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Partial Ribosomal Nontranscribed Spacer Sequences Distinguish *Rhagoletis zephyria* (Diptera: Tephritidae) From the Apple Maggot, *R. pomonella*

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Abstract

The apple maggot, *Rhagoletis pomonella* (Walsh), was introduced into the apple-growing regions of the Pacific Northwest in the U.S.A. during the past 60–100 yr. Apple maggot (larvae, puparia, and adults) is difficult to distinguish from its morphologically similar sister species, *Rhagoletis zephyria* Snow, which is native and abundant in the Pacific Northwest. While morphological identifications are common practice, a simple, inexpensive assay based on genetic differences would be very useful when morphological traits are unclear. Here we report nucleotide substitution and insertion–deletion mutations in the nontranscribed spacer (NTS) of the ribosomal RNA gene cistron of *R. pomonella* and *R. zephyria* that appear to be diagnostic for these two fly species. Insertion–deletion variation is substantial and results in a 49 base-pair difference in PCR amplicon size between *R. zephyria* and *R. pomonella* that can be scored using agarose gel electrophoresis. PCR amplification and DNA sequencing of 766 bp of the NTS region from 38 *R. pomonella* individuals and 35 *R. zephyria* individuals from across their geographic ranges led to the expected PCR fragments of approx. 840 bp and 790 bp, respectively, as did amplification and sequencing of a smaller set of 26 *R. pomonella* and 16 *R. zephyria* flies from a sympatric site in Washington State. Conversely, 633 bp mitochondrial COI barcode sequences from this set of flies were polyphyletic with respect to *R. pomonella* and *R. zephyria*. Thus, differences in NTS PCR products on agarose gels potentially provide a simple way to distinguish between *R. pomonella* and *R. zephyria*.

Key words: Apples, Fruit flies, molecular diagnostics, rDNA, snowberries
*Rhagoletis mendax* Curran is a third species that is very closely related genetically and morphologically similar to *R. pomonella* and *R. zephyria* (Bush 1966). However, *R. mendax* is known only from *Vaccinium* spp. and *Gaylussacia* spp. hosts, and its geographic range is limited to the eastern half of North America (Payne and Berlocher 1995, Smith et al. 2001). Thus, *R. mendax* does not cause a threat to apples or create a problem for the apple industry in the Pacific Northwest.

Because *R. zephyria* is virtually identical morphologically to *R. pomonella* (Bush 1966), this creates problems for morphological identifications in areas where the ranges of these species overlap. Despite these similarities, Westcott (1982) was able to draw upon the work of Curran (1924), Wasbauer (1963), and Bush (1966, 1969) to establish morphological criteria for distinguishing between adults of the two species. Males of the two species can be distinguished from each other based on a difference in the surstylus shape, while females differ in mean ovipositor length. These characters have been used extensively as the basis for species diagnosis of field-caught adult flies in annual apple maggot surveys in Washington State. Ovipositor length can be used to distinguish between females of the two species, but large *R. zephyria* females can be difficult to distinguish from small *R. pomonella* females; males of the two species appear to be reliably distinguished by surstylus shape (Westcott 1982). Additionally, the introduction of *R. pomonella* into the Pacific Northwest over the past 50 yr has created problems for species identification, as introgression appears to have been ongoing (McPheron 1990a), presumably between the relatively abundant *R. zephyria* males and the introduced *R. pomonella* females (Arcella et al. 2015).

A simple, inexpensive diagnostic tool to distinguish between *R. pomonella* and *R. zephyria* would be very useful when morphological characters, especially in females, are unclear (i.e., ovipositor lengths are intermediate between typical specimens). DNA sequencing of the barcode or some other region of the mitochondrial DNA (Hebert et al. 2004) would appear to be the logical choice for this task, but published work to date (Smith and Bush 1997, Schwarz et al. 2005, Michel et al. 2007) has not revealed unique, diagnostic haplotypes for *R. pomonella* and *R. zephyria* based on mitochondrial DNA sequences. It is unclear whether the inability of mtDNA to distinguish between *R. pomonella* and *R. zephyria* is due to insufficient mitochondrial DNA sequence data (more nucleotides need to be sequenced per individual) or if the history and structure of *R. pomonella* and *R. zephyria* populations is such that their mitochondrial DNA haplotypes are shared because of ongoing introgression and/or incomplete lineage sorting.

To date, the use of multilocus genotypes to identify unknown field-caught flies as either *R. zephyria* or *R. pomonella* has had the highest potential for success (Schwarz et al. 2005, Michel et al. 2007). Green et al. (2013) described a quantitative real-time PCR approach for species diagnosis in *R. zephyria* and *R. pomonella* based on four single nucleotide polymorphisms (SNPs) derived from expressed sequence tags (ESTs). While the assay developed by Green et al. (2013) was useful in most cases for distinguishing whether a fly originated from apple, black hawthorn (*Cra taegus douglasi* Lindl.), or snowberry (*S. albus var. laevigatus*), their assay was complicated by the presence of *R. zephyria* alleles that presumably had introgressed into the *R. pomonella* gene pool in areas where the two species were in close proximity. More recently, Doellman et al. (2020) developed a diagnostic test based on PCR amplification of alleles at five nuclear loci followed by restriction enzyme digestion to reveal differences between field-caught *R. pomonella* and *R. zephyria*, that appears to be diagnostic but is somewhat technically demanding.

Here we describe genetic variation in the rRNA gene cistron of *R. pomonella* and *R. zephyria* (Fig. 1) that provides an additional useful tool for distinguishing between individuals in these two fly species, either as field-caught adults or larvae. As a part of a larger study in our lab on species relationships in the genus *Rhagoletis* (Hulbert 2018), we examined molecular variation in a 625-nucleotide fragment of the D2 expansion region of the 28S rRNA gene. This study revealed a single nucleotide insertion mutation in the 28S D2 expansion region of *R. zephyria* individuals that appeared to distinguish these flies from *R. pomonella*. Subsequent assemblies of larger portions of the rDNA cistron of *R. pomonella* and *R. zephyria* using Illumina NextGen sequences (Smith et al. in preparation) indicated that there may be several DNA mutations, both single nucleotide substitutions and larger scale insertion–deletion (indel) mutations, upstream of the 18S structural rRNA gene, in both the external transcribed spacer (ETS) and nontranscribed spacer (NTS) regions of the rDNA cistron.

We report here genetic variation in a portion of the ribosomal NTS region that appears to be useful for distinguishing between *R. pomonella* and *R. zephyria*, with indel mutations that potentially provide the basis for a simple diagnostic test for these two species based on a 49 bp size difference in their PCR amplicons. The ribosomal region that we examined (the 3′ end of the NTS region; Fig. 1) contains both DNA substitutions and indel variation that appear to be fixed between *R. pomonella* and *R. zephyria*. On the other hand, *R. pomonella* and *R. mendax*, the third named species in the *pomonella* group sibling species complex, appear to be polyphyletic with respect to each other based on the NTS gene region, while *R. zephyria* remains monophyletic with respect to both *R. pomonella* and *R. mendax*.

**Materials and Methods**

**Taxon Sample**

The taxon sample for this study consists of both a geographic sample of 101 flies collected from across the northern US, and a sample collected mainly from Beacon Rock State Park near the Columbia River in Washington State. The geographic sample consists of 101 individual flies (38 *R. pomonella*, 35 *R. zephyria*, and 26 *R. mendax*).

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**Fig. 1.** Diagram of the eukaryotic ribosomal DNA repeat unit (cistron), showing the location of the NTS area sequenced in this study. The complete repeating unit of rDNA is shown, showing the relative locations and sizes of the different components. This basic structure, with minor variation, is conserved across the eukaryotes. Our overall rDNA alignment is 9,221 nucleotide positions, which comprise the area outlined with the bold box. We determined sequences (approx. 800 bp) from the 3′ end of the nontranscribed spacer (NTS; shaded area within bold box) within the IGS region for 141 *R. pomonella*, *R. mendax*, and *R. zephyria* flies. Figure adapted from Hwang and Kim (1999).
Table 1. Rhagoletis pomonella, R. zephyria, and R. mendax analyzed in this study, including collection information (locality, host plant, date)

| Taxon       | Designation | State/Province | Locality (County)                  | n  | Host                          | Collector | Date      | Lat   | Long  |
|-------------|-------------|----------------|------------------------------------|----|-------------------------------|-----------|-----------|-------|-------|
| R. mendax   | MNJ         | New Jersey     | Rutgers Expt Stn (Burlington)       | 2* | Vaccinium corymbosum          | OL        | 7/VIII/2000 | 39.72 | −74.51|
| R. mendax   | MEL         | Michigan       | MSU Clinical Center (Ingham)        | 10 | Vaccinium corymbosum          | JEC/BF    | 5/VIII/1999 | 42.72 | −84.46|
| R. mendax   | MJP         | Indiana        | Jasper-Pulaski WR (Jasper)          | 9  | Gaylussacia dumosa            | JS        | 1/VIII/1999 | 41.15 | −86.97|
| R. mendax   | MWB         | Ontario        | Wainfleet Bog (Niagara)             | 3  | Vaccinium corymbosum          | DVJS      | 1/VIII/2001 | 42.91 | −79.30|
| R. pomonella| PIL         | Illinois       | Riverwoods (Lake)                  | 5* | Crataegus mollis             | VG/MJ     | 12/IX/1999 | 42.19 | −87.88|
| R. pomonella| PMA         | Massachusetts  | Amherst (Hampshire)                 | 9* | Crataegus mollis             | AR/GW/VG  | 24/IX/2000 | 42.39 | −72.53|
| R. pomonella| PMN         | Minnesota      | Staples (Todd)                     | 1* | Crataegus mollis             | GLDB      | 5/IX/1994  | 46.40 | −94.93|
| R. pomonella| PNE         | Nebraska       | I-80, Exit 285 (Buffalo)           | 1* | Crataegus mollis             | VG        | 3/IX/1999  | 40.70 | −98.84|
| R. pomonella| PUT         | Utah           | Wellsville (Cache)                 | 4  | Crataegus douglasi            | DA        | 23/VIII/2000 | 41.63 | −111.93|
| R. pomonella| PCO         | Colorado       | Boulder (Larimer)                  | 4  | Crataegus mollis             | VG/BB     | 5/IX/1999  | 40.00 | −105.29|
| R. pomonella| PNY         | New York       | Geneva (Seneca)                    | 7  | Crataegus mollis             | AR/GW/VG  | 24/IX/2000 | 42.87 | −77.01|
| R. pomonella| PPA         | Pennsylvania   | Biglerville (Centre)               | 4  | Malus domestica              | MC        | 18/VIII/1999 | 39.93 | −77.72|
| R. pomonella| PWA         | Washington     | St. Cloud Ranch (Skamania)         | 1* | Crataegus douglasi            | VG        | 12/IX/2000 | 45.60 | −122.11|
| R. pomonella| MK_haw      | Washington     | Beacon Rock St. Pl. (Skamania)      | 26 | Crataegus douglasi            | MK        | IX/2000   | 45.62 | −122.03|
| R. zephyria | ZEL         | Michigan       | East Lansing (Ingham)              | 2* | Symphoricarpus albus var. laevigatus | JS/AR   | 10/VIII/2000 | 42.73 | −84.48|
| R. zephyria | ZMN         | Minnesota      | Hawley (Clay)                      | 2* | Symphoricarpus occidentalis   | GLDB      | 7/IX/1994  | 46.87 | −96.47|
| R. zephyria | ZMT         | Montana        | Melstone (Musselshell)             | 2* | Symphoricarpus occidentalis   | GLDB      | 9/IX/1994  | 46.59 | −107.89|
| R. zephyria | ZND         | North Dakota   | Mandan (Burkei)                    | 1* | Symphoricarpus occidentalis   | GLDB      | 9/IX/1994  | 46.69 | −100.90|
| R. zephyria | ZPA         | Pennsylvania   | State College (Centre)             | 5* | Symphoricarpus albus var. laevigatus | VG     | IX/1995    | 40.79 | −77.86|
| R. zephyria | ZSD         | South Dakota   | Custer (Pennington)                | 1* | Symphoricarpus occidentalis   | VG        | 9/IX/1999  | 43.75 | −103.36|
| R. zephyria | ZNY         | New York       | Geneva (Seneca)                    | 8  | Symphoricarpus albus var. laevigatus | JS      | 18/VIII/1997 | 42.87 | −77.01|
| R. zephyria | ZWI         | Wisconsin      | Waukesha (Waukesha)                | 4  | Symphoricarpus occidentalis   | VG        | 12/IX/1999 | 43.00 | −88.27|
| R. zephyria | ZMA         | Massachusetts  | Amherst (Harshire)                 | 4  | Symphoricarpus occidentalis   | RJP       | IX/1998    | 42.39 | −72.53|
| R. zephyria | ZCO         | Colorado       | Boulder (Larimer)                  | 4  | Symphoricarpus albus var. laevigatus | VG/BB | 3/IX/1999  | 40.01 | −105.27|
| R. zephyria | ZWA         | Washington     | Dixie (Walla Walla)                | 2  | Symphoricarpus albus var. laevigatus | GLDB     | 31/VIII/1997 | 46.14 | −118.17|
| R. zephyria | MK_mendax   | Washington     | Beacon Rock St. Pl. (Skamania)      | 10 | Symphoricarpus albus var. laevigatus | MK      | IX/2000    | 45.62 | −122.03|
| R. zephyria | MK_mendax   | Washington     | St. Cloud Ranch (Skamania)         | 6  | Symphoricarpus albus var. laevigatus | MK      | IX/2000    | 45.60 | −122.11|
| R. pomonella| Mex         | Mexico         | San Diego, Mex. Mex.               | 2* | Crataegus mexicana           | JG        | X/1995     | 19.26 | −99.10|

One individual from each sample with an asterisk (*) was sequenced using Illumina NextGen (Smith et al. in preparation), except R. pomonella Mex., in which both flies were sequenced.

Collectors: DA, Diane Alston; BB, Branislav Blagojevic; DB, Doree Bush; GLB, Guy L. Bush; MC, Marty Condon; JEC, Joe Crossno; DD, Danny Ducat; BF, Ben Fernandez; VG, Vesna Gavrilovic; JG, Jorge Graciano; MJ, Marty Jaycox; MK, Mike Klaus; OL, Oscar Liburd; RJP, Ron J. Prokopy; AR, Angela Roles; JS, Jim Smith; GW, George Weiblen.

Latitudes and Longitudes in italics are approximations.
from populations across their respective geographic ranges (Table 1; Fig. 2), plus two *R. pomonella* Mex. individuals from the Eje Volcanico Trans Mexicano (EVTM; Michel et al. 2007). Following Green et al. (2013), species names were assigned to individuals collected from apple or hawthorn as *R. pomonella*, individuals collected from snowberry as *R. zephyria*, and individuals collected from blueberry as *R. mendax*. This set of 101 flies includes 14 flies from which we have obtained approx. 9,000 bp sequences of the ribosomal DNA cistron (partial NTS, ETS, 18S, ITS1, 5.8S, ITS2, and 28S; GenBank Accession #'s MN507539–MN507552) and 14,000 bp of mitochondrial DNA (all 37 genes; GenBank Accession #'s MN443932–MN443945) via Illumina NextGen sequencing (Table 1; Smith et al. in preparation). The Beacon Rock sample includes 26 individual *R. pomonella* flies collected from *C. douglasii* and 10 individual *R. zephyria* flies collected from *S. albus* at Beacon Rock State Park (WA) in 2000, and also includes six individual *R. zephyria* flies collected nearby from *S. albus* at St. Cloud Ranch (WA) in 2000. Beacon Rock State Park and St. Cloud Ranch correspond to sites 17 and 18, respectively, in Mattson et al. (2022).

**PCR Primers**

We designed PCR primers and developed PCR conditions that reliably amplify an approximately 800 bp portion of the NTS gene region of the rDNA cistron in *R. pomonella*, *R. zephyria*, and *R. mendax* individuals (Fig. 1). In preliminary work, examination of approx. 9,000 bp of the rDNA cistron from 14 individual *Rhagoletis* individuals (six *R. zephyria*, five *R. pomonella*, one *R. mendax*, and two *R. pomonella* Mex. flies; GenBank Accession #’s MN507539–MN 507552) revealed marked indel variation between the *R. pomonella* and *R. zephyria* individuals in the ETS and NTS regions. We examined this further by designing PCR primers that could amplify the DNA segments in which these indels occurred. Two primer pairs were designed using Primer-BLAST as implemented by the NCBI website (Ye et al. 2012). The first pair (PMZrDNA1F/PMZrDNA1R; Table 2) was designed to amplify approx. 800 bp of DNA located at the 3’ end of the nontranscribed spacer (NTS) of the rDNA cistron. The second pair (PMZrDNA2F/PMZrDNA2R) was designed to amplify approx. 800 bp of DNA located at the 3’ end of the external transcribed spacer (ETS) and the 5’ end of the 18S rDNA structural gene. Examination of variation in these two rDNA regions across the 14 individual *Rhagoletis* individuals indicated that the NTS region amplified using PMZrDNA1F/PMZrDNA1R was more likely to provide a marked size difference in fragments between *R. pomonella* and *R. zephyria* individuals. All the work presented here is thus based on amplifications of the NTS region using the PMZrDNA1F/PMZrDNA1R primer pair.

The mitochondrial COI barcode primers of Hebert et al. (2004), LEP F1 and LEP R1 (Table 2), were used to amplify the mitochondrial COI region from the same set of flies from which we amplified the NTS and ETS regions. As observed by Barr et al. (2021) in European cherry fruit fly *Rhagoletis cerasi* (L.), the quality of some COI sequence reads from *Rhagoletis* flies in our sample was low, leading to ‘dirty’ sequences. In two cases, PCR of whole fly *Rhagoletis* extracts using LEP F1 and LEP R1 resulted in the amplification of COI fragments that were very clean upon sequencing, but yielded a DNA sequence that matched exactly the COI sequence from *Wolbachia pipientis* (GenBank Accession CP 042445.1). This phenomenon has been observed before (Smith et al. 2012), and we thus followed the recommendation of Barr et al. (2021) and used the primer pair TY-J-1460 and C1-N-2191 (Simon et al. 1994) for a number of subsequent COI amplifications, modifying the TY-J-1460
primer to the known *Rhagoletis* sequence of the tRNA\(^{\text{Tyr}}\) gene (TY-J-1460_Rhag; Table 2).

**PCR**

All PCR amplification was carried out using a BioRad T100 Thermocycler in 25 μl reactions using GotaqFlexi (Promega, Madison, WI) with the following reagents (and concentrations): reaction buffer (1X), MgCl\(_2\) (8 mM), dNTP (0.5 mM each), forward and reverse primers (0.5 mM each), DNA polymerase (2.5 u), and DNA template (~18–92 ng). The forward and reverse primers used in the PCR are listed in Table 2. Thermocycler conditions used to amplify the approx. 800 bp ribosomal NTS fragment using the PMZrDNA1F/PMZrDNA1R primer pair were as follows: initial denaturation was at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing at 58°C for 30 s, and product elongation at 72°C for 1 min (×35), followed by a final product elongation at 72°C for 5 min. To amplify the approx. 650 bp mitochondrial COI fragment using the LEP F1/LEP R1 primer pair, the conditions used were as described above except the annealing temperature was set at 46°C and the product elongation step was 2 min. These latter conditions were also used to amplify COI using the TY-J-1460_Rhag/CI-N-2191 primer pair.

**Agarose Gel Electrophoresis**

Agarose gel electrophoresis for determining if PCR was successful in preparation for DNA sequencing was carried out with a MiniOne gel apparatus using 1.5–2.0% (w/v) agarose in a gel volume of 15–20 ml. MiniOne gels were run in 1X TBE buffer for 15–20 min. For the higher resolution of PCR products in the 700–900 bp range, larger 2% agarose gels were used and run at 80 V for 4 hr. DNA bands were visualized using SYBR Green (Invitrogen S33102; Waltham, MA) with a UV transilluminator and photographed using a mobile phone. A 100 bp ladder (Gold Biotechnology D001-500; St. Louis, MO) was used to estimate band sizes.

**DNA Sequencing and Sequence Alignment**

PCR products for DNA sequencing were purified using QIAquick PCR Purification Kits (Qiagen; Hilden, Germany) according to the manufacturer’s recommendations. Sanger sequencing was performed at the Michigan State University Research Technology Support Facility via BigDye Terminator Sequencing on an Applied Biosystems 3730xl DNA Analyzer (Foster City, CA, USA) using the PCR primers as sequencing primers. Base calls were made within MEGA (vers. 7.2; Kumar et al. 2016) based on the sequences of both the forward and reverse strands. DNA nucleotide substitutions observed within the data sets were verified by visual examination of .ab1 file tracings within MEGA. DNA sequence alignment of the NTS amplicons was carried out using a TCS alignment (Chang et al. 2015) of 14 complete ribosomal DNA cistron sequences from *R. pomonella*, *R. zephyria*, and *R. mendax* (described above) as a scaffold. All of the NTS sequences generated for this study via Sanger sequencing readily and unambiguously aligned to this scaffold. Mitochondrial COI sequences obtained for this study readily aligned to a set of published COI sequences from *R. pomonella* and *R. mendax* (Frey et al. 2013).

**Phylogenetic Analysis and Network Construction**

Phylogenetic analysis of the aligned NTS sequences was carried out via the neighbor-joining algorithm of Saitou and Nei (1987) as implemented in PAUP*4.0 Version 4.0a (build 167) for Macintosh (X86) (Swofford 2000). The minimum evolution criterion was used as this has been shown to be among the most efficient methods when sequences are closely related (Nei 1991). The NTS sequences from two *R. pomonella* Mex. EVTM were used as the outgroup. Neighbor-joining topologies were examined in Parsimony mode in PAUP to identify unique alleles for generating input files for minimum-spanning network construction. Character changes on branches were identified in PAUP using ‘Show Reconstructions’. Minimum spanning networks (Bandelt et al. 1999) were generated using the computer program PopArt (http://popart.otago.ac.nz) with default settings.

**Results**

Preliminary PCR of the NTS/ETS region of the ribosomal DNA with both primer pairs PMZrDNA1F/PMZrDNA1R and PMZrDNA2F/PMZrDNA2R using a selected number of *R. pomonella* and *R. zephyria* individuals to provide template DNA led to amplification of the predicted NTS and ETS regions of the rDNA cistron sequences that had been obtained by Illumina NextGen sequencing (GenBank Accession #s MN507539–MN507552). Because indel variation in the NTS sequences appeared to provide an opportunity to distinguish between *R. pomonella* and *R. zephyria* based on PCR product length differences, we chose to focus our work on the NTS sequences obtainable using the primer pair PMZrDNA1F/PMZrDNA1R.

**Table 2. DNA sequences of PCR primers used and/or discussed in this study**

| Primers                        | Sequence                                                                 | Source                                      |
|--------------------------------|--------------------------------------------------------------------------|---------------------------------------------|
| a) Nuclear Ribosomal NTS and ETS primers | 5’-TTCTCCGCTGACGTGTTCGTT-3’                                                 | This study                                  |
| PMZrDNA1F                      | 5’-AACTACCTGCTTAAAGACAGGAA-3’                                             | This study                                  |
| PMZrDNA1R                      | 5’-TATGATGGCAACCAATGGATTG-3’                                              | This study                                  |
| PMZrDNA2F                      | 5’-TGGTGGACCGGTTCCTCAGG-3’                                                | This study                                  |
| PMZrDNA2R                      | 5’- AACCTCTGGGATGCAAAAAATCA-3’                                             | This study                                  |
| LCO-1490                       | 5’-GCTAAAAATCATAGATAAGTTG-3’                                               | Folmer et al. (1994)                        |
| LEP F1 Hebert                  | 5’-ATTCAACATGATAAGATAT-3’                                                 | Hebert et al. (2004)                        |
| LEP F1 Smith                   | 5’-ATTCAACATGATAAGATATGG-3’                                                | Smith et al. (2007)                        |
| HCO-2198                       | 5’-TAAACTCGGATGACAAAAATCA-3’                                              | Folmer et al. (1994)                        |
| LEP R1 Hebert                  | 5’-TAAACTGTGGATGCCAAAATTCAACCT-3’                                         | Hebert et al. (2004)                        |
| LEP R1 Smith                   | 5’-TAAACTGTGGATGCCAAAATTCAACCT-3’                                         | Smith et al. (2007)                        |
| CI-N-2191                      | 5’-CCCGGTAAAATATAATATAACCTG-3’                                            | Simon et al. (1994)                        |
| TY-J-1460                      | 5’-TACATTTATCGCCTTAAACTTCAAGC-3’                                          | Simon et al. (1994)                        |
| TY-J-1460_Rhag                 | 5’-TACAGTCTATCGCCTTAAACTTCAGCC-3’                                         | This study                                  |

Nucleotide symbols match IUPAC convention.
**NTS Sequences From the Geographic Sample**

PCR amplification using the primer pair PMZrDNA1F/PMZrDNA1R led to a PCR product of approx. 840 bp in the *R. pomonella* and *R. mendax* individuals tested from the geographic sample (n = 64), and approx. 790 bp in the *R. zephyria* individuals from the geographic sample (n = 35), based on visualization of fragments via agarose gel electrophoresis (Fig. 3) and the size of the sequenced PCR amplicons (see below).

We obtained DNA sequences from the amplified NTS region from 38 *R. pomonella*, 35 *R. zephyria*, and 26 *R. mendax* individuals in the geographic sample. DNA sequencing of each PCR product indicated that these PCR products were 839 bp in *R. pomonella* and *R. mendax*, and 792 bp in *R. zephyria*, respectively, with minor size variation within species due to the presence of short 1–2 bp indels in some sequences. Base calling and alignment of the sequences were straightforward and unambiguous, resulting in 766 aligned nucleotide positions, which is included in .fas format in Supp File 1a (online only).

The PCR products obtained with primers PMZrDNA1F and PMZrDNA1R from all flies studied here yielded DNA sequences that were very clean. There was no evidence of PCR product size heterogeneity, as would be evidenced by sequences that become unreadable at a particular point. This would appear to indicate that the NTS indel structure did not vary within individuals for this PCR amplicon, and that the ribosomal DNA cistrons are well homogenized within the three species examined here. On the other hand, we did observe what could be interpreted as evidence of DNA substitution heterozygosity within *R. mendax*: some changes observed within the *R. mendax* individuals appeared to involve heterozygous states (double peaks; state assigned as the higher of the two peaks). However, no double peaks were observed in the NTS sequences from *R. pomonella* and *R. zephyria* individuals; all of the base calls were clean and we had no difficulty assigning the character states within these sequences.

The overall size difference of the NTS fragment between *R. pomonella* and *R. mendax* individuals compared to *R. zephyria* individuals is accounted for by the presence of a characteristic set of indels in the NTS alignment, each of which appears to have been uniquely derived (relative to the *R. zephyria* Mex. EVTM outgroup) in either all *R. zephyria* individuals, or in all *R. pomonella* and *R. mendax* individuals (Table 3). For example, all *R. zephyria* individuals examined contained a single deletion of 15 nucleotides from positions 318–332 in the NTS alignment relative to *R. pomonella* and *R. mendax* (Fig. 4). Conversely, all *R. pomonella* and *R. mendax* individuals contain a 20-nucleotide insertion from positions 352–371. Consideration of all of the indels in the PCR amplicon corresponding to the NTS region examined accounts for the size difference of 49 nucleotides between the ribosomal NTS PCR products obtained from *R. pomonella* and *R. mendax* individuals, compared to those obtained from *R. zephyria* individuals.

We also observed single-nucleotide substitution variation within the *R. pomonella*, *R. mendax*, and *R. zephyria* individuals of the geographic sample at 12 nucleotide positions in the aligned data, with 11 of these being phylogenetically informative. A minimum evolution neighbor-joining tree based only on DNA substitution mutations shows the relationships of the NTS sequences obtained from the 38 *R. pomonella*, 35 *R. zephyria*, and 26 *R. mendax* individuals in the geographic sample (Fig. 5). Even though DNA substitution variation was slight, the NTS sequences from the 35 *R. zephyria* individuals formed a monophyletic group supported by a bootstrap value of 96. Three of the observed DNA substitutions (position 4, A → T; position 41, G → C; position 113, A → G) are uniquely derived in all of the *R. zephyria* individuals, and only in *R. zephyria* individuals, providing what appear to be diagnostic nucleotide substitutions (character states) for *R. zephyria*, above and beyond the set of indel mutations described above. Within the *R. zephyria* clade, there is also a group of 9 sequences, also with strong bootstrap support, that is mainly composed of *R. zephyria* individuals from the eastern U.S. and differs from the other *R. zephyria* sequences by a single nucleotide change at position 696 in the alignment. While *R. zephyria* was monophyletic in the NTS tree, *R. pomonella* and *R. mendax* were not, appearing polyphyletic with respect to each other in the neighbor-joining tree (Fig. 5). Three *R. mendax* individuals (MJP5, MEL8, and MEL20) share the sequence that is most common for *R. pomonella*, while one *R. pomonella* individual (PNY7) shares its sequence with four *R. mendax* individuals.

Using the neighbor-joining tree as a basis, we defined 15 alleles (genotypes) within the NTS data set. Construction of a minimum-spanning network (Fig. 6) provides a visual representation of the relationships of these 15 alleles and their relative abundance within each of the three ingroup species. Allelic diversity at the NTS locus was higher in *R. mendax* than it was in either *R. zephyria* or *R. pomonella*. While *R. zephyria* and *R. pomonella* had three alleles each, *R. mendax* had nine, despite the fact that there were fewer *R. mendax* individuals in the sample (n = 26) than *R. zephyria* (n = 35) or *R. pomonella* (n = 38). All of the *R. zephyria* individuals

![Fig. 3. Agarose gel showing the NTS size difference between the PCR products obtained from *R. pomonella*, *R. mendax*, and *R. zephyria* individuals.](image-url)
had one of three alleles (Hap1, Hap12, Hap13), and each of these is found in R. zephyria individuals only. R. pomonella individuals also had one of three alleles, with R. pomonella and R. mendax sharing two of these alleles (Hap1 and Hap5). Hap1 was the most frequent allele in the data set, being found in 36 R. pomonella individuals and three R. mendax individuals.

Mitochondrial COI Sequences From the Geographic Sample
A different picture emerged when the geographic sample was analyzed using the mitochondrial COI barcode region. Mitochondrial COI sequences were obtained from the same set of individuals in the Geographic sample from which NTS sequences were obtained. Base calling and alignment of the sequences again were straightforward, resulting in 633 aligned nucleotide positions corresponding to the BOLD barcode region (Ratnasingham and Hebert 2007). The complete set of COI sequences is included in .fas format in Supp File 1b (online only). As was observed in the NTS data set, DNA sequence variation was slight within the COI data set, with observed variation at 14 nucleotide positions within the ingroup, and 8 of these being phylogenetically informative. We included in the COI data set six R. mendax and R. pomonella COI sequences from the literature (Frey et al. 2013) and we observed COI sequences in the geographic sample that matched each of these. Rhagoletis zephyria is polyphyletic in the minimum evolution neighbor-joining COI tree with respect to both R. pomonella and R. mendax (Fig. 7). Three R. zephyria individuals (ZWI10, ZPA2, and ZPA10) appear in a cluster that contains predominantly R. mendax and R. pomonella, while two R. pomonella individuals (PNY5 and PWA19) are grouped with the majority of the R. zephyria individuals in the Geographic sample. While the neighbor-joining tree shows some apparent structure in the R. pomonella, R. mendax, and R. zephyria individuals included in the geographic sample, bootstrap support overall was very weak, with values >50 obtained for only two clusters, one containing three R. mendax individuals and the other containing two R. pomonella individuals.

Using the neighbor-joining tree as a basis, we defined 17 COI haplotypes. Construction of a minimum-spanning network (Fig. 8) shows extensive COI haplotype sharing between R. pomonella, R. mendax, and R. zephyria, with none of the three ingroup species appearing as a monophyletic group. While most of the R. zephyria individuals have ‘private’ haplotypes (Haplotypes 8, 11, or 12), some R. zephyria individuals share haplotypes with R. pomonella (Haplotype 7), R. mendax (Haplotype 2), or both (Haplotype 1). Unlike the NTS locus, where allele diversity was higher in R. mendax than it was in R. zephyria or R. mendax, mitochondrial COI haplotype diversity is highest in R. pomonella, which has 10 haplotypes while R. mendax has six and R. zephyria has five. At the NTS locus, R. pomonella individuals only had one of three closely related alleles, while at the COI locus the ten haplotypes found in R. pomonella individuals are spread across the haplotype network.

Table 3. Insertion/deletion mutations in the NTS region that distinguish between R. pomonella and R. zephyria

| Position(s) | Insertion/Deletion | # bp | Taxa |
|-------------|--------------------|------|------|
| 2–3         | Deletion           | 2    | all R. zephyria |
| 7           | Deletion           | 1    | all R. zephyria |
| 318–332     | Deletion           | 15   | all R. zephyria |
| 345–351     | Deletion           | 7    | all R. zephyria |
| 352–371     | Insertion          | 20   | all R. pomonella |
| 431–434     | Insertion          | 4    | all R. pomonella |

Position(s) refer to the 766 bp NTS alignment; Deletion or insertion is relative to the NTS sequences from R. pomonella Mexico EVTM.

Fig. 4. Selected aligned NTS DNA sequences from the R. pomonella/R. mendax/R. zephyria taxon sets showing consistent differences between R. pomonella and R. zephyria. Shown are three NTS sequences from R. pomonella (PNY2, PL14, PCO1), three from R. zephyria (ZPA10, ZWI10, ZCO16), and two from R. mendax (MW9B, MJ95) from the Geographic sample, plus one sequence from hawthorn (MK92_haw) and one from snowberry (MK53_snow) from the Beacon Rock sample, and two sequences (PMEX4n, PMEX7n) from the R. pom. Mex. outgroup. A) Positions 309–423 in the 766 bp NTS alignment. This segment of the alignment shows a set of indels that appear to be diagnostic between R. pomonella and R. zephyria (R. mendax shares the R. pomonella indels in all instances). B) Positions 40–154 in the alignment. This part of the alignment has four single nucleotide substitution mutations (41, 52, 119, and 152) that appear to be derived (based on outgroup comparison) in R. zephyria (positions 41 and 113) or R. pomonella and R. mendax (positions 119 and 152). MEL 25 and MJF2 share the ancestral state at position 119, and MEL25, MJF2, MEL19, and MEL29 share the ancestral state at position 152, with all R. zephyria. Variation at position 67 is homoplasious.
NTS and COI Sequences From the Beacon Rock Sample

Incorporation into the analysis of a set of 27 individual Rhagoletis flies reared from black hawthorn and 17 individuals reared from snowberry from Beacon Rock State Park and St. Cloud Ranch (the Beacon Rock sample; Supp Table S1 [online only]) served as a preliminary ‘field-test’ of the ability of the NTS PCR product differences to serve as a diagnostic for identification purposes in areas where *R. pomonella* and *R. zephyria* co-exist. All of the flies in the Beacon Rock sample were collected as larvae within infested fruit in 2000 by one of the authors (MK), sent to another of the authors (JJS), overwintered as pupae and reared to adults in the lab (with emerged adults randomly labeled to mask host of origin) and then sent back to the Washington State Department of Agriculture (WSDA) for species identification using morphological characteristics. Locality information and morphological scoring data for these flies is shown in Fig. 5.

Fig. 5. Neighbor joining tree showing relationships of 766 bp NTS sequences obtained from *R. pomonella*, *R. zephyria*, and *R. mendax* individuals in the Geographic Sample. Sequences drawn from *R. pomonella*, “P”; *R. mendax*, “M”; *R. zephyria*, “Z”; and *R. pomonella* Mex. EVTM (PMex; outgroup), are shown. Numbers on branches are bootstrap values (1,000 replicates). Scale bar = p-distance.
Supp Table S1 (online only). All 12 of the males reared from black hawthorn were identified as *R. pomonella* based on morphological criteria, while 6 of the 15 females reared from black hawthorn were identified as *R. pomonella* and the other 9 were identified as *R. zephyria* (based on ovipositor length). On the other hand, all 17 of the flies (9 females and 8 males) reared from snowberry in the Beacon Rock sample were identified as *R. zephyria* based on morphological criteria.

When we sequenced the PMZrDNA1F/PMZrDNA1R PCR product from these flies, all 26 NTS sequences obtained from individuals from Beacon Rock that were reared from black hawthorn had the unique set of NTS indels and DNA substitutions (described above) that were associated with the *R. pomonella* flies in the geographic sample, while all 16 NTS sequences of individuals from Beacon Rock and St. Cloud Ranch that were reared from snowberry had the unique set of NTS indels and DNA substitutions that were associated with all of the *R. zephyria* flies in the geographic sample (Supp Figs. S1 and S2 [online only]). Thus, the 49 bp PCR-amplicon length difference that was observed between *R. pomonella* and *R. zephyria* individuals in the geographic sample was also observed.

Fig. 6. Minimum spanning network showing relationships of rDNA NTS sequences drawn from *R. pomonella*, *R. mendax*, and *R. zephyria*. The snowberry fly, *R. zephyria*, is distinguished from *R. mendax* and *R. pomonella* by four nucleotide substitutions, while *R. pomonella* and *R. mendax* share alleles (1 and S).
in the flies reared from black hawthorns (*R. pomonella*) and flies reared from snowberries (*R. zephyria*) in the Beacon Rock sample.

A neighbor-joining tree with the combined Geographic sample and Beacon Rock sample is shown as Supp Fig. S1 (online only), and the NTS allele network including flies from both the Geographic sample and the Beacon Rock sample is shown as Supp Fig. S2 (online only). All of the individuals reared from snowberry in the Beacon Rock sample clustered with *R. zephyria* in the NTS NJ tree, while all of the individuals reared from black hawthorn in the Beacon Rock sample clustered with *R. pomonella* and *R. mendax* (Supp Fig. 1 [online only]). There were no unique NTS sequences (alleles) observed in the Beacon Rock sample (Supp Fig. 2 [online only]). Twenty-five

**Fig. 7.** Neighbor-joining tree showing relationships of 633 bp mitochondrial COI sequences obtained from *R. pomonella*, *R. zephyria*, and *R. mendax* individuals in the Geographic Sample. Sequences drawn from *R. pomonella* individuals, “P”; *R. mendax*, “M”; *R. zephyria*, “Z”. *R. pomonella* Mex. EVTM is shown as the outgroup (*R. pom Mex*). Numbers on branches are bootstrap values (500 replicates). Scale bar = p-distance.
of the 26 individuals reared from black hawthorn in the Beacon Rock sample carried NTS allele 1, while one individual (MK34) carried allele 9, which is shared with two *R. mendax* individuals (MEL19 and MEL29). Conversely, all of the 16 individuals reared from snowberry from Beacon Rock State Park and St. Cloud Ranch carried NTS allele 13, which was the main allele exclusively associated with *R. zephyria* in the geographic sample.

The COI neighbor-joining tree based on the combined Geographic and Beacon Rock samples is shown as Supp Fig. S3 (online only), and the COI haplotype network including flies from both the Geographic sample and the Beacon Rock sample is shown as Supp Fig. S4 (online only). No new COI sequences were observed when the Beacon Rock sample was added to the analysis, and the neighbor-joining tree shows that the COI sequences from the flies reared from black hawthorn and snowberry were not strictly associated with COI sequences drawn from *R. pomonella* and *R. mendax* respectively (Supp Fig. S3 [online only]). While most Beacon Rock sample flies from snowberry (12/16) carried COI haplotype 8, MK97, which was reared from snowberry and identified as *R. zephyria* based on morphology, carried COI haplotype 1, which was predominantly associated with *R. pomonella* and *R. mendax* (ZWI10 also had COI haplotype 1). Similarly, Beacon Rock sample flies reared from black hawthorn carried four haplotypes. While most of these flies carried COI haplotype 1 (16/26), others showed COI haplotypes that were...
more closely related to those carried mainly by \( R. \) \emph{zephyria} flies in the geographic sample (e.g., haplotype 10; Supp Figs. S3 and S4 [online only]).

**Discussion**

**NTS Sequences Distinguish \( R. \) \emph{pomonella} From \( R. \) \emph{zephyria}**

All of the \( R. \) \emph{zephyria} individuals analyzed in this study formed a monophyletic group based on DNA sequences of an 800 bp PCR product amplified from the nuclear rDNA NTS region ([Supp Fig. 1 [online only]]. This monophyletic group was supported unambiguously by three DNA substitutions in the 766 bp NTS alignment (positions 4, 41, and 113). In addition, \( R. \) \emph{zephyria} was supported as a monophyletic group by a set of four unique insertion–deletion mutations within the alignment (Table 3), which serve as synapomorphies defining this clade, using \( R. \) \emph{pomonella} Mex. EVTM as the outgroup, and appear to unambiguously distinguish \( R. \) \emph{zephyria} from \( R. \) \emph{pomonella} and \( R. \) \emph{mendax}.

The indel structure occurring between positions 318 and 371 in the alignment contributes the bulk of the size difference of 49 nucleotides in the PCR products obtained from \( R. \) \emph{zephyria} individuals compared to the PCR products obtained from \( R. \) \emph{pomonella} and \( R. \) \emph{mendax} individuals ([Fig. 4]). This DNA size fragment difference between \( R. \) \emph{zephyria}, \( R. \) \emph{pomonella}, and \( R. \) \emph{mendax} individuals is readily apparent on agarose gels of the PCR products from these three different fly species ([Fig. 2]), and provides the potential for the development of a rapid, simple, inexpensive agarose gel-based test to use in efforts to distinguish between \( R. \) \emph{zephyria} and \( R. \) \emph{pomonella} in the apple-growing regions of the Pacific Northwest. A method based on these NTS differences would allow discrimination between \( R. \) \emph{pomonella} and \( R. \) \emph{zephyria} simply by scoring size differences between amplified PCR products via agarose gel electrophoresis.

The availability of the rDNA NTS region adjacent to the ETS as a molecular marker will complement and extend other tools available to distinguish \( R. \) \emph{pomonella} from \( R. \) \emph{zephyria} for quarantine purposes. Traditionally, adults obtained via trap catches in apple maggot surveys have been analyzed for morphological differences in ovipositor length and surstylus shape ([Westcott 1982, Yee et al. 2011]). The PCR assay described here based on size differences in the NTS PCR amplicon would be particularly useful for individuals in the ‘grey area’ in these analyses. The \( R. \) \emph{pomonella} individuals and \( R. \) \emph{zephyria} individuals from Beacon Rock State Park and St. Cloud Ranch analyzed in this paper were scored in the WSDA lab ([Supp Table 1 [online only]], which resulted in scoring nine \( R. \) \emph{pomonella} females as \( R. \) \emph{zephyria} (ovipositor length below threshold) as judged by the NTS sequence data. While the advanced morphometric techniques now available based on wing shape and aculeus shape ([Yee et al. 2009, 2011]) would likely correctly identify these flies as \( R. \) \emph{pomonella}, all of the flies reared from black hawthorn in the Beacon Rock sample carry the set of diagnostic DNA nucleotide substitutions and indel mutations characteristic of \( R. \) \emph{pomonella} and not \( R. \) \emph{zephyria}.

Green et al. (2013), and more recently Doellman et al. (2020), described SNP-based diagnostic tools that would also identify all individuals in this study to their correct species. The method described by Doellman et al. (2020) is based on PCR-amplification and restriction digestion of PCR-amplified products at five nuclear loci. While accurate, the method is potentially more time and labor intensive than the assay described here. Agarose gel separation of DNA fragments following PCR amplifications is standard, and the possibility of errors associated with a restriction digestion step would be avoided in an NTS-based assay. No DNA sequencing would be required for distinguishing between \( R. \) \emph{pomonella} and \( R. \) \emph{zephyria}, and the known sequence differences between the two species provide opportunity for the development of a qPCR assay.

While successful PCR-amplification is required for the NTS assay to distinguish between \( R. \) \emph{zephyria}, \( R. \) \emph{pomonella}, and \( R. \) \emph{mendax}, no further processing is necessary beyond running and visualizing the agarose gel. The primers designed for use in the current study (PMZrDNA1F and PMZrDNA1R), and the PCR conditions used, appear to be specific with respect to amplification of NTS only from \( R. \) \emph{zephyria}, \( R. \) \emph{pomonella}, and \( R. \) \emph{mendax}. However, one shortcoming of the current study is that the specificity of these primers beyond \( R. \) \emph{zephyria}, \( R. \) \emph{pomonella}, and \( R. \) \emph{mendax} has not been determined. There is one mismatch between the sequence of the forward primer PMZrDNA1F and the corresponding sequence in \( R. \) \emph{pomonella} Mex. EVTM, and either four mismatches (\( R. \) \emph{tabellaria} (Fitch) and \( R. \) \emph{electromorpha} Berlocher) or five (\( R. \) \emph{persimilis} Bush) in PMZrDNA1R in \( R. \) \emph{zephyria} species group flies ([Supp Table 2 [online only]], which is a sister group to the \( R. \) \emph{pomonella} species group within the genus \( R. \) \emph{zephyria} Hulbert (2018). Sequences corresponding to the PMZrDNA1F primer have not yet been found in either \( R. \) \emph{cornivora} Bush or \( R. \) \emph{bushi} Hulbert and Smith. Whether the PMZrDNA1F and PMZrDNA1R primer pair can be used to amplify the NTS region in \( R. \) \emph{zephyria} Mex. EVTM, \( R. \) \emph{cornivora}, and the four \( R. \) \emph{tabellaria} group species, and under what conditions, remain to be empirically determined.

**NTS as a Molecular Marker**

One significant aspect of the current study is the possible use of the NTS region of the ribosomal DNA just upstream from the ETS to explore systematics and evolutionary questions in other insect groups. The NTS region may prove to be particularly useful in circumstances like \( R. \) \emph{pomonella} and \( R. \) \emph{zephyria}, where closely related and/or cryptic species present taxonomic problems, and positive species ID is necessary for pest regulatory purposes. However, the usefulness of NTS as a marker may be limited by the history of gene flow between the species examined. While NTS appears to have fixed differences between \( R. \) \emph{zephyria} and both \( R. \) \emph{pomonella} and \( R. \) \emph{mendax}, the NTS region is not diagnostic for these latter two sister species, based either on indel variation or DNA substitutions. Until the introduction of \( R. \) \emph{pomonella} into western North America, \( R. \) \emph{zephyria} had been geographically separated from \( R. \) \emph{pomonella} except for along a narrow band of overlap from Minnesota into the eastern U.S. ([Gavrilovic et al. 2007, Arcella et al. 2015]). This historical lack of gene flow may have been necessary for the NTS divergence observed in this study to have occurred. However, there has been some gene flow between \( R. \) \emph{mendax} and \( R. \) \emph{zephyria} where they overlap in eastern North America ([Schwarz et al. 2005], and NTS remained diagnostic in eastern North American samples of those species examined here. On the other hand, \( R. \) \emph{mendax} and \( R. \) \emph{pomonella} are broadly sympatric in eastern North America, with ample opportunities for gene flow between them, and the NTS region is not diagnostic for these two sister species, either based on indel variation or DNA substitutions.

Nonetheless, analysis of NTS regions may be useful for addressing questions in instances where there are shared mitochondrial DNA haplotypes across species boundaries (e.g., the mealybugs \( Planococcus \) \emph{citri} Russo (Hemiptera: Pseudococcidae) and \( P. \) \emph{minor} (Maskell), [Rung et al. 2009]; several species of \( Neodiprion \) sawflies (Hymenoptera: Diprionidae), [Linnen and Farrell 2007]; the blow
flies *Lucilia coerulescens* Macquart (Diptera: Calliphoridae) and *Lucilia Mexicana* Macquart, Debruyne et al. 2013), and in insect taxa where two or more divergent mitochondrial lineages exist within a single species (e.g., the tree weta *Hemideina crassidens* (Blanchard) (Orthoptera: Anostostomatidae), Morgan-Richards et al. 2017).

While the 18S and 28S ribosomal RNA structural genes, and the internal transcribed spacers (ITS1 and ITS2), of the ribosomal DNA (rDNA) cistron have been used extensively for phylogenetics and population-level studies (the review of Hillis and Dixon (1991) has been cited over 2000 times), the external transcribed spacer (ETS) and nontranscribed spacer (NTS) regions, which together comprise the ribosomal intergenic spacer (IGS), have been relatively underutilized in systematics and species identification in insects, and exploration of these uses may prove fruitful. Shaivevich et al. (2013) described IGS sequences from mosquitoes (*Culex* spp.; Diptera: Culicidae) and indicated that these might prove useful for population genetics and phylogenetic analyses, but this study has been cited only once in the literature since publication (Web of Science; accessed 22 June 2021). On the other hand, the NTS and ETS gene regions seem to have found some use in phylogenetics studies, mainly in plants, with most of these studies employing the ETS region (e.g., Linder et al. 2000, Valuenae et al. 2017).

For reasons that are not entirely clear, the work of Shaivevich et al. (2013), who showed that the IGS regions of *Culex* species provided promising molecular markers for population genetics studies and phylogenetic analysis, has not seen broad applicability. This may be, in part, because the ribosomal IGS regions are not as easy to use as the 18S, 28S, ITS1, and ITS2 sequences, which are readily accessible and easy to amplify via PCR, and have proven to be successful in a range of phylogenetics studies (e.g., Si et al. 2020). In addition, because they are not transcribed, the NTS region will not be included in results obtained using RNASeq protocols. Our work allowed us to find the NTS regions by virtue of the use of whole genome NextGen sequencing, originally targeting high coverage sequences to assemble nearly complete mtDNA sequences (Smith et al. in preparation). The IGS regions also appear to be fast evolving (Stage and Eckbush 2007), so they are not readily obtained via homology searches. The DNA sequences of the NTS regions analyzed in this study from *R. pomonella*, *R. mendax*, and *R. zephyria* are apparently unique: BLAST searches of GenBank using the NTS PCR product sequences did not return any matches. Nonetheless, we were able to align our NTS sequences, at least partially, to the NTS sequence from *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) (Gichrist et al. 2014) and used this as the basis for hypothesizing the position of the ribosomal binding site.

The data presented here also illustrate why mtDNA has not proven useful for species diagnostics in *R. pomonella* and *R. zephyria*, as there are shared COI mtDNA haplotypes between these two species (Fig. 8). One possible explanation for this observation is that we simply have not sequenced enough mtDNA characters; the dataset here includes only 633 of the mitochondrion's approximately 15,000 bp. However, NextGen sequences of complete coding regions of the mtDNA (37 genes, 14,942 bp) from five *R. pomonella*, six *R. zephyria*, and one *R. mendax* individuals included in this study (GenBank Accession #’s MN443934–MN443945) did not provide species-level resolution (Smith et al. in preparation); instead, we observed what appears to be cytonuclear discordance between *R. zephyria* and *R. mendax* in the eastern U.S. This discordance presumably arises via introgression of *R. mendax* mtDNA into *R. zephyria*, which was described by Schwarz et al. (2005) in their report of the ‘speciation by hybridization’ of an (undescribed) *Rhagoletis* species, the *Loniceria* fly. The extent to which these introgressions have occurred (and are occurring) in *R. pomonella*, *R. mendax*, and *R. zephyria* is unclear and further work remains to be done to provide a clearer picture of these dynamics.

**Future Work**

Another shortcoming of the current study is that we have not determined the extent of fixation of the IGS differences in a large sample such as was employed by Doellman et al. (2020). In our study, we analyzed 38 *R. pomonella*, 26 *R. mendax*, and 35 *R. zephyria* in the geographic sample, and an additional 27 *R. pomonella* and 17 *R. zephyria* in the Beacon Rock sample. We also have not yet analyzed F1 hybrids between *R. pomonella* and *R. zephyria* (Yee and Goughnour 2011, Yee et al. 2013), or backcross individuals. Work to expand the sample size and experiments to determine the outcomes given hybrid and introgressed individuals remains to be carried out. Results of such studies may inform questions about the inheritance of rDNA cistrons and the homogenization of the different regions within them.

We also did not take into consideration or try to determine the nature of the variation leading to the dual NTS sequence signals observed at some nucleotide positions in some *R. mendax* sequences. It is unclear whether these dual signals occurred because of variation within the multiple copies of the ribosomal DNA cistron within the same haploid genome or if there are differences at some positions due to heterozygosity. In either case, we did not observe any differences in the indel patterns within any individual sequences, which would have been observable as sequences that fell out of register from each other at a certain point in a particular read. Because *R. mendax* was not a major focus of this study, and because the sequences in question did not impact the finding that *R. zephyria* is diagnosable based on its indel pattern, we did not pursue these lines of inquiry here and leave them to a future study.

**Supplementary Data**

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

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