CONTINUING (Final) REPORT **YEAR 3/3**

TITLE: Development of Genetic Markers to Identify Problematic Pests in Deciduous Fruits Intercepted at Foreign Quarantine Inspection Stations

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OBJECTIVES (2005):

- Complete development and validation of molecular protocol to discriminate between apple maggot (*Rhagoletis pomonella*) and snowberry maggot (*R. zephyria*) captured in traps for accurate monitoring and quarantine actions.
- Develop DNA extraction method suitable for flies captured in traps (conservation of specimens far from optimum: the fly dries out and it is immersed in a sticky fluid).
- Complete characterization of putative new *Grapholita* species in roses (rose-hip worm).

Significant Findings:

- A 90% diagnostic system based on PCR-RFLP of a mitochondrial and a nuclear gene has been developed to discriminate *Rhagoletis pomonella* and *R. zephyria*.
- A pseudogene of mitochondrial COI that seems to be exclusive of *R. pomonella* has been discovered.
- Molecular diagnosis between *Rhagolettis basiola* (rose-hip maggot) and *R. pomonella* could be easily performed by amplification of mitochondrial COI or COII genes by real time PCR, or PCR-RFLP with conventional PCR.

Methods for 2005:

1. The simple and inexpensive method of Chelex will be tested to obtain DNA from legs and or heads of flies from sticky traps. If this does not work, we will try other methods that have been successful for poorly conserved DNA.
2. Single Stranded Conformational Polymorphism (SSCP) will be tested as an alternative cheaper and faster method to detect polymorphism in the diagnostic genes (obviating restrictions).
3. Characterize and optimize detection of the newly discovered pseudogene of mitochondrial COI.

Results and Discussion (Based on 2004 objectives in bold):

1. **Complete discovery and development of molecular protocol to discriminate between apple maggot (*Rhagoletis pomonella*) and snowberry maggot (*R. zephyria*) captured in traps for accurate monitoring and quarantine actions**.

   Backgroud: The different species of flies that are captured in traps can be classified by the wing pattern. However, *R. pomonella* and *R. zephyria* are identical and need to be taken to the lab for microscopic analysis. Adult female flies are distinguished by the size of the ovipositor, *R. zephyria* 0.9 mm or less, *R. pomonella* 1.0 mm or more. Flies with ovipositors that fall between 0.9 and 1.0 are considered to fall in a "gray area". A definitive ID is attempted by measuring wing band ratio and wing length, but these characters may also fall in a gray area. Adult males are separated by genital structure; *R. pomonella* has a parallel surstyli with broad surfaces facing directly lateral, *R zephyria* has divergent surstyli witn broad surfaces arranged obliquely. Similarly, this character 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. A DNA diagnostic protocol that could discriminate between the species in the "gray area" is being developed.

   Follow up on the sequence data presented last year:

   1) McPheron 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 Alu1 to distinguish between the two forms. However, further sequencing by us proved that the polymorphism is interspecific and hence can not be used for diagnosis.

   2) We found one base-pair difference between *R. pomonella* and *R. zephyria* in a 780 bp fragment of mitochondrial CytB, 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.

   3) 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. Analysis of specimens from Skamania, Cowlitz, Pierce, Thurston and Clark counties (*R. pomonella*) and from Pierce, Chelan, Kittitas and Yakima counties (*R. zephiria*) showed three haplotypes, only two of which are diagnostic (Table 1).

COI Haplotype	A	B	C
R. pomonella N=24	0.75	0.25	0.00
R. zephyria N=20	0.00	0.90	0.10

From this work, it became obvious that the two species are extremely similar and we needed to sequence more diverse genes, so we changed our target to introns of nuclear genes.

   4) 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.

   5) We sequenced 180bp of nuclear intron EF-1A and found one difference, which can be resolved by the restriction enzyme SCrF1. We screened the same individuals as above and found that *R. pomonella* is monomorphic for genotype 1 but share the character with

10% of *R. zephyria*. Genotype 2 is exclusive of *R. zephyria* and can be used for diagnosis (Table 2).

Intron EF1A genotype	1	2
R. pomonella N=24	1.00	0.00
R. zephyria N=20	0.10	0.90

If we consider that diagnosis is economically critical in areas where the frequency of *R. pomonella* is 10% or less of the trap catches, this means that with the combination of the two genes we can determine accurately 88.5% to 90% of the flies in the gray area, while the rest remains as unknown, with a probability of 78-100% of being *R. zephyria*. The test would not allow any *R. pomonella* to go undetected .

We recently discovered a pseudo gene of mitochondrial COI that seems to be exclusive of *Rhagoletis pomonella*. Pseudo genes are non-functional 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. We obtained PCR products for almost 300 snowberry maggots and none showed evidence of the pseudo gene. In contrast, most of the PCR products of 60 apple maggots show the pseudo gene. We developed pseudo gene 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.

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.

To validate this protocol and to estimate the frequencies of the mitochondrial and nuclear genes in different geographic areas throughout Washington, we made extensive collections of both species (Table 3). We will wait until the emergence of adult flies in late winter (February 2005) in order to correlate host plant, adult morphology and molecular data. This will allow us to validate our protocols with flies from the "gray area" and look for evidence of hybridization.

Table 3. Individuals of Rhagoletis to be characterized molecularly from a larger collection from Washington and Oregon.

Species	Region	County	Quarantine?	2003	2004
				n	
R. zephyria	West	Whatcom	YES	0	8
	West	Skagit	YES	0	13
	West	Snohomish	YES	0	20
	West	King	YES	0	6
	West	Pierce	YES	4	0
	Central	Chelan	NO	2	17
	Central	Kittitas	YES	3	20
	Central	Yakima	NO	11	80
	East	Stevens	NO	0	20
	East	Spokane	YES	0	10
	East	Columbia	NO	0	9
Total				20	203

R. pomonella	West	Whatcom	YES	0	10
	West	Pierce	YES	5	0
	West	Thurston	YES	2	0
	West	Cowlitz	YES	5	0
	West	Clark	YES	4	20
	West	Skamania	YES	8	0
	Central	Kittitas	YES	0	20
	Central	Yakima	NO	0	40
	East	Spokane	YES	0	20
	Oregon	Pendelton			6
Total				24	86

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. 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 conventional PCR it is necessary to do PCR-RFLP, for which we suggest Alu1.

2. **Continue collections of spider mites from different locations, design primers and test reliability and robustness of molecular protocols to discriminate among mites of quarantine importance, mainly Pacific spider mite (*Tetranychus pacificus*) and McDaniel spider mite (*T. mcdanieli*).**

We found a “hot spot” in mitochondrial gene COI suitable to design species-specific primers but were unable to continue the project due to the lack of specimens from different geographic populations to validate the methodology. We have recently been made aware of a source

3. **Acquire samples of world lepidoptera attacking apple, pear, and cherry, sequence mtDNA and develop protocols to identify them (emphasis on exotic fruit boring species).**

No further work was done due to lack of specimens from exotic species. However, the possible discovery of a new species of *Grapholita* in Washington was pursued as follows:

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.

BUDGET

$ 0.0

Current funding extends through July of 2005 and will be enough to complete the 2005 goals, so no further funding is being requested for this project. If there is no objection by the Commission, the poster and oral presentation will be given as a continuing project, and the final comprehensive report will be presented next year.