LAMP reactions were performed using ISO-001 mix (Pro-Lab Diagnostics, Round Rock, TX). The ISO-001 master mix uses an engineered LF (Large Fragment) DNA polymerase isolated from *Geobacillus* sp. SSD, GspSSD. The mix includes dye (measured using FAM channel), $MgCl_2$, and polymerase all at unreported concentrations. Reactions were performed in 25 µl volumes that were diluted in sterile water with final concentrations of 1× ISO-001 mix, 0.3 µM primer F3, 0.3 µM primer B3, 1.2 µM primer FIP, and 1.2 µM primer BIP. The primer ratio of inner (FIP, BIP) to outer (F3, B3) was 4:1. All primers were synthesized as salt-free oligos (Operon and Biosearch) and resuspended in 1× TE. All LAMP experiments were performed using 1 µl of template DNA or sterile water as a negative control.

The LAMP tests were performed on two different instruments: the Genie III (OptiGene Ltd., Horsham, UK) and the Bio-Rad CFX96 Touch Real-time PCR Detection System using Bio-Rad CFX Maestro Software (Bio-Rad Laboratories, Inc., Hercules, CA). In addition to calculating the time for amplification success the software of each instrument was used to collect melting temperatures of the products. All Genie III reactions were run for 3 min at 45°C as a preheat step and then for 30 min at 63°C and read in channel 1-Blue. An annealing melt analysis was tested with Genie III using range of 98°C to 70°C and ramp rate of 0.05 s. For the Bio-Rad CFX96 tests, the software was at default settings: Cq Determination Mode–Single Threshold, Baseline settings–Baseline subtracted curve fit. Bio-Rad runs were performed using three step program: step 1) 63°C for 1 min, step 2) go to step 1 for 29 repeats, step 3) perform melt analysis using range of 98°C to 70°C and ramp rate of 0.05 s and increments of 0.5°C on plate read. The ITS1 and 18S reactions were always performed in separate tubes but performed on same runs. Amplification of the 18S control reaction was required for the sample run to be scored as valid for all LAMP runs.

Initial testing was performed using both the Genie III and the Bio-Rad CFX instruments. A comparison was performed using the same five *R. cerasi* samples but in separate runs conducted on both instruments and by three different operators. This was to test for an effect on identifications and the time required to reach positive result. This study was designed and analyzed in JMP 13.1 (SAS Institute, Cary, NC) using Design of Experiments, *F*-statistics for variance, and Factorial ANOVA under the standard least square option. The factors Device and Operator were tested as was the interaction of these factors. The time value is measured as seconds on the Genie III but as quantification cycle (Cq) on the Bio-Rad CFX. The Cq values were converted to seconds for comparison between instruments. The correlation between ITS1 and 18S LAMP Cq values was calculated in JMP for each instrument tested. All subsequent runs of the LAMP test were performed on a Bio-Rad CFX instrument.

Twenty *R. cerasi* larvae from a lab colony were treated in the DNeasy extraction procedure by incubating for either 10, 30, 60, or 180 min. Each group of five flies were compared using ANOVA in

JMP 13.1 to determine if there was a statistical difference in mean values of DNA concentration (ng/µl), and Cq value (ITS1 and 18S) on the Bio-Rad instrument. The analysis was performed on means treating variables separately.

mcPCR Test for *R. cerasi*

An mcPCR experiment was performed on all samples using the outer LAMP primers for ITS1 (C5F3 and C5B3) and outer LAMP primers for 18S (18sFF2-F3 and 18sFF2-B3) (Table 2). The expected product sizes of the ITS1 and 18S primer pairs are 248 and 180 bp, respectively. Reactions were performed on GeneAmp PCR system 9700 instruments in 25 µl volumes of sterile water with final concentrations of 1× buffer, 0.2 mM each dNTP, 0.4 µM of each primer, and 0.625 U of Ex Taq HS DNA polymerase. Amplification conditions were 3 min at 94°C followed by 30 cycles of 20 s at 94°C, 20 s at 60°C, 30 s at 72°C, and an extension of 5 min at 72°C. The entire PCR product (25 µl) was visualized using 2% TAE agarose gels (90 min, 120 V) prestained with ethidium bromide. The size of products was compared to TriDye 100-bp ladder (New England Biolabs, Beverly, MA) to inspect fragment size. Presence of the 18S amplicon was required to interpret the sample run as valid.

Serial Dilution

Sensitivity was evaluated for both LAMP and multiplex PCR tests using a series of serial dilutions of five *R. cerasi* DNA extractions in concentrations from 1 ng/µl to 0.0001 ng/µl. Dilutions were prepared in water. LAMP and multiplex PCR conditions were identical to those listed above. All extractions included in the experiment were successful in prior LAMP and mcPCR tests. Three of the extractions were from legs of field trapped flies that represent specimens with estimated DNA concentrations of approximately 5 ng/µl. Two of the flies were from a lab colony: one extract was from a whole adult fly (estimated starting concentration of 55 ng/µl) that has been used as a control in the studies and the second from a larva (estimated starting concentration of 180 ng/µl). Each extraction dilution was tested in triplicate resulting in a total of 75 reactions for each test method. The number of successful reactions was summed for each dilution concentration and for each fly extraction. The limit of detection for LAMP and mcPCR test methods was determined by using the concentration at which all reactions were successful as the cutoff value. The Cq values estimated from the LAMP reactions on the Bio-Rad instrument were used to estimate mean, SD, and coefficient of variation (CV) of triplicate readings. In addition to Nanodrop readings, double-stranded DNA concentrations were estimated using Qubit dsDNA HS Assay kit on an Invitrogen Qubit Fluorometer (ThermoFisher, Waltham, MA) for the five flies included in the serial dilution test.

Diagnostic Sensitivity and Specificity

Rates of false negatives for the LAMP and PCR multiplex tests were estimated by performing each protocol on all 223 *R. cerasi* specimens in the study (Table 1). It is calculated as 1 – Diagnostic Sensitivity (DSe), where DSe equals the number of correctly identified *R. cerasi* specimens divided by the total number of *R. cerasi* specimens. The rates of false positives for the LAMP and PCR multiplex tests were estimated by performing each protocol on all 138 specimens of the nontarget species (Table 1). It is calculated as 1 – Diagnostic Specificity (DSp), where DSp equals the number of correctly identified nontarget specimens divided by the total number of nontarget specimens. The 95% Exact Binomial Confidence Limits were estimated for these values using the Clopper-Pearson Exact Method in JMP 13.1.0.

For LAMP, these tests were performed using the Bio-Rad instrument. All runs included an extraction of a *R. cerasi* specimen (BX190524-001) from the Greek lab colony as a positive control, a negative control of the PCR using water as template, and a second negative control for DNA extraction process (i.e., a blank nucleic acid isolation reaction run alongside specimens during each DNeasy processed batch). The positive control had to amplify the ITS1 target and negative controls had to not amplify product, as expected, for a run to be treated as valid. If the 18S product failed to amplify for a sample, then the sample in the run was scored as a reaction failure (i.e., inconclusive). If both markers generated amplicons, then the test sample was scored as *R. cerasi*. If only the 18S marker generated an amplicon, then the sample was scored as nontarget (i.e., not *R. cerasi*).

The difference between ITS1 and 18S Cq values were calculated for each tested sample using the formula ITS1 Cq – 18S Cq = sample difference. The results were grouped using intervals of 1 Cq and plotted on a graph. The data used for this graph were derived from the initial testing of samples to calculate DSe and DSp values.

A sample run was repeated if the sample failed to amplify the control 18S marker or resulted in determinations that disagreed with known identity of the sample. This repeat was not included in calculation of false positives or false negatives. The diagnostic sensitivity and specificity values were based on first run of each test. The repeats were performed to determine if the outcomes were reproducible. The melting temperatures of all ITS1 and 18S products were recorded as well to compare with molecular determinations.

Results

DNA Sequence Data

Representative 18S sequences of *R. cerasi* (MW088962–65), *R. indifferens* (MW088966–67), and *R. pomonella* (MW088968–69) generated in the study were submitted to GenBank. As expected, the 18S data are highly conserved in the genus. ITS1 sequences of *R. cingulata*, *R. indifferens*, and *R. fausta* specimens used in the study were submitted to GenBank (MW183266–72). The new *R. cingulata* and *R. indifferens* records from this study matched the previously reported *R. cingulata* ITS1 record from GenBank. The three *R. fausta* records from this study matched each other and represent the first reported ITS1 records for the species. The McKenzie et al. (1999) primers used to amplify ITS1 from those three species were not successful at amplifying the longer fragment from *R. cerasi*. The new primers ITS1-RC363F and ITS1-RC363R were successful in PCR amplification and DNA sequencing for a few *R. cerasi* in the study but still failed to amplify ITS1 from all *R. cerasi* specimens. These new primers were also more successful than the longer McKenzie et al. (1999) primers at sequencing specimens of other species (e.g., *R. cingulata* GenBank MW183267). The amplified *R. cerasi* fragment was about 340 bp and contained four insertions/deletions and over 30 substitution sites that could be suitable for developing diagnostic primers or probes for distinguishing *R. cerasi* and other species. Using the primer set baITS1f and ITS1-RC363R, it was possible to amplify a larger fragment of ITS1 from *R. cerasi*. DNA sequencing of this fragment required cloning to generate a high-quality sequence reads in both directions. Based on the cloning experiments, the *R. cerasi* genome includes an ITS1 copy that is double the length of ITS1 copies in the other *Rhagoletis* species examined from North America. Amplification and cloning from

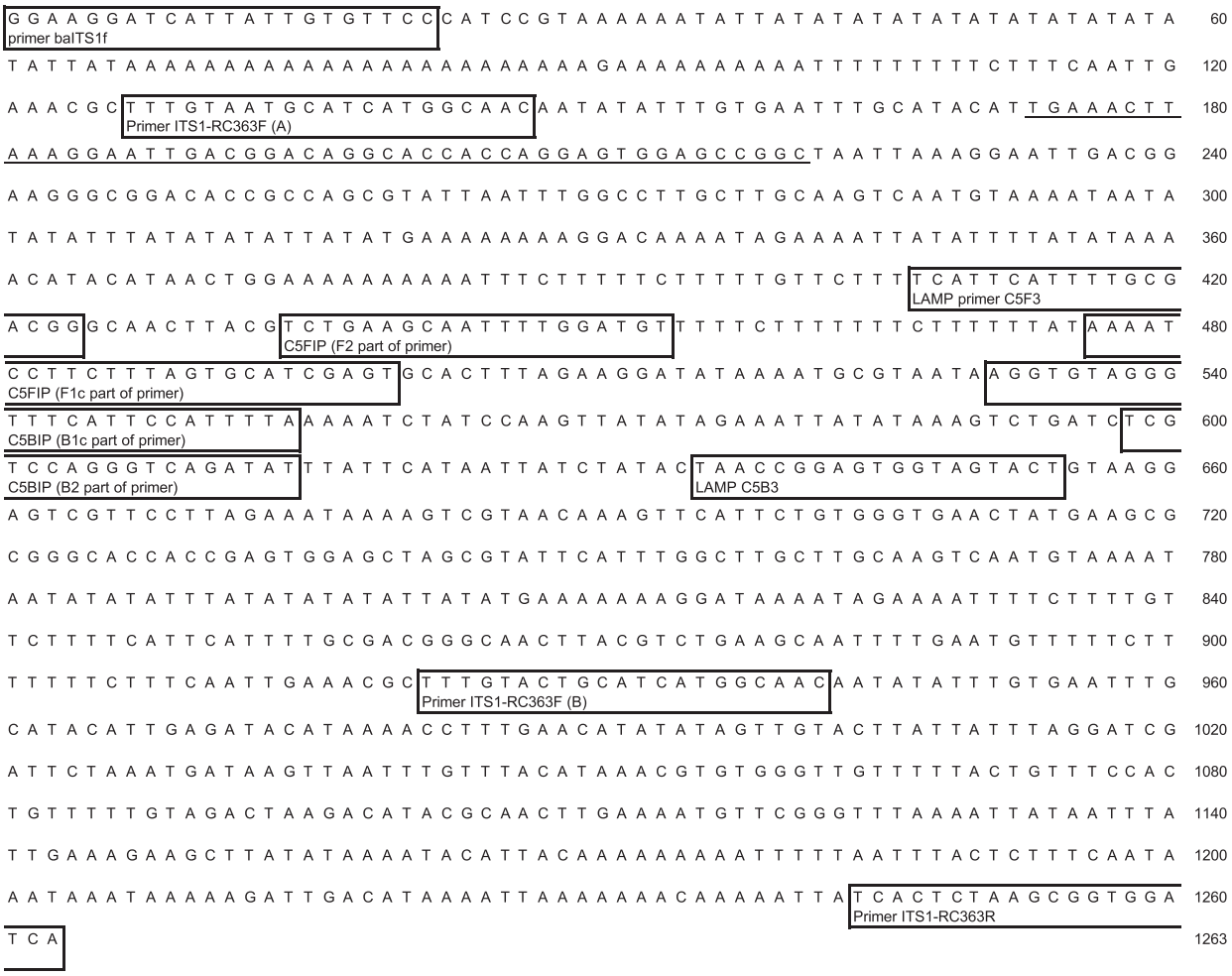

Fig. 1. Cloned *R. cerasi* ITS1 sequence with primer locations indicated. The ITS1-RC363F primer is located at two locations (A and B). The second location (B) is present in other *Rhagoletis* species. A segment of 49 bases is underlined because it matches 18S sequences of other *Rhagoletis*.

one extract (BX190502-001, GenBank MW183264) generated an amplicon insert (clone 5.01) of 1,263 bp (including the PCR primers), after removal of the plasmid DNA at ends (Fig. 1). This ITS1 fragment included 49 bases that matched the 18S sequence of other species (e.g., when compared to MN507538). The short 18S-like segment in the cloned fragment was located within the IT1 sequence (Fig. 1). The ITS1-RC363F primer sequence is present at least twice in the ITS1 sequence. This observation might explain why the primer did not work reliably for amplification on all *R. cerasi* samples. Using BLAST, the *R. fausta*, *R. cingulata*, and *R. indifferens* ITS1 sequences were matches to 100- to 200-bp segments of the entire *R. cerasi* sequence (measured as coverage) but highly divergent to other segments of the *R. cerasi* sequence. Pairwise similarities between the three species and *R cerasi* ranged from 86 to 87% for those sites that were included in coverage.

LAMP Primer Design for ITS1

The PrimerExplorer program selected LAMP primers for both 18S and ITS1 targets that included four primers (two inner and two outer) for each target (Table 1; Fig. 1). Although LAMP can include one or two additional loop primers for a reaction, neither search found a suitable set of loop primers. The selected LAMP 18S primers target a 180-bp fragment of the gene. The selected LAMP ITS1 primers target a 248-bp fragment of the spacer. Additional ITS1 primers have been developed and tested for *R. cerasi* in the region between nucleotides 922 and 1263 but these were determined to be nonspecific (data not shown). The mcPCR protocol was performed using the F3 (i.e., C5F3) and B3 (i.e., C5B3) primer sets for the two genes and generated products of expected sizes (Fig. 2).

Experimentation on Different Instruments

The LAMP 18S and ITS1 reactions were performed on *R. cerasi* specimens to test the effect of operator (repeatability) and instrument on ability to diagnose samples as *R. cerasi* and on the time required to generate product. The 18S (F-ratio 4.5460, P = 0.1213) and ITS1 (F-ratio 0.4986, P = 0.7682) data sets were normally distributed. The experiment did not detect any difference in ability to diagnose the specimen correctly to the species *R. cerasi*. All runs generated the correct determination (N = 30). Comparison of the time required to generate a positive result, however, was statistically different for both the operator and instrument variables based on ITS1 ($F_{(2, 22)}$ = 14.8315, P < 0.002; $F_{(1, 22)}$ = 687.4273, P < 0.0001, respectively) and 18S ($F_{(2, 22)}$ = 47.1429, P < 0.001; $F_{(1, 22)}$ = 1221.4890, P < 0.0001, respectively) markers (Supp Fig. S1 [online only]). The Bio-Rad instrument consistently had earlier amplification times than the Genie III in our test but this does not equate to better performance in completing correct identification. The interaction for these two factors, however, was also statistically significant for ITS1 and

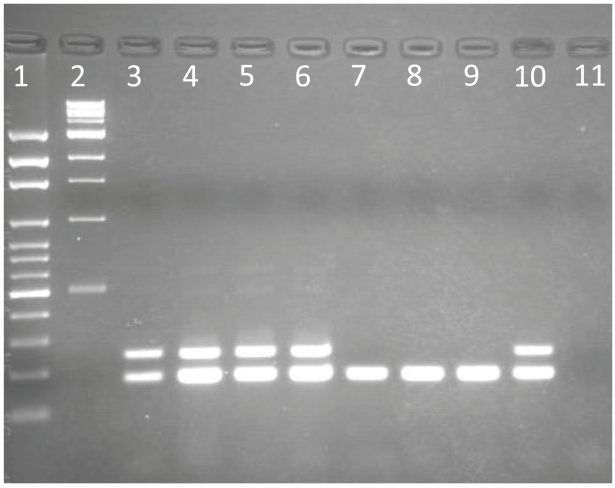

Fig. 2. Gel image of multiplex PCR test result on 2% TAE agarose. Lanes 1 = 100-bp ladder, 2 = 1-Kb ladder, 3–6 = *R. cerasi* from field collections, 7–8 = *R. cingulata*, 9 = *R. indifferens*, 10 = control of *R. cerasi* from lab colony, 11 = negative control.

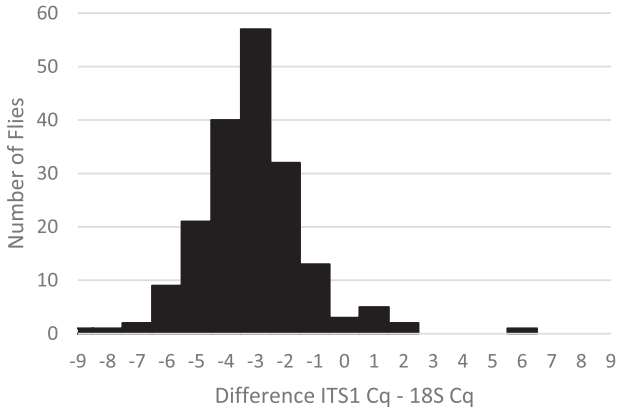

Fig. 3. Distribution of *R. cerasi* specimen counts according to differences between ITS1 and 18S Cq values.

18S ($P = 0.0007$ and $P < 0.0001$, respectively) precluding a simple interpretation of an additive relationship between device and operator. The outcome of the comparison is that, under the conditions tested in the study, values can statistically vary for instrument and operator. Additional analysis would be required to characterize within-laboratory variation of values. Although statistically significant, the observed variation did not have a biologically significant impact on the pest identification results.

Correlations for ITS1 and 18S values on the Bio-Rad and Genie III were $r = 0.902$ and $r = 0.770$, respectively (Supp Fig. S2 [online only]) demonstrating a connection between values. Using the Bio-Rad data generated to calculate DSe, the difference between Cq values for ITS1 and 18S from *R. cerasi* specimens (using absolute values) was five or less for approximately 93% of the flies. This percentage only includes specimens that generated readings for both markers ($N = 190$). The most extreme absolute difference was equal to 8.56. In most cases the ITS1 amplified before the 18S marker (Fig. 3). This suggests that ITS1 is more sensitive than the control 18S target. Using the DSe data set, the melt temperatures for ITS1 products ranged from 80 to 84°C with most sample temperatures equal to 81°C (31%) and 81.5°C (60%). The melt temperatures for 18S products ranged from 83.5 to 85°C with most sample temperatures equal to 84.5°C (93%). Although correlation between the DNA targets for mcPCR test was not measured because gel images were scored as binomial presence or absence states, the 18S band was less intense than ITS1 band when dilution studies were performed.

Effect of Incubation Time on DNA Extractions From Larvae

The four extraction treatments which varied incubation time for *R. cerasi* larvae collected from a lab colony did not detect a statistical difference in the final DNA concentrations ($F_{(3, 16)} = 0.3002$, $P = 0.8248$), ITS1 Cq values ($F_{(3, 16)} = 2.3455$, $P = 0.1114$), or 18S Cq values ($F_{(3, 16)} = 1.8847$, $P = 0.1729$). All 20 flies generated correct identifications in experiments using LAMP and mcPCR tests. The mean DNA concentration and standard deviation for the lab-reared larvae extracted using the 3-h incubation was 192 ± 40 ng/µl ($n = 5$). In comparison, the *R. cerasi* larvae reared from fruits collected in New York in 2019 and processed using 3-h incubation time had a lower mean DNA concentration (65 ± 21 ng/µl, $n = 55$). The two larval data sets, however, had much higher concentration means than the *R. cerasi* leg extractions of trapped flies captured in New York in 2018 and treated under the same extraction incubation conditions (4 ± 1 ng/µl, $n = 56$). Higher nucleic acid extraction yields in larval samples compared to adult leg samples is consistent with less tissue in a leg versus a larval segment. The study did not control for mass of tissue types.

Serial Dilution

One of the extractions included in the serial dilution test failed to generate successful reactions at all dilutions and for both LAMP and mcPCR methods. The nondiluted extraction of this fly was successful for both test methods. This extract was from one of the three fly leg samples analyzed in the experiment. Although this sample had an estimated nucleic acid concentration similar to the other two leg samples (5 ng/µl), it had an A260/280 reading (0.96) lower than other samples (Supp Table S1 [online only]). Based on consistent failure at 1 ng/µl and evidence of poor purity, this extract was excluded from subsequent analysis of test results. Of the other four remaining extractions, all test reactions were successful at 1 ng/µl dilution (Fig. 4). For the mcPCR test, all samples were successful at the 0.1 ng/µl dilution. The extraction from the larva failed to amplify in mcPCR at the 0.01 ng/µl dilution. A similar result was observed for the LAMP test; the larva was the first extraction to generate inconsistent amplification success, but that failure started at the 0.1 ng/µl dilution. Including all four extractions, the limit of detection for the mcPCR test method is 0.1 ng/µl and the LAMP test method is 1 ng/µl. The mean LAMP Cq values based on triplicate runs generated similar values (Supp Table S1 [online only]). The CV values for the experiment ranged from 0.4 to 1.5% (mean CV 1.1%).

Diagnostic Sensitivity and Specificity for LAMP Test

The LAMP test generated conclusive data for 323 of the 361 flies in the study on the first run (Table 3). Of the 223 *R. cerasi* specimens tested, one larva and 33 adults trapped as part of a domestic survey in New York failed to generate interpretable data. These inconclusive results were the result of 12 adult flies failing to generate 18S even though the ITS1 target did amplify and the 22 remaining flies failing to generate any amplification products. Repeating reactions did not resolve the problem of 18S failure for these flies. Subsequent analyses were performed on the 189 *R. cerasi* flies that had successful 18S

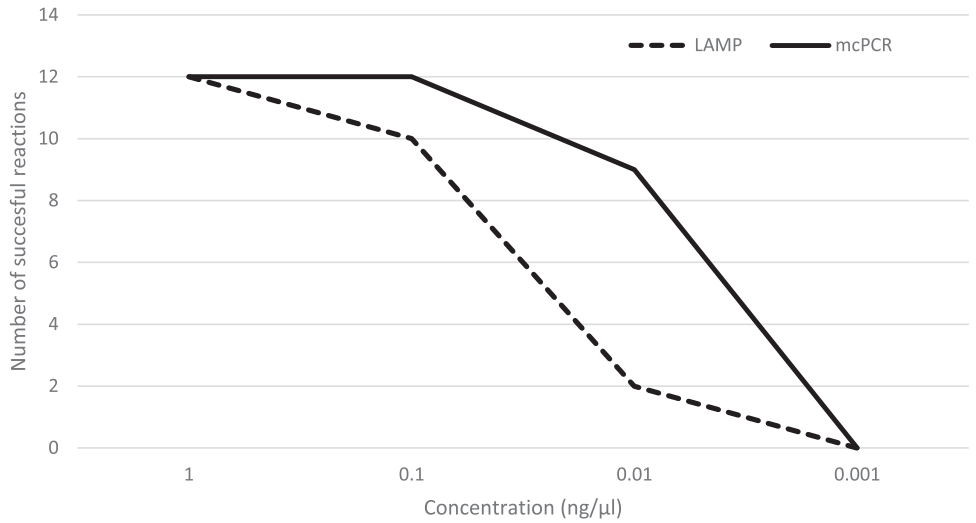

Fig. 4. Reaction success for LAMP and mcPCR testing of *R. cerasi* at different concentrations of DNA extractions using serial dilutions. A total of 12 reactions were tested for each concentration from four different nucleic acid extractions (each extract tested in triplicate).

amplification. The DSe for this LAMP experiment was 98.4% (CI 95.4–99.7%) and the false-negative rate was 1.6%. This error rate was the result of one pupa and two adults from New York failing to amplify ITS1 on the first run that was used to estimate sensitivity. These samples were correctly identified when the reaction was repeated for a second time and had DNA extraction concentrations similar to other samples (>1 ng/µl).

The test was performed on 138 specimens that were grouped as nontarget species (i.e., not *R. cerasi*). Of these flies, four failed to amplify the 18S target. The DSp of the 134 specimens in the LAMP data set was 97.8% (CI 93.6–99.5%) and the false-positive rate 2.2%. There were two *R. cornivora* specimens (isolates BX190702-016 and BX190702-019) that generated late ITS1 amplifications (Cq > 28). Relative to 18S values (Cq ~ 16), the ITS1 readings for these *R. cornivora* specimens were much higher than expected based on *R. cerasi* specimens (Fig. 3). It is possible that these were the result of contamination in the runs. Analysis of melt temperatures revealed one with 80°C and the second at 83.5°C. Based on samples in the study, the most common melt temperature for a *R. cerasi* ITS1 product was around 80.5 to 81°C. However, our study has reported readings of 83 to 84°C. A repeat of the LAMP test for these two flies generated the expected identifications as nontargets. The third false-positive was a *R. cingulata* (isolate BX190304-029) from Florida that had an unusually high ITS1 Cq (29.59) relative to its 18S Cq (18.83). The ITS1 amplicon had no measurable melting temperature. Repeating this test for the three aforementioned nontarget samples generated the correct identification as a nontarget species.

Diagnostic Sensitivity and Specificity for mcPCR Test

The mcPCR test generated conclusive data for 317 of the 361 flies in the study, on the first run (Table 3). Of the 223 *R. cerasi* specimens tested, 32 adults trapped as part of a domestic survey in New York failed to generate interpretable data because the 18S marker did not amplify. Like the LAMP test, six of the flies failed to amplify 18S but did amplify the ITS1 target. All of these flies were excluded from calculations of false-negative rate. Repeating reactions did not resolve the problem of 18S failure for these flies. The DSe for this mcPCR experiment was 99.5% (CI 97.1–100%) and the false-negative rate was 0.5%. This error rate was the result of one larva (isolate BX190724-021) from New York failing to amplify ITS1. This sample was retested three times and did not generate the ITS1 target. Repeating the protocol using only the ITS1 primer set without the 18S primers did generate the expected product for *R. cerasi*. It is possible that the 18S primers are interfering with ITS1 amplification in the multiplex reaction for this specimen. Of the 138 nontarget specimens, the mcPCR test failed to generate interpretable results for 12 flies because 18S control marker did not amplify. The DSp of the mcPCR data set was 100% (CI 97.7–100%) and the false-positive rate 0% for the 126 specimens in the data set.

Discussion

This study is the first to report ITS1 sequence records for *R. cerasi* and another cherry pest, *R. fausta*. In comparison to ITS1 sequences from other *Rhagoletis* species in North America, the *R. cerasi* record is much longer and includes regions of moderate (85%) to low similarity. The previously published primer sets tested in the study were not successful at amplifying the entire ITS1 sequence from a *R. cerasi* specimen. Initial experiments generated reaction failures and occasionally multiple bands in PCR products. The relatively long length of the *R. cerasi* sequence and the presence of homopolymeric regions could have prevented successful amplification and sequencing of the target in some of the attempted reactions (Sutton et al. 2015). Repeating the experiment on multiple *R. cerasi* specimens did not result in successful amplification. Primers for a shorter fragment of ITS1 nearer to the 5.8S gene did provide more reliable amplification success from the *R. cerasi* specimens in the study.

As part of the rDNA array, the ITS1 is expected to be present in multiple copies in the genome of a fly. But these copies are usually located in a tandem array of rDNA and assumed to be identical or nearly identical because of convergent evolution within a single individual (Elder and Turner 1995, Potts et al. 2014). This can result in *R. cerasi* individuals sharing the same sequence (i.e., low intraspecific variation). It is possible, however, for a specimen or a species to have multiple copies of ITS1 that are distinct from each other (Douglas et al. 2004). The current study did not explore the possibility of multiple copies resulting from incomplete convergent

Table 3. ID success rates of the two test methods

Species	Source	Life stage	Total no. of flies	LAMP test results			mcPCR test results		
				European cherry fruit fly	Not-European cherry fruit fly	Failed	European cherry fruit fly	Not-European cherry fruit fly	Failed
R. cerasi	Colony	Larvae	21	21	0	0	21	0	0
	Colony	Adults	5	5	0	0	5	0	0
	Field	Larvae	55	54	0	1	54	1	0
	Field	Pupae	11	10	1	0	11	0	0
	Field	Adults	131	96	2	33	99	0	32
R. cingulata	Field	Larvae	3	0	3	0	0	3	0
	Field	Adults	49	1	45	3	0	38	11
R. indifferens	Field	Adults	16	0	16	0	0	16	0
R. fausta	Field	Pupae	3	0	3	0	0	3	0
	Field	Adults	11	0	11	0	0	11	0
R. meigenii	Field	Larvae	1	0	1	0	0	1	0
R. cornivora	Field	Adults	5	2	3	0	0	5	0
R. zephyria	Field	Larvae	25	0	25	0	0	25	0
	Field	Adults	10	0	10	0	0	10	0
R. pomonella	Field	Adults	15	0	14	1	0	14	1
Sums			361	189	134	38	190	127	44

evolution or presence of rDNA arrays on different chromosomes. The ITS1 primer sets developed for pest diagnosis did reliably detect ITS1 in a large series of *R. cerasi* specimens. This supports the presence of the target sequence in all *R. cerasi* specimens.

DNA sequencing of the ITS1 target can provide adequate information useful for identification of *R. cerasi* in North America; however, this process can take more than a day to complete. There is interest in diagnostic protocols that are appropriate for analysis within a day; in clinical studies these are termed as STAT tests. The LAMP protocol reported here is one such technology. It can be completed in less than an hour (including reagent setup), has been shown to be successful on multiple instrument platforms, and detects DNA within the range expected for real-world fly specimens at both larval and adult stages. Initial testing of the method as a qualitative diagnostic (i.e., measures presence or absence of reaction product) has shown it to be repeatable at the lab. There was no evidence of interference that would affect analytical sensitivity using DNA extracted from animal tissues.

In general, the time required to complete DNA extraction increases the total time required to complete LAMP analysis of a detected fly. Under the recommended incubation time of 3 h for an extraction, the LAMP would still be completed in less than half a day. Larvae from a lab colony were tested using shorter incubation times and all were successfully diagnosed. This suggests that incubation time could be reduced. Further analysis might be needed, however, to confirm that shorter incubation times performed on adult legs from specimens that were collected from traps are appropriate for the LAMP test.

The DSe of the LAMP test was estimated using a collection of *R. cerasi* from various sources including a lab colony. Therefore, it was a composite collection rather than an estimate from a single geographic population. It did include a series of flies from the New York outbreak that span over 3 yr. Similarly, the DSp was estimated using multiple collections from species expected to be confused with *R. cerasi* in North America. Again, this was done to maximize variation but is not an estimate of nontarget variation from a geographic population. That nontarget set also included some species that are not pests of cherries. Although this could have inflated the values, there is no reason to believe the *R. cerasi* ITS1 target would be present in additional specimens of species that use cherry as a host versus species that do not use the host. Therefore, including additional species likely provides additional confidence.

The DSe and DSp values of the LAMP test are relatively high (98.4 and 97.8%, respectively). Three *R. cerasi* specimens generated false negatives and three nontarget specimens generate false positives based on the first run. Repeating the test on these flies demonstrated that these were the result of technical error and not because of specimen DNA. The three false-positive results each exhibited atypical DNA results when compared to other samples because of late readings and/or failure to have product melting temperatures. The DSe and DSp values reported here can be used to select sampling strategies for future surveys of the pest in regions of North America. The prevalence of the pest is expected to vary for different geographic populations and to change overtime. Consequently, the positive predictive value (PPV) and negative predictive value (NPV) are not estimated in the study.

In addition to the LAMP test, an mcPCR test is also reported. This second test uses the ITS1 and 18S primers developed for the LAMP protocol to diagnose *R. cerasi*. This technology is also classified as STAT. The test can be completed in 5 h, once the DNA has been extracted, but it is not as fast as LAMP. The benefits of mcPCR are that samples can be processed in a single reaction (as opposed to running 18S and ITS1 separately in LAMP) and only requires basic molecular biological equipment that is available to most laboratories. The disadvantages of the protocol in comparison to LAMP is that reactions must be scored after separation on an electrophoresis apparatus (a process that must be performed at a laboratory) and the time required to complete analysis is longer. The DSe (99.5%) and DSp (100%) values for the test were like those for LAMP. Given the confidence intervals for these two tests, neither is superior to the other. The mcPCR test generated one false-negative sample and this was not the result of technical error. PCR and sequencing (MW183265) confirmed that the sample has the ITS1 target but mcPCR test cannot detect it.

The two test methods are appropriate for successful identification of field collected material, as demonstrated in the study. The limit of detection is not equivalent for the methods; the mcPCR test method can reliably identify at concentrations of 0.1 ng/µl and the LAMP test method at of 1 ng/µl. This difference is likely a consequence of selecting a short isothermal amplification time for the

LAMP test relative to the longer amplification cycling of the mcPCR test. As expected, adjustments to either test method could impact these limits of detection. The results also suggest that the sensitivities of tests are greater for adult tissue than for larvae. This, however, is based on one larval specimen and additional testing would be needed to confirm if inhibitors are more common in larvae than in adult tissues. As demonstrated in the extraction tests, larvae generate a relatively high concentration of DNA from field collected samples that are above the established limits of detection.

In conclusion, this study provides new resources and protocols that will assist in the diagnosis of an important invasive pest. The protocols are designed to diagnose *R. cerasi* in North America and further testing might be needed to apply it to other regions. The flies tested in the study include European populations and specimens collected from both cherry and honeysuckle hosts. Population genetics of the pest is an ongoing area of study (Augustinos et al. 2019) and outside the scope of the ITS1-based diagnostic reported. Future work on introgression of gene pools between *R. cerasi* and other species will be important to update the protocols presented here (Johannesen et al. 2013, Barringer 2018).

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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