

FINAL PROJECT REPORT

TITLE: Genetic Markers to Identify Pests of Quarantine Importance.
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OBJECTIVES (2002-2005):

- 1) Develop species-specific primers for key problematic lepidopteran pests.
- 2) Develop and test procedures suitable for any diagnostic lab conducting PCR.
- 3) Develop sequence information for mites, mealybug complex, or other pests

SIGNIFICANT FINDINGS:

1. Tortricidae internal fruit feeders.

- DNA diagnostics for the lepidopteran fruit boring complex [codling moth (CM), oriental fruit moth (OFM), lesser apple worm (LAW) and cherry fruit worm (CFW)] were developed and the technology validated by and transferred to Mexico.
- DNA sequencing of “filbertworms” (*Cydia latiferreana*) from filberts (*Corylus* spp.) in Oregon and from oak acorns (*Quercus* spp.) in Washington demonstrated that they are different species. A new *Cydia* species (“oakworm”) should be described for the Pacific Northwest.
- DNA sequence of putative “filbertworm” intercepted in nectarines in California was identical to the sequence of filbertworm collected in filberts in Oregon. The same species might be responsible for the recent findings of “filbertworms” in pears in Washington.
- A set of diagnostic primers for *Cydia latiferreana* was designed and tested with several filbertworm specimens. Once validation is completed, the primers will be included in the *Cydia-Grapholita* diagnostic kit for the Pacific Northwest.
- DNA sequencing indicate that *Grapholita* spp. larvae found in rose-hips (similar to LAW as larvae and to CFW as an adult) is a new, undescribed species (“rose-hip worm”).

2. Apple maggot/snowberry maggot complex.

- A 95%-99% diagnostic system based on PCR-RFLP of a mitochondrial and a nuclear gene was developed to discriminate apple maggot (*Rhagoletis pomonella*) and snowberry maggot (*R. zephyria*).
- Current morphological criteria for discriminating between *Rhagoletis pomonella* and *R. zephyria* require revision because 18% of *R. pomonella* from hawthorns would be misclassified as *R. zephyria*.
- A molecular method to discriminate between rose-hip maggot (*Rhagolettis basiola*) and apple maggot (*R. pomonella*) was developed to allow us to determine the identity of maggot infestations without waiting six months for adult emergence.

3. Spider mites

- Sequences for the COI gene of the mtDNA were collected, aligned, and analyzed for the Pacific Northwest spider mites and a hot spot has been detected for species specific primer design and/or PCR-RFLP diagnosis.

METHODS:

1. Tortricidae internal fruit feeders.

Specimens from the target species were collected from lab colonies, field collections, museum specimens, etc. Our collection included representative specimens from around the world. Existing information on DNA sequences of target and related species were retrieved from NCBI genebank. Mitochondrial COI was selected for the development of the DNA diagnostic tool. Mitochondrial COI gene was amplified by PCR and sequenced for specimens of diverse geographic origin of the target species using universal primers C1-J-1718 and C1-N-2191 (Simon, 1994). DNA sequences were aligned and potential diagnostic regions determined. Several sets of potential diagnostic primers were designed and tested, and reliable and robust combinations were identified. Conventional PCR and real time PCR methods were developed and optimized. The molecular kit was tested with specimens from diverse geographic locations. Methods for quick and reliable DNA extraction from small larvae in alcohol were also developed and optimized.

2. Apple maggot/snowberry maggot complex.

Extensive collections of the target species were made throughout Washington and parts of Oregon. Existing information on DNA sequences of target and related species were retrieved from NCBI genebank. No potential diagnostic genes were found among existing sequences (COI-COII and 16s rDNA). Thus, we sequenced other genes including different region of COI, 12s rDNA, Cytochrome B of the mtDNA and introns for Tubulin 3 and Elongation Factor alpha 1 from the nuclear genome. From these sequences restriction site differences were discovered and PCR-RFLP markers were developed and screened for over 350 flies for COI and elongation factor. Methods for DNA extraction from badly preserved specimens from sticky traps were developed.

3. Spiders mites.

Specimens of *T. urticae* and *P. ulmi* from WA, *T. pacificus* and *T. turkestanii* from CA, and *T. mcdanieli* from Vancouver, WA were collected for analysis. Existing information on DNA sequences of target and related species were retrieved from NCBI genebank. Genbank DNA sequences were aligned and a potential diagnostic PCR-RFLP method designed.

RESULTS AND DISCUSSION

1. Tortricidae internal fruit feeders.

1.1 Molecular diagnosis of the Washington fruit boring complex.

The PCR protocol for diagnosis of the Washington fruit boring complex: codling moth (CM), oriental fruit moth (OFM), lesser apple worm (LAW), and cherry fruitworm (CFW) was successfully developed, validated and transferred to Mexico [Barcenas et al. (2005). J. Econ. Entomol. 98(2): 299-306, <http://www.bioone.org/pdfserv/i0022-0493-098-02-0299.pdf>].

The diagnostic method works as follows. When a living larva of an internal fruit feeding Tortricidae is found during inspection, it is fixed in 70% ethanol, and observed under a microscope. If the larva is in 4th instar or older, it can be identified to species by morphology. The most likely species to be found from Washington exports is codling which pose no quarantine restrictions to Mexico. However, if the larva is younger, it can only be identified as a member of the *Cydia/Grapholita* complex, and the shipment is rejected because the specimen has

the characteristics of a quarantine pest. The specimen is then mailed to the Sanidad Vegetal Diagnostic Laboratory in Mexico City for further analysis. Upon arrival, the taxonomist confirms that it is a tortricid and proceeds to cut the specimen in 3 sections. The anterior and posterior sections are saved as a voucher specimen (since it has characters used for morphological classification) and the middle part is used for DNA extraction. The optimized quick and inexpensive method of Chelex is used to prepare DNA for molecular analysis in 1-2 hours. The DNA of the unknown species is exposed to five primers sets in independent PCR reactions: a positive control where any of the *Cydia/Grapholita* complex would amplify a 330 bp section of the mitochondrial gene COI (CG primers), and the four diagnostic primer sets for CM, OFM, LAW and CFW, which would amplify a 115 bp section of the target species. Only one of the four primer sets will successfully amplify. A negative control with no DNA is also included in the analysis (to test for possible contamination). Example results are summarized in Table 1.

PCR reactions	Negative control	Positive Control	Diagnostic primers			
			Test Species	CG primers with water	CG primers	CM
CM	-	+	+	-	-	-
OFM	-	+	-	+	-	-
LAW	-	+	-	-	+	-
CFW	-	+	-	-	-	+

The method was optimized for both conventional PCR (results in 8-12 hours after DNA extraction) and real time PCR (2-3 hours). Species specific primers were tested with populations as diverse as possible and they are highly robust.

The molecular technique was validated by Enrique Vega (taxonomist), and Dr. Juan P. Martínez (Molecular Biologist and Director of the Laboratorio de Diagnóstico Fitosanitario, SAGARPA, México). They also received training to use the real time PCR protocol. However, since this equipment is not yet available at the Mexican Laboratory, the protocol was adapted for traditional PCR so they can start using it right away.

1.2 Discovery of new species of *Cydia* and *Grapholita* in WA.

DNA sequence analyses during our studies of internal fruit feeders Tortricidae led to the discovery of two new species in Washington: *Grapholita* sp. The rose-hips of multiflora rose (*Rosa* sp.) used in Unruh's studies of habitat modification were found to host a tortricid that resembles LAW as a larvae and CFW as an adult. Specimens of this moth were sequenced and found to be a distinct from both *G. packardi* and *G. prunivora* (Figure 1). We are collaborating with a taxonomist to have this new species, the rose-hip worm, described. We have no evidence that this species attacks apples or other pome fruits.

2003 Background: A moth from Indiana classified by taxonomists as cherry fruit worm (CFW) had a distinct sequence from two other CFW moths from Michigan and Washington (28 out of 420 base pair differences as opposed to 2/420 between WA and MI), suggesting a different race or even a different species. This "CFW" proved identical in DNA sequences to two moths collected in rose-hips in ecological studies in Washington using rose as a host habitat for leafroller parasitoids. The rose-hip form looks like LAW as a larva (but has 33/420 base pair differences from LAW) and the adult resembles CFW (but has 28/420 differences from CFW). We think that this group represents an undescribed species of "rose-hip-worm". We will make more collections and do more sequencing next year to clarify this issue. We find no evidence that this rose form is a pest but it may be mistaken for CFW in traps.

Collections of specimens of the putative new species of *Grapholita* (rose-hip worm) in Washington were made during 2004. A total of 488 specimens from five counties (Okanagan,

Douglas, Kittitas, Yakima and Whitman) were collected in three species of roses (*Rosa woodsii* 96.5%, *R. nutkana* 2.3%, and *R. canina* 1.2%). One third of the specimens were fixed in ethanol 70% as larvae, and the rest are being kept alive waiting for emergence in spring of 2005. Sample larvae and adults will be sent to taxonomist Dr. William Miller, University of Minnesota, for morphological characterization. Hopefully morphological characters will be discovered that could discriminate between this “rose-hip worm”. If they can not be discriminated by morphological characters, it would be easy to design species specific primers if correct diagnosis becomes a quarantine issue, specifically if we find evidence of it in LAW or CFW traps.

Moths will also be compared to specimens of *Grapholita rosana*, a species that is known to attack roses in Europe but has not been previously reported in America. If the Washington specimens key out to this species, and molecular analysis confirm the identity, it would mean that *G. rosana* has been introduced and spread in America. If the moth turns out to be morphologically and or genetically separate from the European rose-hip moth, we can conclude that we discovered a new species of *Grapholita* in Washington based on molecular data. Fortunately, the new species does not seem to be of economic importance to the region.

***Cydia* sp. near *latiferreana*.** According to the literature, *C. latiferreana* is found in filberts (*Corylus* spp.) and in acorns from many oak species (*Quercus* spp.). We acquired specimens from filberts in Oregon and from oaks in Washington and sequenced a segment of the mitochondrial COI. This revealed that the two populations were very distinct species (63/421 bp differences). The species that feeds on oak (*Quercus* spp.) is not *Cydia latiferreana* as previously thought, but a distinct new species that must be described (“oakworm”). Molecular analysis of putative “filbertworms” intercepted in nectarines in California revealed that the DNA sequence is identical to the sequence of the filbertworm collected in filberts in Oregon (Figure 1). Additional specimens have been collected from filberts and oaks in Oregon and additional sequencing will help clarify species limits.

1.2 Expanding the molecular diagnosis kit for the pome fruit-boring complex.

The interception of putative “*Cydia latiferreana*” (filbertworm) in pears in Washington and in nectarines in California underlines the need to include this species in the diagnostic kit for the internal fruit feeders of pome fruits. A set of diagnostic primers for *Cydia latiferreana* was designed and tested with several filbertworm specimens but additional sequencing of specimens of diverse geographic origin from known host plants is necessary to complete the validation. Once this validation is completed, the primers will be included in the *Cydia-Grapholita* diagnostic kit. In addition, it is urgent to determine with DNA technology if claims of “filbertworms” attacking pears in Washington are valid or mistaken identifications.

2. Apple maggot and Snowberry maggot flies DNA diagnostics

These species were not considered in our original proposal but their study was undertaken after evidence of apple maggot (AM) establishment in Kittitas County. Since first detected in 1979 in Oregon, the AM *Rhagoletis pomonella*, has spread and infested apples in many parts of the Pacific Northwest. It is now considered established in the 19 Washington counties where tree fruits are NOT abundantly produced, but recently has been found in Kittitas (2003) and Yakima (2004) counties, creating quarantines in the main apple production areas. AM flies caught near apple orchards pose a quarantine problem for export to California as well as virtually all of our export markets abroad and may require frequent OP application and intensive monitoring for certification. This monitoring is performed with sticky traps, however, *R. pomonella* flies are morphologically near identical to its sister species *Rhagoletis zephyria* (snowberry maggot, SBM), a native species that feeds on snowberry, not on apples, but which is abundant in these problem areas. Current ID methods use male genitalia shape and female ovipositor length to discriminate these species. Adult female flies are distinguished by the length of the ovipositor, *R. zephyria* 0.9 mm or less, *R. pomonella* 1.0 mm or more. Flies with ovipositors between 0.9 and 1.0 mm are considered to fall into a “gray area”.

Additional measure to discriminate these species may include measuring wing band ratio and

wing length, but these characters are also continuous and overlap between species. Adult males are more reliably separated by genital structure; *R. pomonella* has a parallel surstyli with broad surfaces facing directly lateral, while *R. zephyria* has divergent surstyli with broad surfaces arranged obliquely. Unfortunately, this character also has a continuous distribution and there are specimens that fall in a “gray area” as well (Westcott, 1982; and Mike Klaus, (pers. comm.). Depending in the collection area, specimens in the gray area could represent 0.1 to 11% of the flies. Our objective was to find molecular markers that distinguish between the apple maggot and the closely related snowberry maggot.

DNA sequencing efforts toward understanding the evolution of the *Rhagoletis pomonella* species group have shown that *R. pomonella* and *R. zephyria* have identical or almost identical haplotype/genotypes for most genes analyzed. We explored the possibilities of using the few polymorphisms found by us and others (Table 2) for molecular diagnosis.

Mitochondrial genes:

- a) McPherson and Han (1997) reported one base-pair difference between *R. pomonella* and *R. zephyria* in a 460bp long fragment of the mitochondrial 16s rDNA gene. We found that we can use RFLP-PCR with primers LR-J-12887 and LR-N-13398 and AluI to distinguish between the two forms. However, further sequencing by us proved that the polymorphism is shared in both species and hence can not be used for diagnosis.
- b) We sequenced a 780 bp fragment of mitochondrial CytB and found one base-pair difference between *R. pomonella* and *R. zephyria*. However this is not enough to design reliable species specific primers and there is no restriction enzyme that specifically cuts in this region. Therefore, the gene was discarded as potential for diagnosis.
- c) We found 3 base-pair differences in a 526bp mitochondrial COI gene, one of which can be resolved by PCR-RFLP with primers C1-J-1718 and C1-N-2191 and AluI. Screening of specimens collected throughout Washington revealed four haplotypes that can be discerned with AluI, two species-specific (C for *zephyria* and D for *pomonella*) and two shared (Table 3). Furthermore, we found that the distribution of these haplotypes in *R. zephyria* is geographic dependent, with the population of western and eastern Washington being very different. Central Washington is intermediate but closer to the western population. This marker showed potential for diagnosis if combined with another marker.

Nuclear genes:

- a) We discovered a pseudo gene of mitochondrial COI that seems to be exclusive of *Rhagoletis pomonella*. Pseudo genes are non-functional nuclear copies of genes that because of their lack of function allow a more rapid evolution and diversification of gene sequences. When *R. pomonella* DNA is exposed to primers C1-J-1718 and C1-N-2191 two products become evident, the expected 525bp fragment of mitochondrial COI, and an extra 360bp fragment. DNA sequencing of the latter aligned with COI but showed 2 deletions 134 and 31 bp long and 25 individual base-pair differences. The location of this putative pseudogene is most likely nuclear. We obtained PCR products for almost 300 snowberry maggots and none showed evidence of the pseudogene. In contrast, around 70% of the PCR products of 120 apple maggots show the pseudogene. We developed pseudogene specific primers and validated its presence but have not been able to amplify it in all the *R. pomonella*. Further work needs to be done to assess the utility of this character.
- b) We sequenced 350bp of the intron of Tubulin-3 and found only one difference, which can not be resolved by any known restriction enzyme. Not useful for diagnosis.
- c) We sequenced 180bp of nuclear intron EF-1A and found one difference, which can be resolved by the restriction enzyme S_{Cr}F1 (allele 1 and 2). We screened the same individuals

as above and found that allele 1 is the most common in *R. pomonella* while allele 2 is almost the unique in *R. zephyria* (Table 3), which makes it very promising for diagnosis.

Development and validation of DNA diagnostics.

Although neither COI nor EF1A can be used alone for a reliable molecular diagnosis, the two of them combined make a powerful tool. If we consider that diagnosis is economically critical in areas where the frequency of AM is 1% or less of the trap catches, with the combination of the two genes we can determine accurately 96.7% of the flies in the gray area, while 3.3% remain unknown. Of those still unknown, 98.7% would be SBM. The hypothetical probability of error is very low: 1 in 2,300 SBM flies would be mistakenly called AM; and 1 in 50,000 AM flies would be called SBM.

Given the geographic distribution of the COI haplotypes in SBM, the power of the molecular diagnosis is different for each region: 95.1% for western, 96.6 for central, and 99.3% for eastern Washington. The overall hypothetical power is 96.7%.

In order to validate the molecular tool, we recorded ovipositor lengths for 40 female flies reared out from apples, pears, plums and hawthorns. Of these, only 70% had ovipositor lengths larger than 1mm, 12% would have been considered in the morphological gray area (0.9-1.00 mm), and 18% would have been misidentified as *R. zephyria* (0.76 to 0.9). This highlights the urgent need to revise the morphological key for the identification of these flies.

The molecular technique has been validated with flies reared out from known hosts under the assumption that any fly reared out of snowberries is *R. zephyria* and any fly reared out of apple, pears, cotoneaster, or hawthorns is *R. pomonella*. In the case of 249 *R. zephyria* flies reared out of snowberries, 97.2% were identified correctly, while 2.8% remained unknown. None of them was misidentified. In the case of 107 *R. pomonella* reared out of apple, hawthorns, pears and plums, 93.5% was correctly identified as AMs, 5.6% remained unknown, and 1.7% was misidentified as *R. zephyria*. The frequency of misidentification was higher than expected. What is most surprising is that the “misidentified” flies had ovipositor lengths of 0.76 and 0.84 mm, which would have also classified them as *R. zephyria*. This raises the question: is *R. zephyria* occasionally laying eggs in hawthorns?

In a study based on allozyme and mitochondrial DNA variation in 1105 flies collected throughout the Northern US, Feder et al. (1999) found evidence that two flies reared from snowberries and one fly reared from hawthorns had genotypes indicative of them being *R. pomonella* and *R. zephyria*, respectively (the opposite genotypes expected). He suggested that AM and SBM adults may therefore occasionally frequent each others host plant, providing the opportunity for hybridization. He also found four flies collected from hawthorns and one from snowberries that had genotypes that made them likely to be F1 hybrids. In our own studies with COI and EF1A, the “intermediate” genotypes (22A and 12B) were observed in 5.6% of the flies while only 4.2% were expected. This might also be indicative of some level of hybridization. More detailed statistical analysis will be performed to test these hypotheses.

The lack of a fully diagnostic method could be the result of genetic introgression between the two species, in which case, it is impossible to have a 100% diagnostic method. This hypothesis will be tested by genetic analysis of population of *R. zephyria* in areas free of *R. pomonella* versus areas where the two species occur in sympatry. A new proposal is being submitted to continue with this research.

***Rhagoletis pomonella* versus *R. basiola*.** A molecular method was developed to discriminate between the apple maggot and the rose-hip maggot. This work was performed to aid Dr. Wee Yee (USDA-ARS-YARL) in discriminating these flies as pupae without having to wait several months for emergence and positive identification. Given that these flies are phylogenetically well separated, the diagnosis is easy and straightforward. Real time PCR melting profiles of PCR products of COI (primers C1-J-1718 and C1-N-2191) and COII (primers C1-J-2792 and C1-N-3287) genes are

distinct; with *T. basiola* showing lower Tm's (the temperature at which 50% of the PCR product melts). The method was validated with rose-hip maggots collected in six Washington counties (Okanagan, Chelan, Kittitas, Yakima, Stevens and Whitman). If no real time PCR equipment is available, the same conventional PCR-RFLP method used for the *R. pomonella/zephyria* complex will discriminate between the three species (*pomonella*, *zephyria* and *basiola*).

3. Spiders mites.

Our work focused in literature and gene bank sequences. A 390 bp fragment of COI has been sequenced by others for 33 populations of *T. urticae* from around the world, *T. mcdanieli*, *T. pacificus*, *Paninychus ulmi* and *Eotetranychus carpini*, all known to occur in the Pacific Northwest. As with the internal fruit feeding moths, there is a hot spot in CO1 (base pairs 2310-2336) that looks promising, especially because it is conserved in *T. urticae* (only one bp polymorphic among 33 mites populations from around the world). Future work should start with extensive collections of mites for DNA sequencing to ensure differences are conserved and to validate diagnostic primers or PCR-RFLP methods.

BUDGET:

Project Duration: 3 years (2002-2005)

Total Cost 92,200 (reduced from \$100,400 originally requested)

Item	2002	2003	2004 ¹
Salaries	20,000 ²	27,000 ²	21,500
Benefits	6,000	8,200	6,500
Supplies	2,000	1,000	1,000
Total	28,000	36,200	28,000

¹ Budget was reduced in year 3. ² Salary for Nina Bárcenas. WSU.

Table 2. DNA sequencing in *Rhagoletis pomonella* (Rpo) and *R. zephyria* (Rze), showing number of sequences collected and their source. Also shown are number of base-pair (bp) differences observed between these two very closely related species.

Mitochondrial (Mt) or nuclear (N) DNA	Gene (region)	Fragm. Size (bp)	# Rpo	# Rze	Bp diff.	Reference
Mt	COI (1718-2191)	501	4	6	3-5	herein
Mt	COI (2183-2329)	192	2	2	0	herein
Mt	COI/COII (2792-3722)	946	35	10	0	Smith et al.'02
Mt	CytB (10612-11367)	780	2	2	1	herein
Mt	12s rDNA (14233-14594)	405	2	2	0	herein
Mt	16s rDNA (12920-13760)	848	2	2	1	McPherson&Han'97
N	G6PDH	460	1	1	5	Soto-Adames'94
N	Intron EF1A	180	2	2	1	herein
N	Intron Tub3	350	2	2	1	herein

Table 3. Haplotype and gene frequencies for the COI mitochondrial gene and the intron-EF1A among populations of *Rhagoletis pomonella* and *R. zephyria* in Washington..

Species (population)	n	COI mitochondrial haplotypes				Intron EF1A alleles	
		A	B	C	D	1	2
<i>R. pomonella</i>	125	0.822	0.150	0.0	0.028	0.883	0.117
<i>R. zephyria</i> (overall)	250	0.004	0.735	0.261	0	0.020	0.980
<i>R. zephyria</i> Western	64	0.016	0.984	0	0	0.016	0.984
<i>R. zephyria</i> Central	133	0	0.894	0.106	0	0.019	0.981
<i>R. zephyria</i> Eastern	53	0	0.038	0.962	0	0.128	0.972

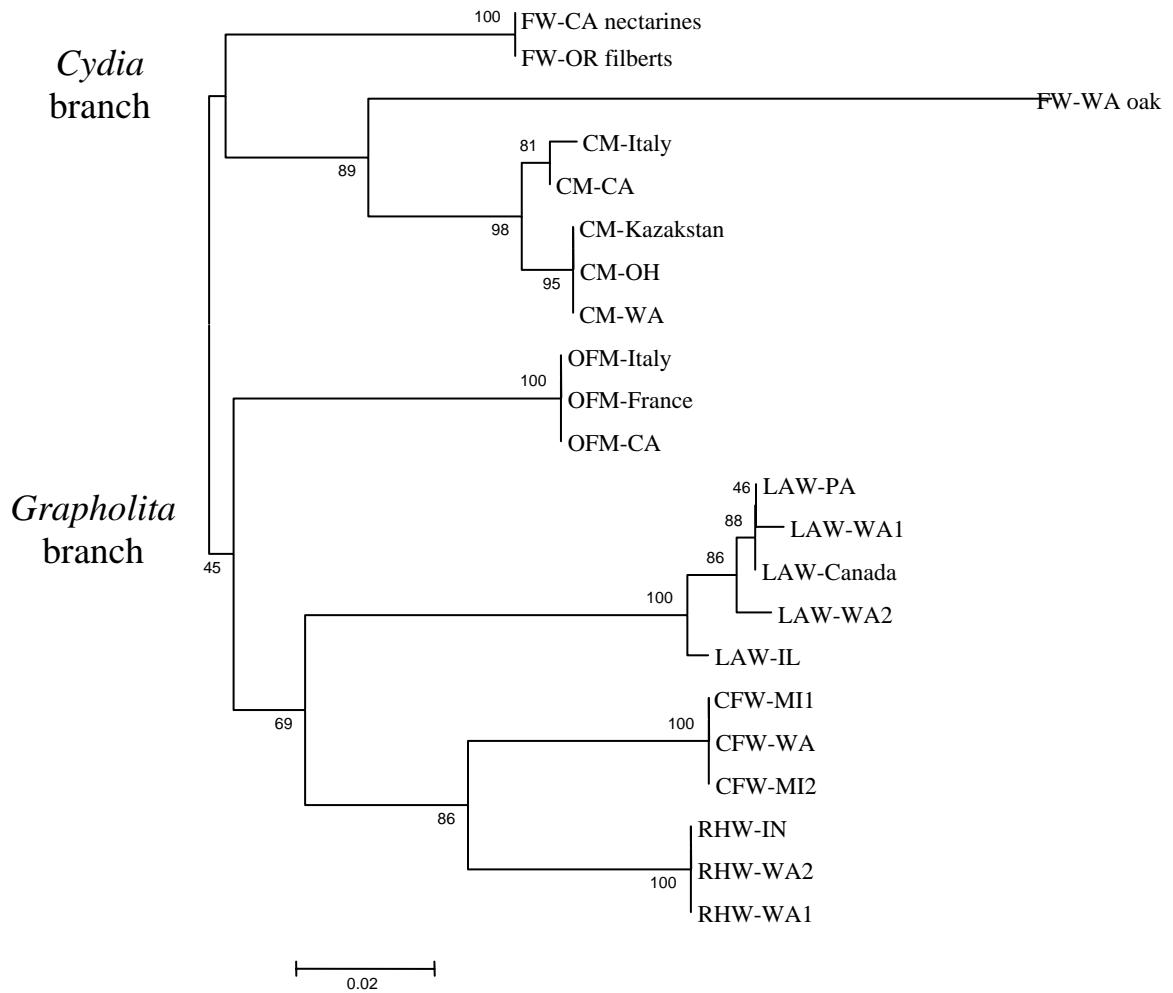


Figure 1. Neighbor-joining tree depicting DNA sequence differences among *Grapholita* and *Cydia* of export concern and other close relatives. Clusters of populations in the same species are highly supported by reliability tests (bootstrap). Note that filbertworms from filberts are distinct from “filbertworms” from oaks. FW: filbertworm *Cydia latiferreana* (filbert) or *C. near latiferreana* (oak); CM Codling moth *C. pomonella*; OFM oriental fruit moth *Grapholita molesta*, LAW: lesser apple worm *Grapholita prunivora*; RHW: rose-hip worm *Grapholita* sp.