



FINAL REPORT

Pretesting of molecular identification tests for *Rhagoletis pomonella* (Walsh, 1867)

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1. Introduction

The European Reference Laboratory for Insects and Mites has to select, adapt or develop reliable identification tests for the phytosanitary insect and mite species that are relevant for the European Union (included in the Commission Delegated Regulation (EU) 2019/1702 and in the EURL for Insects and Mites working programmes). One of the tasks of the EURL is to validate available diagnostic protocols before recommending their use to the National Reference Laboratories of the European Union. Pretesting of available tests is necessary to select the most reliable ones for the validation study.



Fig. 1 *R. pomonella* adult

Rhagoletis pomonella (Fig. 1) is, as its common name ‘apple maggot fly’ suggests, largely characterized by the infestation of some Rosaceae including domesticated apples (*Malus domestica*) and *Crataegus* spp., which seems to be the trait separating it from other closely related sibling species in the complex (*R. mendax*, *R. zephyria*, ‘flowering dogwood fly’ and *R. cornivora*) with hosts from the Ericaceae, Caprifoliaceae and Cornaceae family, respectively. Morphological distinction of adults is challenging, especially if information on the host plant is missing. Molecular identification employing barcoding of the *COI* locus or sequencing of other nuclear loci has so far not been able to sufficiently resolve this species complex either, excluding *R. cornivora*, which can be separated by e.g sequencing the *COI* locus (Xie *et al.* 2008).

R. pomonella is primarily found in North America, with restricted distribution in adjacent parts of Central America (Fig. 2). So far it has not been introduced to any other continents but it is a European Union regulated species, listed among the EU quarantine pests (Annex II of the Commission Implementing Regulation (EU) 2019/2072) and among the EU priority pests (Commission Delegated Regulation (EU) 2019/1702).

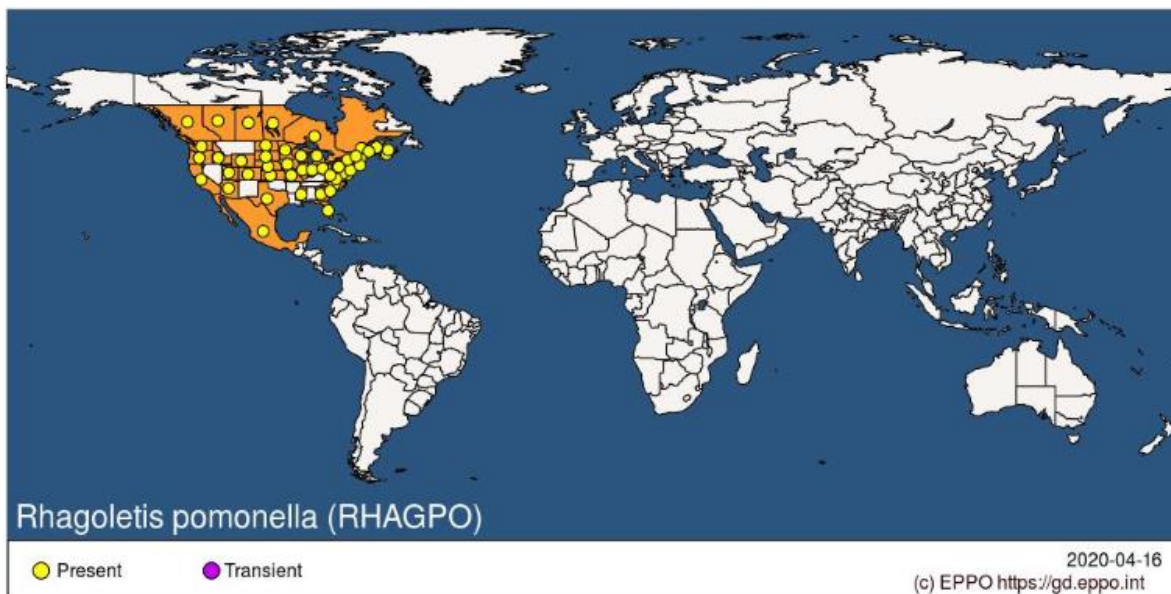


Fig. 2: Distribution of *R. pomonella* according to the EPPO global database

2. Scope of pretesting

The scope of this preliminary study was to identify molecular tests which are appropriate for the identification of *R. pomonella*. Additionally, a database inventory for sequence records should shed light on the application possibilities of barcoding as identification method.

3. Test selection

For this pest species no published specific diagnostic protocols in an international standard are available yet.

However, identification of these quarantine pests is covered in the EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021) (Appendix 1), which includes tests for the DNA barcoding of arthropods in general.

Specific identification tests in scientific literature for the identification of *R. pomonella* i.e.:

- Burgher-MacLellan *et al.* (2009): three SYBR Green PCRs targeting the *COI* locus
- Velez *et al.* (2006): three SYBR Green PCRS targeting microsatellite DNA; it has to be noted that the aim of this publication was to evaluate the usefulness of microsatellite markers for “mapping host-plant-associated adaptations in *Rhagoletis* that generate reproductive isolation and facilitate speciation, as well as for resolving the genetic structure and evolutionary history of fly populations”.

4. Composition of samples

For pretesting of tests indicated above, DNA was extracted from parts of single adult specimens (legs) of *R. pomonella* and *Rhagoletis* sp. non-targets (see Table 1). All specimens included in this study were morphologically confirmed to the species level (pers. comm. A. Egartner, AGES). However, the sample 66/19 could only be assigned as either *R. pomonella* or *mendax*. Additionally, the condition of this sample was questionable, as *R. pomonella* samples in Europe are rare to begin with, therefore all results have to be taken with a grain of salt until further tests with fresh specimens can be conducted.

Table 1: Details on single adult specimens for pretesting *Rhagoletis* spp. identification (sample set)

| Sample | Species | Host plant | Origin | Source |
|---------|------------------------------------|---------------|--------------|----------------|
| 66/19 | <i>Rhagoletis pomonella/mendax</i> | na | Michigan, US | AGES, Egartner |
| 2247/22 | <i>Rhagoletis cingulata</i> | Juglans regia | Austria | AGES, Egartner |
| 1928/22 | <i>Rhagoletis meigenii</i> | Juglans regia | Austria | AGES, Egartner |
| 1233/22 | <i>Rhagoletis alternata</i> | Malus sp. | Austria | AGES, Egartner |
| 1231/22 | <i>Rhagoletis cerasi</i> | Malus sp. | Austria | AGES, Egartner |
| 2552/21 | <i>Rhagoletis meigenii</i> | Juglans regia | Austria | AGES, Egartner |

*na: not available

5. Specification of pretesting procedures

DNA extraction

For DNA extraction the DNeasy Blood and Tissue (Qiagen) on *Rhagoletis* spp. legs was used. Samples indicated in Table 1 were tested undiluted and with at least one additional dilution (usually 1:20).

Real-time PCR

Following SYBR Green real-time PCR tests for the identification of *R. pomonella* were evaluated:

- Tests according to Burgher-MacLellan *et al.* (2009) targeting the *COI* locus
- Tests according to Velez *et al.* (2006) targeting microsatellite DNA

Other molecular tests

Included in EPPO PM 7/129 (2) DNA barcoding (EPPO, 2021):

- Barcoding for arthropods (Appendix 1); was tested for the identification of *R. pomonella* and related non-target species.

6. Database inventory for sequence records

DNA barcoding relies on PCR of predetermined marker genes (e.g. for the *COI* gene), DNA sequencing and comparison of those sequences to a database of reference sequences (Armstrong and Ball 2005). Applying barcoding for insect identification requires enough sequence records from the species within the genus for a reliable comparison. Not only the number, but also the genetic and geographic diversity of the records and the quality are potential issues that should be taken into account.

Three different databases (NCBI GenBank, Bold and EPPO Q-Bank) were consulted for the inventory. As search parameters the genus and species name and the gene locus (*COI* and synonyms) were used. In addition the reliability of the records were checked and, if reasonable, the search was extended to other gene loci.

Results

Due to the extraordinary amount of sequence data deposited in the NCBI GenBank, additional restrictions have to be made to evaluate the availability and suitability of sequences, especially for additional loci.

Items: 1 to 20 of 123279

Additionally, accession descriptors such as the one depicted below make the evaluation of sequence availability difficult.

- [Rhagoletis pomonella isolate G09_DW_012 cytochrome oxidase subunit I \(COI\) gene, partial cds](#);
 17. [tRNA-Leu gene, complete sequence, and cytochrome oxidase subunit II \(COII\) gene, partial cds](#);
[mitochondrial](#)
 871 bp linear DNA
 Accession: KF877743.1 GI: 569345324
[Protein](#) [PubMed](#) [Taxonomy](#)
[GenBank](#) [FASTA](#) [Graphics](#) [PopSet](#)

In all three consulted databases sequence records for *R. pomonella* on the *COI* locus are available. GenBank also offered 8 sequence records for the complete mitochondrial genome and a large number of accessions from whole genome shotgun sequencing projects. Additionally, sequences on other loci (e.g., *COII*, *ITS2*, *12S* rDNA, *16S* rDNA, *18S* rDNA) have been deposited in the NCBI GenBank (Query date October 2022).

Table 2: Number of *Rhagoletis pomonella* sequence records per gene for each database (data accessed November 2022).

| Gene | GenBank | Q-Bank | Bold |
|---------------------------------|----------|--------|-----------|
| <i>COI</i> | 53(13*) | 11 | 37(28***) |
| <i>COII</i> | 66(21**) | 0 | 0 |
| <i>ITS2</i> | 8 | 0 | 0 |
| <i>12S</i> | 2 | 0 | 0 |
| <i>16S</i> | 4 | 0 | 0 |
| <i>18S</i> | 15 | 0 | 0 |
| Complete genome (mitochondrium) | 8 | 0 | 0 |
| Whole genome shotgun sequencing | 36 796 | 0 | 0 |

*sequences of appropriate length for standard barcode

**sequences solely identified as *COII* locus

***sequences >500bp

Despite the large amount of sequence data in the NCBI GenBank, only part of the deposited *COI* sequences are of the expected length for barcoding, with many others being much longer and stretching into additional gene loci. Out of the 37 *R. pomonella* specimens listed in the Bold database, only 28 have appropriate sequences deposited, a large part of which were mined from NCBI GenBank. Regarding the geographic variation of the sequence records for *R. pomonella* in Bold, all records originate from North or Central America, with most of them being from Canada and Mexico. A similar distribution is seen in the 11 records found in EPPO Q-Bank, with the majority of them having been sampled in Mexico.

Bold Data base lists 680 specimen records for the *Rhagoletis* genus (about 70 species), 531 of which have barcodes. *R. cingulata* makes up the bulk of them with 115 records, followed by *R. basiola*, *meigenii*, *pomonella* and *completa*. None of the other *Rhagoletis* species relevant for this pretesting have any whole genome shotgun sequencing data deposited in the NCBI genbank, and only for *Rhagoletis cerasi* complete mitochondrial genome sequences are available (see Table 3). This is not relevant for the *in silico* evaluation of real-time PCRs targeting the *COI* locus, as there are sequences available for all species on this locus, but it has to be taken into account when reviewing *in silico* data for PCRs targeting microsatellite DNA.

Table 3: Number of sequence records available in NCBI GenBank for selected *Rhagoletis* species.

| Species | Sequences COI | Complete mitochondrial genome | Whole genome sequencing |
|-------------------------------|---------------|-------------------------------|-------------------------|
| <i>Rhagoletis cingulata</i> | 41 | 0 | 0 |
| <i>Rhagoletis meigenii</i> | 39 | 0 | 0 |
| <i>Rhagoletis alternata</i> | 4 | 0 | 0 |
| <i>Rhagoletis indifferens</i> | 5 | 0 | 0 |
| <i>Rhagoletis cerasi</i> | 21 | 6 | 0 |

Tree-based identification

To evaluate the species divergences within the genus, a Neighbor Joining (NJ) tree of distance was constructed using NCBI GenBank (max. seq. diff. of 0.75). Fig 3 showcases further complications that are known to arise with the species *R. mendax* and *R. zephyria*, which are both part of the *R. pomonella* complex.



Fig 3: Neighbor joining tree for *R. pomonella* on the *COI* locus, showing the species of the *R. pomonella* locus (*R. pomonella*, *R. zephyria* and *R. mendax*) clustering

7. Results

In silico data

Burgher-MacLellan *et al.* 2009: Primer BLAST and *in silico* PCRs of the ID-1F/ID-1R/ID-3R primers indicated them as specific for *R. pomonella* complex species. Both the ID-8F and ID-9R primer BLAST led to trees where *R. pomonella* grouped with many other species of the *Rhagoletis* genus. *In silico* PCR on this primer pair resulted in all three species of the *R. pomonella* complex showing as potential targets, in addition to singular results of fly species belonging other families such as *Clusiodes* sp., *Lonchaeidae* sp., and *Teleopsis* sp., but no other *Rhagoletis* species.

Velez *et al.* 2006: All three primer pairs (P6F/P6R, P9F/P9R, P12F/P12R) showed *in silico* specificity for the *R. pomonella* complex. The *in silico* PCR resulted in exactly one hit each, which were sequences deposited by the authors. It has to be noted, that whole genome sequences are lacking for most closely related *Rhagoletis* species, making evaluation of such results difficult.

Details on the *in silico* data see Appendix 2.

SYBR Green real-time PCRs according to Burgher-MacLellan *et al.* (2009) and Velez *et al.* (2006)

Identification of *R. pomonella* with these SYBR Green real-time PCRs targeting the *COI* locus and microsatellite DNA proved to be difficult with the sample set used in this pretesting. As Table 4 and 5 show, unspecific signals were observed for most of the PCRs with the melting temperature being unsuitable to give further insight into the specificity in most cases. Only in the real-time PCR ID-8F/ID-9R (Burgher-MacLellan *et al.* 2009) a slight difference in melting temperature between the target sample and non-target samples could be observed.

Table 4: SYBR Green PCR results of pretesting with the sample set; results for *Rhagoletis* spp. according to assigned values

| Sample | Species | Burgher-MacLellan <i>et al.</i> 2009 tests 1-3 | | | Velez <i>et al.</i> 2006 tests 4-6 | | |
|---------|----------------------------|--|-------------------------|-------------------------|------------------------------------|---------------------|-----------------------|
| | | Test 1 (ID-8F/ID-9R) | Test 2 (ID-1F/ID-1R) | Test 3 (ID-1F/ID-3R) | Test 4 (P6F/P6R) | Test 5 (P9F/P9R) | Test 6 (P12F/P12R) |
| 66/19 | <i>R. pomonella/mendax</i> | + | (-) | (-) | (-) | (-) | (-) |
| 2247/22 | <i>R. cingulata</i> | (-) | (-) | (-) | (-) | (-) | (-) |
| 1928/22 | <i>R. meigenii</i> | (-) | (-) | (-) | (-) | (-) | (-) |
| 1233/22 | <i>R. alternata</i> | (-) | (-) | (-) | (-) | (-) | (-) |
| 1231/22 | <i>R. cerasi</i> | (-) | (-) | (-) | (-) | (-) | (-) |
| 2552/21 | <i>R. meigenii</i> | (-) | (-) | (-) | (-) | (-) | (-) |

Table 5: Result summary and verdict for the eight real-time PCRs tested

| Author | Test | Verdict |
|--|--------|---|
| Burgher-MacLellan <i>et al.</i> (2009) | Test 1 | <i>R. pomonella/mendax</i> does have a slightly different melting temperature than all other tested non-targets. |
| | Test 2 | <i>R. pomonella/mendax</i> and <i>R. cerasi</i> show lower Ct values than the other samples, however with the same melting temperature. |
| | Test 3 | <i>R. pomonella</i> and <i>R. cerasi</i> show lower Ct values than the other samples, however with the same melting temperature. |
| Velez <i>et al.</i> (2006) | Test 4 | There is no distinction possible on the basis of either Ct or melting temperature. |
| | Test 5 | <i>R. pomonella</i> and <i>R. cingulata</i> show lower Ct values than the other samples, however with the same melting temperature. |
| | Test 6 | There is no distinction possible on the basis of either Ct or melting temperature. |

Barcoding according to EPPO PM7/129(2)

Sequencing on the *COI* locus was able to distinguish all non-targets of the sample set used in this pretesting (Tab. 6). The target sample could be identified down to the *Rhagoletis pomonella* complex, which includes *R. mendax* and *R. zephyria*. Additionally, the sequence quality did not meet the expected quality criteria.

Table 6: Results of barcoding with the pretesting sample set

| Sample | Assigned value | Result of pretesting (LepF/R) |
|---------|------------------------------------|-------------------------------------|
| 66/19 | <i>Rhagoletis pomonella/mendax</i> | <i>Rhagoletis pomonella</i> complex |
| 2247/22 | <i>Rhagoletis cingulata</i> | <i>Rhagoletis cingulata</i> |
| 1928/22 | <i>Rhagoletis meigenii</i> | <i>Rhagoletis meigenii</i> |
| 1233/22 | <i>Rhagoletis alternata</i> | <i>Rhagoletis alternata</i> |
| 1231/22 | <i>Rhagoletis cerasi</i> | <i>Rhagoletis cerasi</i> |
| 2552/21 | <i>Rhagoletis meigenii</i> | <i>Rhagoletis meigenii</i> |

8. Discussion

Molecular identification of *Rhagoletis pomonella* pretested in this study highlighted some of the difficulties that arise with this species complex. *In silico* data (database inventory, primer blast, including fast minimum evolution tree analysis, and *in silico* PCR) already indicated the impossibility of resolving the species within the *R. pomonella* complex with the tests included in this pretesting, and occasionally led to doubts in regards to the specificity of the tests as well.

The *in silico* specificity of the tests according to Velez *et al.* (2006) targeting microsatellite DNA could not be evaluated, as there seem to be no sequences available for the non-target *Rhagoletis* species included in this study on these gene loci.

For one real-time PCR targeting the *COI* locus (Test1 according to Burgher-MacLellan *et al.* 2009) *R. pomonella/mendax* was distinguishable via differences in melting temperature. None of the other real-time PCRs could be confirmed as suitable for the identification of *R. pomonella* in this pretesting. Nevertheless, in Test2 and 3, significantly lower Ct values were achieved for *R. pomonella/mendax* and *R. cerasi*, which could be an indication of these tests not being exclusive for all tested *Rhagoletis* species. It has to be noted however, that due to the limited availability of *R. pomonella* specimens, the target in this pretesting was of questionable condition.

COI barcoding could be confirmed as suitable to identify targets down to the level of *Rhagoletis pomonella* complex, but distinction among species in this complex remains difficult.

Further studies will have to be conducted both with an extended sample set for non-targets and more target specimens of sufficient quality. Special attention will have to be given to *Rhagoletis cerasi* specimen, as well as sufficient representation of all species included in the *Rhagoletis pomonella* complex (*R. pomonella*, *R. mendax*, *R. zephyria*, *R. cornivora*).

Participating staff:

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Date: December 12th, 2022



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EURL Deputy Director



Richard Gottsberger
Scientific Project Leader – Molecular Unit

Appendix 1 - References

EPPO (2021). EPPO standards PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests. Bulletin OEPP/EPPO Bulletin, 51 (1): 100–143.

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. (2006). DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences, 103(4), 968-971.

Burgher-MacLellan, K. L., Gaul, S., MacKenzie, K., & Vincent, C. (2009). The use of real-time PCR to identify blueberry maggot (Diptera: Tephritidae, *Rhagoletis mendax*) from other *Rhagoletis* species and in lowbush blueberry fruit (*Vaccinium angustifolium*). Acta Horticulturae International Society of Horticultural Sciences, 810, 265-274.

Velez, S., Taylor, M. S., Noor, M. A. F., Lobo, N. F., & Feder, J. L. (2006). Isolation and characterization of microsatellite loci from the apple maggot fly *Rhagoletis pomonella* (Diptera: Tephritidae). Molecular Ecology Notes, 6(1), 90-92.

Xie, X., Michel, A. P., Schwarz, D., Rull, J., Velez, S., Forbes, A. A., Aluja, M., Feder, J. L. (2008) Radiation and divergence in the *Rhagoletis pomonella* species complex: inferences from DNA sequence data. J Evol Biol. 21(3), 900-913.

Appendix 2 – In silico data

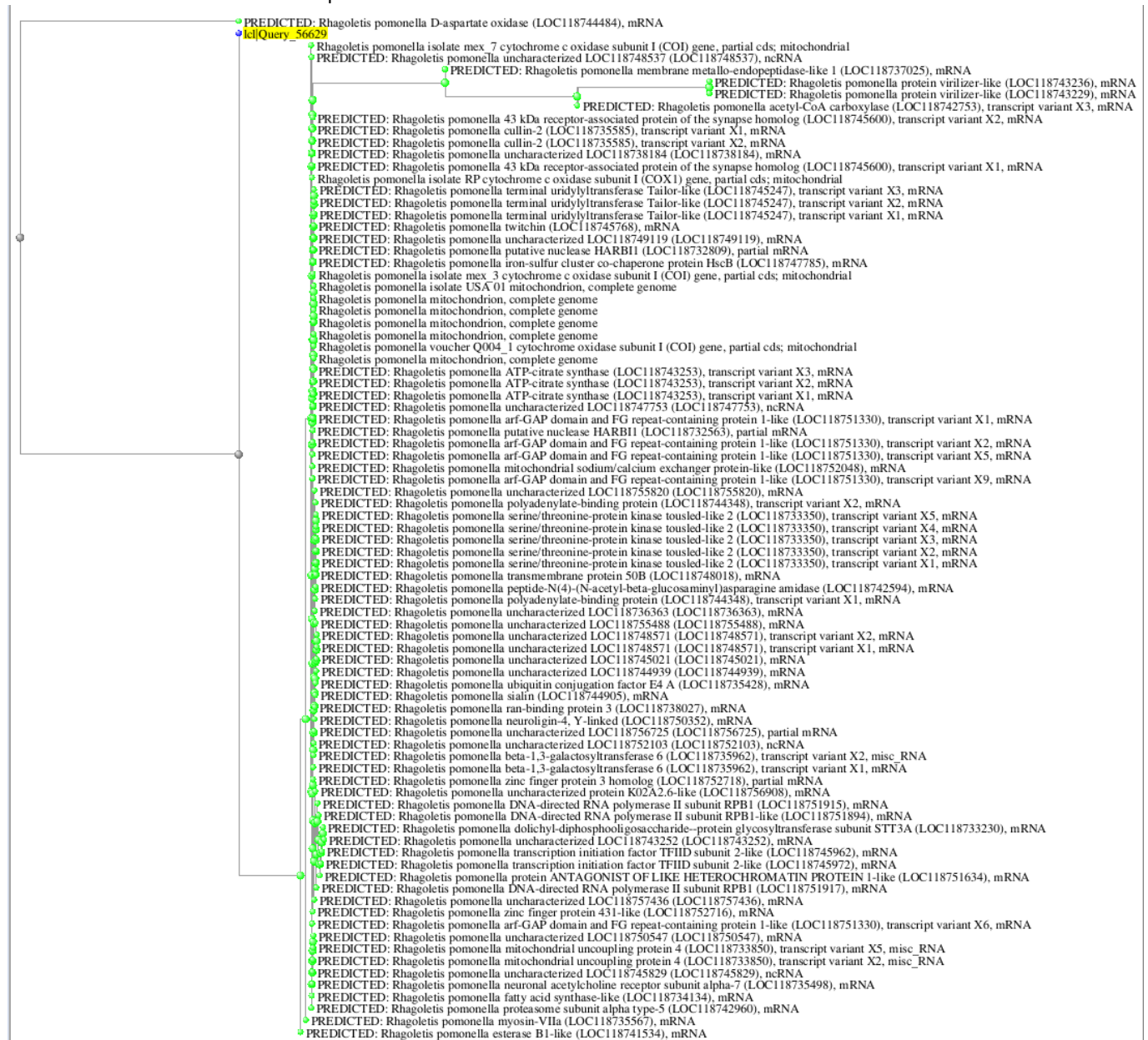
In silico data PCR assay 1

EPPO (2021). EPPO standards PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests. Bulletin OEPP/EPPO Bulletin, 51 (1): 100–143.

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. (2006). DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences, 103(4), 968-971.

Search was restricted to *Rhagoletis pomonella*

Fast Minimum Evolution tree for LepF



Fast Minimum Evolution tree for LepF

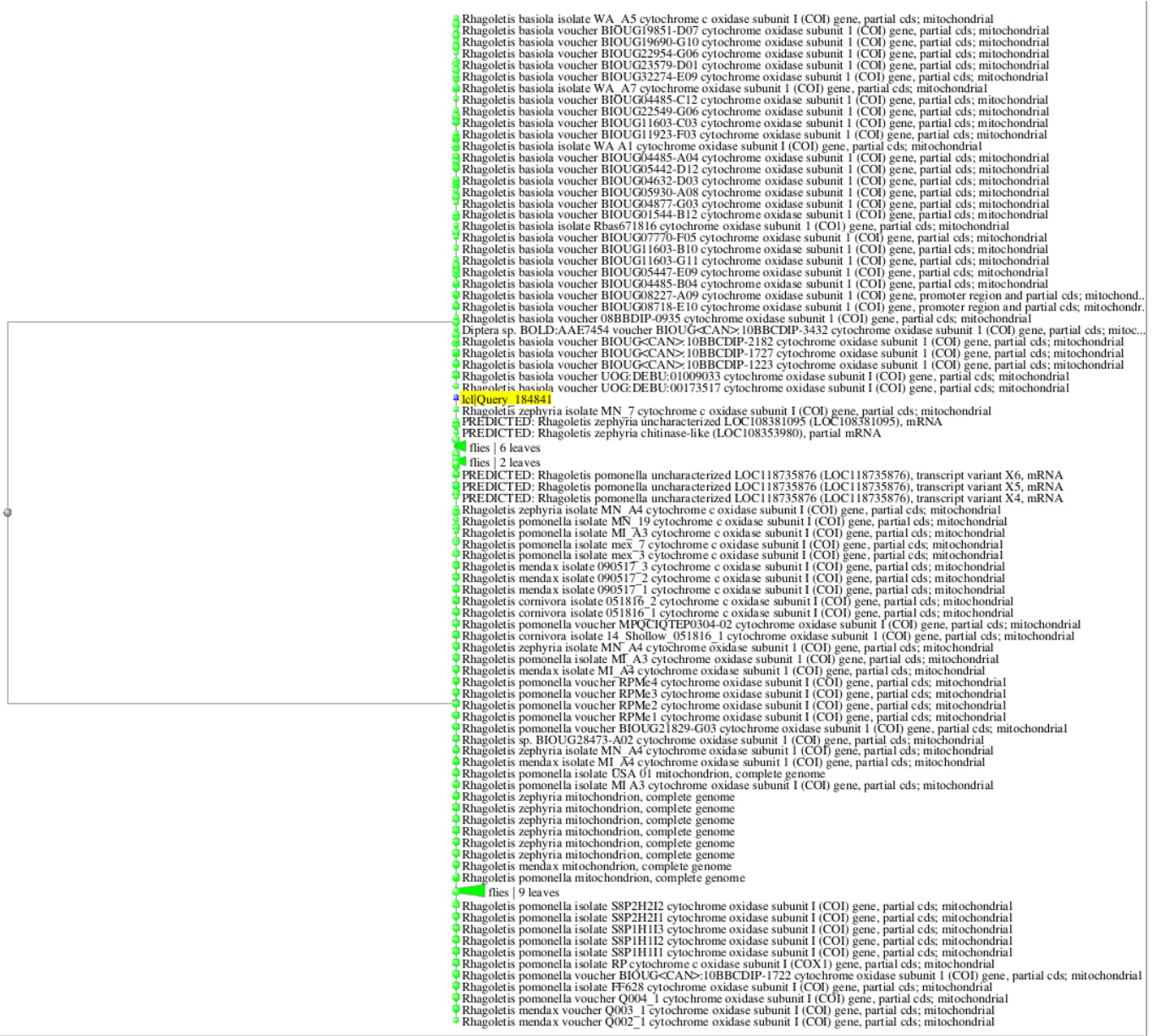


***In silico* data PCR assay 2-4**

Burgher-MacLellan, K. L., Gaul, S., MacKenzie, K., & Vincent, C. (2009). The use of real-time PCR to identify blueberry maggot (Diptera: Tephritidae, *Rhagoletis mendax*) from other *Rhagoletis* species and in lowbush blueberry fruit (*Vaccinium angustifolium*). *Acta Horticulturae International Society of Horticultural Sciences*, 810, 265-274.

Search was restricted to *Rhagoletis*

Fast Minimum Evolution tree for ID-8 F:



Fast Minimum Evolution tree for ID-9R:



Fast Minimum Evolution tree for ID-1R:

- flies | 15 leaves
- Rhagoletis zephyria haplotype 15 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 14 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 13 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 12 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis mendax haplotype 11 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis mendax haplotype 10 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis mendax haplotype 9 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis pomonella haplotype 8 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 7 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis mendax haplotype 6 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis pomonella haplotype 5 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 4 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis zephyria haplotype 3 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis zephyria haplotype 2 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis zephyria haplotype 1 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis pomonella cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate USA 01 mitochondrion, complete genome
- Rhagoletis pomonella isolate B08_A_013 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu gen...
- Rhagoletis pomonella isolate G09_MF_013 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu g...
- Rhagoletis pomonella isolate G08_BB_017 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu ge...
- Rhagoletis pomonella isolate M06_I_012 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu gen...
- Rhagoletis pomonella isolate B07_SC_009 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu ge...
- Rhagoletis pomonella isolate B08_A_022 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu gen...
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis mendax mitochondrion, complete genome
- Rhagoletis pomonella mitochondrion, complete genome
- Rhagoletis pomonella mitochondrion, complete genome
- Rhagoletis pomonella mitochondrion, complete genome
- Rhagoletis pomonella mitochondrion, complete genome
- Rhagoletis pomonella mitochondrion, complete genome
- Rhagoletis pomonella mitochondrion, complete genome
- Rhagoletis pomonella isolate Rp_H_NY3 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate Rp_H_TX_HNG cytochrome oxidase subunit II (COII) gene, partial cds; mitoch...
- Rhagoletis pomonella isolate Rp_H_NY5 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate Rp_H_NY2 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate Rp_H_NY4 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis aff. pomonella 'flowering dogwood fly' cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-
- Rhagoletis aff. pomonella 'flowering dogwood fly' haplotype 2 cytochrome oxidase subunit I (COI) gene, parti...
- Rhagoletis pomonella cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu gene, complete sequenc...
- Rhagoletis pomonella voucher Rp1 cytochrome c oxidase subunit 2 gene, partial cds; mitochondrial
- Rhagoletis pomonella voucher PSU93032912 cytochrome oxidase subunit II (COII) gene, partial cds; mitochon...
- Rhagoletis pomonella isolate NSSPA20 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate NSSMP13 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate NSSML5 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate NSSMA14 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis zephyria isolate PA1300 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis zephyria isolate PA2900 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate PA1 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate PA1300 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate PA200 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate MI1 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis aff. pomonella 'flowering dogwood fly' isolate GA cytochrome oxidase subunit II (COII) gene, parti...
- Rhagoletis pomonella isolate NY18013 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate TX2100 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate MIA4011 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate NY1 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate MC6030 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate CP2 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate CP3 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate SJ1 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate SJ4 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate SJ3 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis aff. pomonella JLF-2002 isolate Cfloridaa_8 cytochrome oxidase subunit I gene, partial cds; tRNA-...
- Rhagoletis pomonella isolate PomTXH2_3 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis pomonella isolate CB1 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis mendax cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, complete sequence; and cy...
- Rhagoletis nr. mendax JJS-1996 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu (trnL) gene, ...
- Rhagoletis pomonella isolate MexHiJ2_4 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, com...
- Rhagoletis pomonella isolate MC5 cytochrome oxidase subunit I and cytochrome oxidase subunit II genes, parti...
- Rhagoletis pomonella isolate MC4 cytochrome oxidase subunit I and cytochrome oxidase subunit II genes, parti...
- Rhagoletis pomonella isolate MC2 cytochrome oxidase subunit I and cytochrome oxidase subunit II genes, parti...
- Rhagoletis mendax isolate Rmendax5_6 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, compl...
- Rhagoletis zephyria cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu (trnL) gene, complete seq...
- Rhagoletis pomonella isolate PomMexHiH5_1 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene...
- Rhagoletis pomonella isolate PomMexHiH4_8 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene...
- Rhagoletis pomonella isolate PomMexHiH2_3 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene...
- Rhagoletis pomonella isolate MexHiI1 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, complet...
- Rhagoletis pomonella isolate PomMIA45ma_4 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene...
- Rhagoletis pomonella isolate PomHNYH19 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis pomonella isolate PomHNYH2 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis pomonella cytochrome oxidase subunit II gene, partial cds; tRNA-Leu gene, complete sequence; and

kl|Query_349771

Fast Minimum Evolution tree for ID-3R:

- flies | 12 leaves
- Rhagoletis zephyria haplotype 18 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 17 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 16 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 15 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 14 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 13 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 12 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis mendax haplotype 11 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis mendax haplotype 10 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis mendax haplotype 9 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis pomonella haplotype 8 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 7 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis mendax haplotype 6 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis pomonella haplotype 5 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, c...
- Rhagoletis zephyria haplotype 4 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis zephyria haplotype 3 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis zephyria haplotype 2 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis zephyria haplotype 1 cytochrome c oxidase subunit I (COX1) gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis pomonella cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate USA 01 mitochondrion, complete genome
- Rhagoletis pomonella isolate B08_A 013 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu gen.
- Rhagoletis pomonella isolate G09_MF 013 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu g.
- Rhagoletis pomonella isolate G08_BB 017 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu ge.
- Rhagoletis pomonella isolate M06_I 012 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu gen.
- Rhagoletis pomonella isolate B07_SC 009 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu ge.
- Rhagoletis pomonella isolate B08_A 022 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu gen.
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis zephyria mitochondrion, complete genome
- Rhagoletis mendax mitochondrion, complete genome
- Rhagoletis pomonella mitochondrion, complete genome
- Rhagoletis pomonella mitochondrion, complete genome
- Rhagoletis pomonella mitochondrion, complete genome
- Rhagoletis pomonella mitochondrion, complete genome
- Rhagoletis pomonella mitochondrion, complete genome
- Rhagoletis pomonella isolate Rp_H_NY3 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate Rp_H_TX HNG cytochrome oxidase subunit II (COII) gene, partial cds; mitoch.
- Rhagoletis pomonella isolate Rp_H_NY5 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate Rp_H_NY2 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate Rp_H_NY4 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis aff. pomonella 'flowering dogwood fly' cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-
- Rhagoletis aff. pomonella 'flowering dogwood fly' haplotype 2 cytochrome oxidase subunit I (COI) gene, partia.
- Rhagoletis pomonella cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu gene, complete sequenc.
- Rhagoletis pomonella voucher Rn1 cytochrome c oxidase subunit 2 gene, partial cds; mitochondrial
- Rhagoletis pomonella voucher PSU93032912 cytochrome oxidase subunit II (COII) gene, partial cds; mitochon.
- Rhagoletis pomonella isolate NSSPA20 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate NSSMP13 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate NSSML5 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate NSSMA14 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis zephyria isolate PA1300 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis zephyria isolate PA2900 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate PA1 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate PA1300 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate PA200 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis mendax isolate M11 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis aff. pomonella 'flowering dogwood fly' isolate GA cytochrome oxidase subunit II (COII) gene, parti.
- Rhagoletis pomonella isolate NY18013 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate TX2100 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate MIA4011 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate NY1 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate MC6030 cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate CP2 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate CP3 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate SJ1 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate SJ4 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis pomonella isolate SJ3 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis aff. pomonella JLF-2002 isolate Cfloridaa_8 cytochrome oxidase subunit I gene, partial cds; tRNA-...
- Rhagoletis pomonella isolate PomTXH2_3 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis pomonella isolate CB1 cytochrome oxidase subunit II gene, partial cds; mitochondrial
- Rhagoletis mendax cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, complete sequence; and cy.
- Rhagoletis nr. mendax JJS-1996 cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu (trnL) gene, ...
- Rhagoletis pomonella isolate MexHiJ2_4 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, com.
- Rhagoletis pomonella isolate MC5 cytochrome oxidase subunit I and cytochrome oxidase subunit II genes, parti.
- Rhagoletis pomonella isolate MC4 cytochrome oxidase subunit I and cytochrome oxidase subunit II genes, parti.
- Rhagoletis mendax isolate Rmendax5_6 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, compl.
- Rhagoletis zephyria cytochrome oxidase subunit I (COI) gene, partial cds; tRNA-Leu (trnL) gene, complete seq.
- Rhagoletis pomonella isolate PomMexHiH5_1 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene.
- Rhagoletis pomonella isolate PomMexHiH4_8 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gen.
- Rhagoletis pomonella isolate PomMexHiH2_3 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene.
- Rhagoletis pomonella isolate MexHI1 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, complet.
- Rhagoletis pomonella isolate PomMIA45ma_4 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene.
- Rhagoletis pomonella isolate PomHNYH19 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, co.
- Rhagoletis pomonella isolate PomHNYH2 cytochrome oxidase subunit I gene, partial cds; tRNA-Leu gene, co...
- Rhagoletis pomonella cytochrome oxidase subunit II gene, partial cds; tRNA-Leu gene, complete sequence; and.
- ICIQuery_333685

In silico PCR for primers ID-8F/ID-9R:

>MH998965.1 *Rhagoletis zephyria* isolate MN_A4 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 206
Forward primer 1 TGGAGGGGCATCTGTTGA 18
Template       381 ..... 398

Reverse primer 1 TAATGGCTCCTGCTAATACTGGT 23
Template       586 ..... 564
```

>MH998964.1 *Rhagoletis pomonella* isolate MN_19 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 206
Forward primer 1 TGGAGGGGCATCTGTTGA 18
Template       378 ..... 395

Reverse primer 1 TAATGGCTCCTGCTAATACTGGT 23
Template       583 ..... 561
```

>MH998963.1 *Rhagoletis pomonella* isolate MI_A3 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 206
Forward primer 1 TGGAGGGGCATCTGTTGA 18
Template       378 ..... 395

Reverse primer 1 TAATGGCTCCTGCTAATACTGGT 23
Template       583 ..... 561
```

>MH998960.1 *Rhagoletis mendax* isolate 090517_3 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 206
Forward primer 1 TGGAGGGGCATCTGTTGA 18
Template       375 ..... 392

Reverse primer 1 TAATGGCTCCTGCTAATACTGGT 23
Template       580 ..... 558
```

>JF873670.1 *Clusiodes melanostoma* voucher BIOUG:10JSROW-1054 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial

```
product length = 206
Forward primer 1 TGGAGGGGCATCTGTTGA 18
Template       367 ..... 384

Reverse primer 1 TAATGGCTCCTGCTAATACTGGT 23
Template       572 ..... 550
```

>KP978468.1 *Teleopsis pallifacies* voucher RMNH.INS.555138 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial

```
product length = 206
Forward primer 1 TGGAGGGGCATCTGTTGA 18
Template       367 ..... 384

Reverse primer 1 TAATGGCTCCTGCTAATACTGGT 23
Template       572 ....A..... 550
```

>KR395792.1 *Clusiodes orbitalis* voucher BIOUG12107-B07 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial

```
product length = 206
Forward primer 1 TGGAGGGGCATCTGTTGA 18
Template       366 ..... 383

Reverse primer 1 TAATGGCTCCTGCTAATACTGGT 23
Template       571 ....C..... 549
```


>KM939719.1 Lonchaeidae sp. BOLD:AAP8893 voucher BIOUG05093-F07 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial

```
product length = 206
Forward primer 1 TGGAGGGGCATCTGTTGA 18
Template 367 ..... 384

Reverse primer 1 TAATGGCTCCTGCTAATACTGGT 23
Template 572 ....T..... 550
```

>KM929290.1 Clusiodes clandestinus voucher BIOUG05088-H06 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial

```
product length = 206
Forward primer 1 TGGAGGGGCATCTGTTGA 18
Template 366 ..... 383

Reverse primer 1 TAATGGCTCCTGCTAATACTGGT 23
Template 571 .....C..... 549
```

In silico PCR ID-1F/ID-1R:

>MN443940.1 Rhagoletis zephyria mitochondrion, complete genome

```
product length = 152
Forward primer 1 GACGGATTTTCGATTATTAGATGT 23
Template 3463 ..... 3485

Reverse primer 1 TGATTTAGACGACCAGGAGTT 21
Template 3614 ..... 3594
```

>MN443939.1 Rhagoletis mendax mitochondrion, complete genome

```
product length = 152
Forward primer 1 GACGGATTTTCGATTATTAGATGT 23
Template 3463 ..... 3485

Reverse primer 1 TGATTTAGACGACCAGGAGTT 21
Template 3614 ..... 3594
```

>MN443938.1 Rhagoletis pomonella mitochondrion, complete genome

```
product length = 152
Forward primer 1 GACGGATTTTCGATTATTAGATGT 23
Template 3463 ..... 3485

Reverse primer 1 TGATTTAGACGACCAGGAGTT 21
Template 3614 ..... 3594
```

In silico PCR ID-1F/ID-3R:

>MN443940.1 Rhagoletis zephyria mitochondrion, complete genome

```
product length = 151
Forward primer 1 GACGGATTTTCGATTATTAGATGT 23
Template 3463 ..... 3485

Reverse primer 1 GATTTAGACGACCAGGAGTTC 21
Template 3613 ..... 3593
```

>MN443939.1 Rhagoletis mendax mitochondrion, complete genome

```
product length = 151
Forward primer 1 GACGGATTTTCGATTATTAGATGT 23
Template 3463 ..... 3485

Reverse primer 1 GATTTAGACGACCAGGAGTTC 21
Template 3613 ..... 3593
```

>MN443938.1 Rhagoletis pomonella mitochondrion, complete genome

```
product length = 151
Forward primer 1 GACGGATTTTCGATTATTAGATGT 23
Template 3463 ..... 3485

Reverse primer 1 GATTTAGACGACCAGGAGTTC 21
Template 3613 ..... 3593
```

>MN443937.1 Rhagoletis pomonella mitochondrion, complete genome

```
product length = 151
Forward primer 1 GACGGATTTTCGATTATTAGATGT 23
Template 3437 ..... 3459

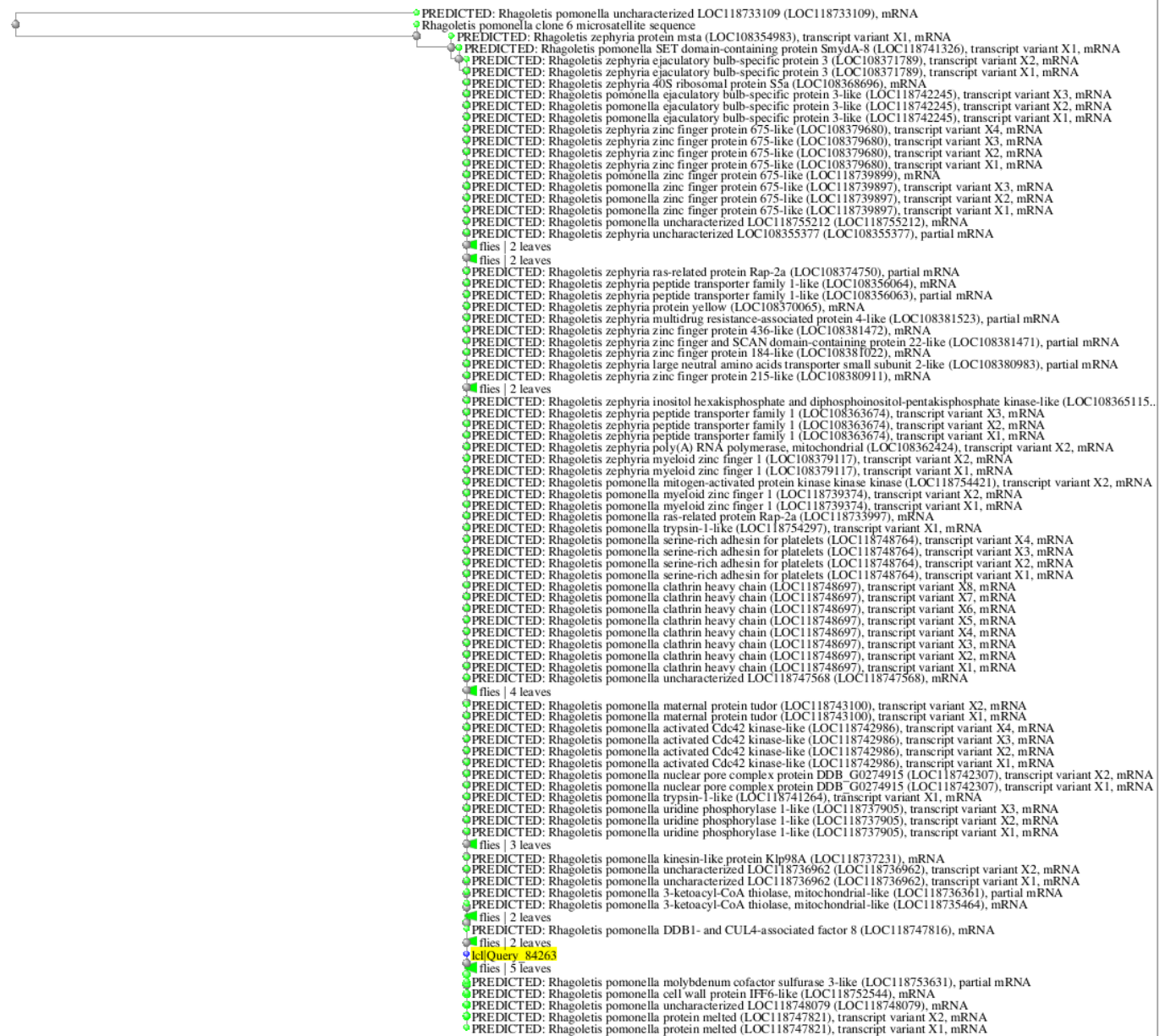
Reverse primer 1 GATTTAGACGACCAGGAGTTC 21
Template 3587 ..... 3567
```

In silico data PCR assay 5-7

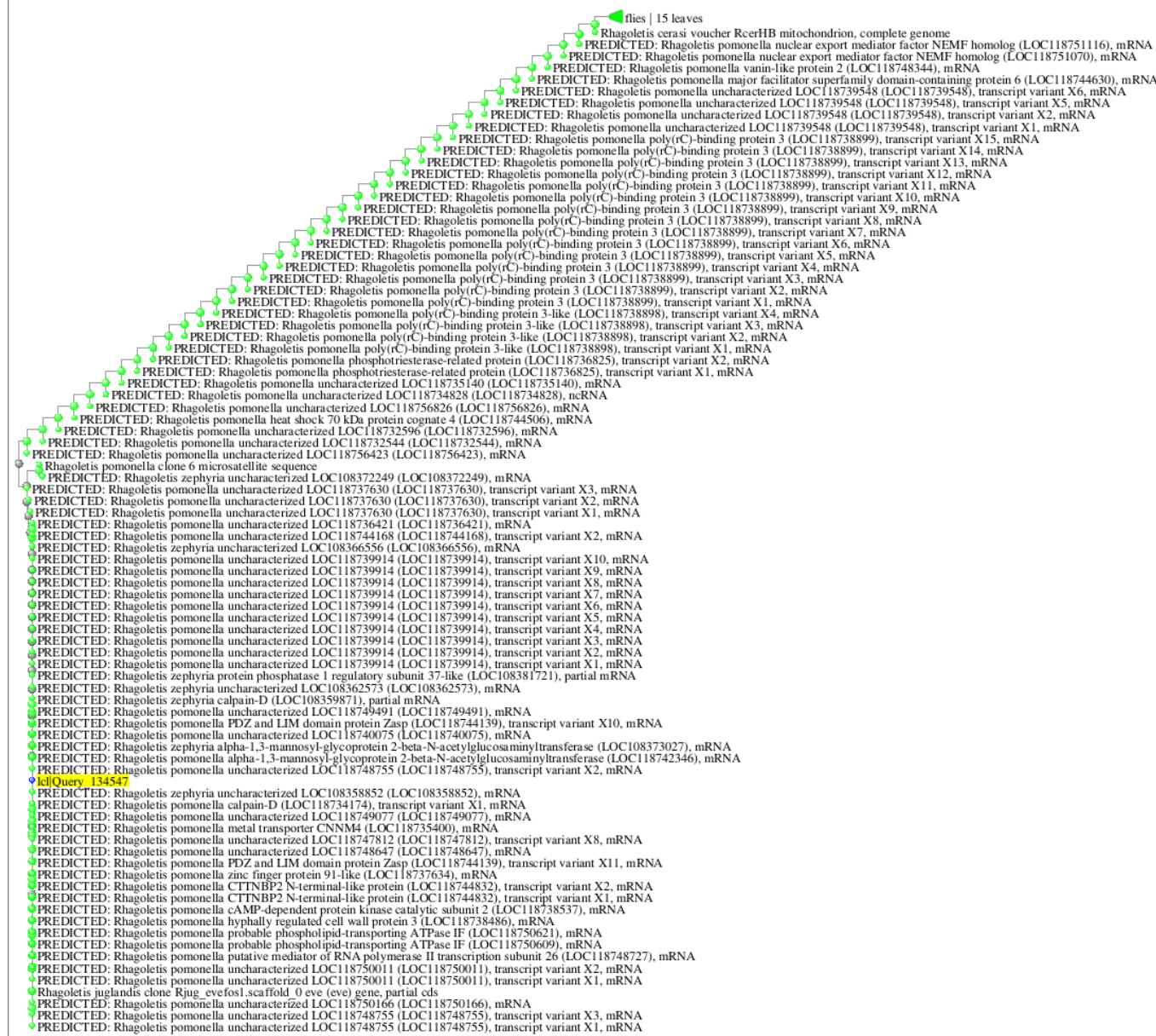
Velez, S., Taylor, M. S., Noor, M. A. F., Lobo, N. F., & Feder, J. L. (2006). Isolation and characterization of microsatellite loci from the apple maggot fly *Rhagoletis pomonella* (Diptera: Tephritidae). *Molecular Ecology Notes*, 6(1), 90-92.

Search was restricted to *Rhagoletis*

Fast Minimum Evolution tree for P6_F



Fast Minimum Evolution tree for P6_R



In silico PCR P6_F/R

| Primer pair 1 | | | | | |
|--|-------------------------|--------|-------|-------|----------------------|
| | Sequence (5'->3') | Length | Tm | GC% | Self complementarity |
| Forward primer | AGTCAGAGTGC GGCAAAAGT | 20 | 60.18 | 50.00 | 5.00 |
| Reverse primer | CGGTAGACCTCAGGCTGATAG | 21 | 59.12 | 57.14 | 7.00 |
| Products on target templates | | | | | |
| >AY734890.1 Rhagoletis pomonella clone 6 microsatellite sequence | | | | | |
| product length = 162 | | | | | |
| Forward primer | 1 AGTCAGAGTGC GGCAAAAGT | 20 | | | |
| Template | 119 | 138 | | | |
| Reverse primer | 1 CGGTAGACCTCAGGCTGATAG | 21 | | | |
| Template | 280 | 260 | | | |

Fast Minimum Evolution tree for P9_F



Fast Minimum Evolution tree for P9_R



In silico PCR P9_F/R

Primer pair 1

| | Sequence (5'→3') | Length | Tm | GC% | Self complementarity | Self 3' complementarity |
|----------------|----------------------|--------|-------|-------|----------------------|-------------------------|
| Forward primer | CGGCAGGTAATGACCAAAA | 20 | 56.61 | 45.00 | 5.00 | 0.00 |
| Reverse primer | GCAATGACCGTTGGCTATTA | 20 | 56.20 | 45.00 | 4.00 | 3.00 |

Products on target templates

>AY734893.1 Rhagoletis pomonella clone 9 microsatellite sequence

product length = 158

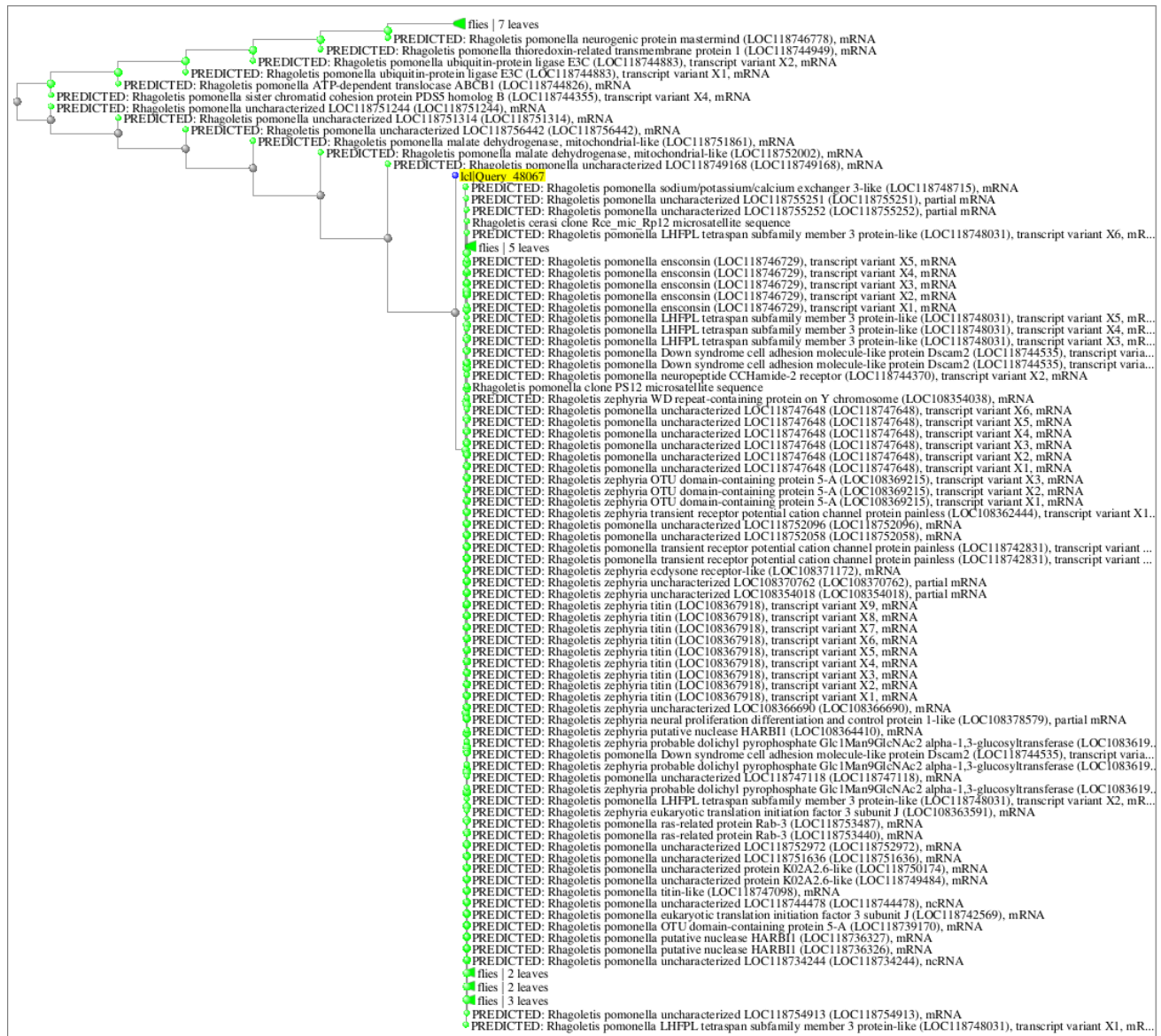
Forward primer 1 CGGCAGGTAATGACCAAAA 20
 Template 24 43

Reverse primer 1 GCAATGACCGTTGGCTATTA 20
 Template 181 162

Fast Minimum Evolution tree for P12_F



Fast Minimum Evolution tree for P12_R



In silico PCR P12_F/R

Primer pair 1

| | Sequence (5'→3') | Length | Tm | GC% | Self complementarity | Self 3' complementarity |
|-----------------------|--------------------------|--------|-------|-------|----------------------|-------------------------|
| Forward primer | GGGTGTTTCATGGTAGTTGTAGAT | 23 | 57.90 | 43.48 | 4.00 | 2.00 |
| Reverse primer | ACTAGTAAAGGAAAGCGCAAT | 22 | 58.05 | 40.91 | 6.00 | 2.00 |

Products on target templates

>JX870459.1 Rhagoletis cerasi clone Rce_mic_Rp12 microsatellite sequence

product length = 224

```
Forward primer 1 GGGTGTTTCATGGTAGTTGTAGAT 23
Template      229 ..... 207
```

```
Reverse primer 1 ACTAGTAAAGGAAAGCGCAAT 22
Template      6 ..... 27
```

>AY734896.1 Rhagoletis pomonella clone PS12 microsatellite sequence

product length = 250

```
Forward primer 1 GGGTGTTTCATGGTAGTTGTAGAT 23
Template      1 ..... 23
```

```
Reverse primer 1 ACTAGTAAAGGAAAGCGCAAT 22
Template      250 ..... 229
```

Appendix 3 – Specifications and parameters for the molecular tests

Specification of the PCR Assay 1 (COI Barcoding according to EPPO PM7/129(2))

Name of the primer incl. sequence, literature reference, fragment length in bp:

LepF: 5'- ATTCAACCAATCATAAAGATATTGG-3'

LepR: 5'- TAAACTTCTGGATGTCCAAAAAATCA-3'

Literature: Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. (2006). DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Sciences*, 103(4), 968-971.

Fragment length: 709bp

PCR - Parameters:

Thermocycler used: Biometra T3000 Thermal cycler

Mastermix: 5x HOT FIREPol® Master Mix, Solis Biodyne:

| Composition: | | Final concentration: |
|--------------|------------------------|----------------------|
| | Volume per reaction µl | |
| Water | 6 | |
| Mastermix | 2 | 1x |
| Primer1: | 0,5 | 0,5µM |
| Primer2: | 0,5 | 0,5µM |
| ∑ | 9 | |
| DNA | 1 | |

PCR conditions:

| | °C | Duration (min., sec.) | Nr. of Cycles |
|-----------------|----|-----------------------|---------------|
| Start | 95 | 15 min | 1 |
| Denaturation | 95 | 45 sec | 5 |
| Annealing | 44 | 45 sec | |
| Extension | 72 | 45 sec | |
| Denaturation | 95 | 45 sec | 35 |
| Annealing | 49 | 45 sec | |
| Extension | 72 | 45 sec | |
| Final extension | 72 | 7 min | 1 |
| Cooling | 15 | ∞ | |

Specification of the PCR Assay 2-4 (SYBR Green real-time PCRs according to Burgher-McLellan *et al.* 2009)

Name of the primer incl. sequence, literature reference, fragment length in bp:

ID-8 F: 5'- TGGAGGGGCATCTGTTGA-3'

ID-9R: 5'- TAATGGCTCCTGCTAATACTGGT-3'

ID-1 F: 5'- GACGGATTTGATTATTAGATGT-3'

ID-1 R: 5'- TGATTTAGACGACCAGGAGTT-3'

ID-3 R: 5'- GATTTAGACGACCAGGAGTTC-3'

Burgher-McLellan, K. L., Gaul, S., MacKenzie, K., & Vincent, C. (2009). The use of real-time PCR to identify blueberry maggot (Diptera: Tephritidae, *Rhagoletis mendax*) from other *Rhagoletis* species and in lowbush blueberry fruit (*Vaccinium angustifolium*). *Acta Horticulturae International Society of Horticultural Sciences*, 810, 265-274.

Fragment length:
 ID-8F/ID-9R: 206bp
 ID-1F/ID-1R: 152bp
 ID-1F/ID-3R: 151bp

PCR - Parameters:

Analytic Jena qTower³ G (230 V) with accompanying software, Bio Molecular Systems Magnetic Induction Cycler (MIC) with accompanying software.

Mastermix: HOT FIREPol[®] EvaGreen[®] qPCR Mix (Solis Biodyne)

| Composition: | | Final concentration: |
|--------------|-----------------------------|----------------------|
| | Volume per reaction μ l | |
| Water | 6 | |
| Mastermix | 2 | 1x |
| Primer1: | 0,5 | 0.5 μ M |
| Primer2: | 0,5 | 0.5 μ M |
| Σ | 9 | |
| DNA | 1 | |

PCR conditions ID-8F/ID-9R:

| Step | $^{\circ}$ C | Duration (min., sec.) | Nr. of Cycles |
|------------------------------------|--------------|-----------------------|--------------------|
| Start | 95 | 12 min | 1 |
| Denaturation | 95 | 15 sec | 45 |
| Annealing | 60 | 30 sec | |
| Extension and fluorescence reading | 72 | 30 sec | |
| Melting curve | 72-95 | 0.1 $^{\circ}$ C/s | Stepwise increment |

PCR conditions ID-1F/ID-1R:

| Step | $^{\circ}$ C | Duration (min., sec.) | Nr. of Cycles |
|------------------------------------|--------------|-----------------------|--------------------|
| Start | 95 | 12 min | 1 |
| Denaturation | 95 | 15 sec | 45 |
| Annealing | 50 | 30 sec | |
| Extension and fluorescence reading | 72 | 30 sec | |
| Melting curve | 72-95 | 0.1 $^{\circ}$ C/s | Stepwise increment |

PCR conditions ID-1F/ID-3R

| Step | $^{\circ}$ C | Duration (min., sec.) | Nr. of Cycles |
|------------------------------------|--------------|-----------------------|--------------------|
| Start | 95 | 12 min | 1 |
| Denaturation | 95 | 15 sec | 45 |
| Annealing | 58 | 30 sec | |
| Extension and fluorescence reading | 72 | 30 sec | |
| Melting curve | 72-95 | 0.1 $^{\circ}$ C/s | Stepwise increment |

Specification of the PCR Assay 5-7 (SYBR Green real-time PCRs according to Velez *et al.* 2006)

Name of the primer incl. sequence, literature reference, fragment length in bp:

P12_F: 5'- GGGTGTTTCATGGTAGTTGTAGAT-3'
P12_R: 5'- ACTAGTAAAGGAAAGGCGCAAT-3'
P6_F: 5'- AGTCAGAGTGCGGCAAAAGT -3'
P6_R: 5'- CGGTAGACCTCAGGCTGATAG -3'
P9_F: 5'- CGGCAGGTAAATGACCAAAA -3'
P9_R: 5'- GCAATGACCGTTGGCTATTA -3'

Velez, S., Taylor, M. S., Noor, M. A. F., Lobo, N. F., & Feder, J. L. (2006). Isolation and characterization of microsatellite loci from the apple maggot fly *Rhagoletis pomonella* (Diptera: Tephritidae). *Molecular Ecology Notes*, 6(1), 90-92.

Fragment length:

P12_F/R: 250bp

P6_F/R: 155bp

P9_F/R: 156bp

PCR - Parameters:

Analytic Jena qTower³ G (230 V) with accompanying software, Bio Molecular Systems Magnetic Induction Cyclers (MIC) with accompanying software.

Mastermix: HOT FIREPol[®] EvaGreen[®] qPCR Mix (Solis Biotec)

| Composition: | | Final concentration: |
|--------------|-----------------------------|----------------------|
| | Volume per reaction μ l | |
| Water | 6 | |
| Mastermix | 2 | 1x |
| Primer1: | 0,5 | 0.5 μ M |
| Primer2: | 0,5 | 0.5 μ M |
| Σ | 9 | |
| DNA | 1 | |

PCR conditions:

| Step | $^{\circ}$ C | Duration (min., sec.) | Nr. of Cycles |
|------------------------------------|--------------|-----------------------|--------------------|
| Start | 95 | 12 min | 1 |
| Denaturation | 95 | 15 sec | 45 |
| Annealing | 56 | 30 sec | |
| Extension and fluorescence reading | 72 | 30 sec | |
| Melting curve | 72-95 | 0.1 $^{\circ}$ C/s | Stepwise increment |

Appendix 4 – Real-time PCR results

Ct values for each of the samples

| Author | Burgher-MacLellan <i>et al.</i> (2009) | | | Velez <i>et al.</i> 2006 | | |
|---------|--|---------------------|---------------------|--------------------------|---------------------|---------------------|
| Primer | ID-8F/ID-9R | ID-1F/ID-1R | ID-1F/ID-3R | P6F/P6R | P9F/P9R | P12F/P12R |
| Target | <i>R. pomonella</i> | <i>R. pomonella</i> | <i>R. pomonella</i> | <i>R. pomonella</i> | <i>R. pomonella</i> | <i>R. pomonella</i> |
| 66/19 | 21.08 | 20.22 | 20.61 | 28.44 | 27.75 | 28.58 |
| 2247/22 | 16.05 | 34.23 | 30.18 | 24.48 | 26.46 | 30.14 |
| 1928/22 | 21.59 | 32.68 | 29.88 | 24.49 | 33.11 | 24.26 |
| 1233/22 | 19.16 | 33.27 | 28.58 | 29.01 | 35.82 | 30.50 |
| 1231/22 | 17.32 | 25.00 | 22.96 | 29.74 | 38.97 | 26.31 |
| 2552/21 | 22.56 | 34.31 | 31.77 | 26.20 | 34.39 | 27.07 |

Melting temperature (Tm) for each of the samples

| Author | Burgher-MacLellan <i>et al.</i> (2009) | | | Velez <i>et al.</i> 2006 | | |
|---------|--|---------------------|---------------------|--------------------------|---------------------|---------------------|
| Primer | ID-8F/ID-9R | ID-1F/ID-1R | ID-1F/ID-3R | P6F/P6R | P9F/P9R | P12F/P12R |
| Target | <i>R. pomonella</i> | <i>R. pomonella</i> | <i>R. pomonella</i> | <i>R. pomonella</i> | <i>R. pomonella</i> | <i>R. pomonella</i> |
| 66/19 | 80.55/79.54 | 79.97/78.85 | 79.89/78.89 | -/83.26 | -/84.14 | -/- |
| 2247/22 | 78.79/78.81 | 79.84/- | 79.81/79.78 | 83.96/84.38 | 84.53/84.53 | -/- |
| 1928/22 | 78.64/78.74 | 79.73/80.08 | 80.00/79.89 | 84.49/84.50 | -/- | 85.53/85.67 |
| 1233/22 | 77.95/77.95 | 79.28/76.35 | 79.03/79.44 | -/- | -/- | -/75.12 |
| 1231/22 | 78.37/78.43 | 78.98/79.11 | 79.17/79.09 | -/- | -/- | 84.29/84.29 |
| 2552/21 | 78.42/78.54 | 79.67/79.67 | 79.91/78.99 | 84.33/- | 84.08/- | 85.39/85.56 |