FINAL REPORT **DURATION: 1 YEAR**

Project Title: DNA and morphometric diagnostics for apple and snowberry maggot flies

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Total Project Request: **Year 1:** $25,000

Other funding Sources: none

Budget 1:

Organization: USDA-ARS	**Contract Administrator:** Bobbie Bobango		
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Item	**2008**		
Salaries	0		
Benefits	0		
Wages	$12,700		
Benefits	$1,270		
Equipment	0		
Supplies	$800		
Travel	$230		
Miscellaneous	0		
Total	$15,000		

Budget 2:

Organization: Univ. Notre Dame	**Contract Administrator:** Rick Hilliard		
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Item	**2008**		
Salaries			
Benefits			
Wages			
Benefits			
Equipment			
Supplies	$9,000		
Travel	$1,000		
Miscellaneous	0		
Total	$10,000		

RECAP OF ORIGINAL OBJECTIVES

1. Increase sample sizes for morphometric diagnostics between apple and snowberry maggot.
2. Determine if host races of apple maggot exist in Washington using microsatellite variation.

SIGNIFICANT FINDINGS

● Wing shape identified female apple maggot flies with 97% accuracy.

● Wing shape misidentified 3% of apple maggot flies as snowberry maggots flies.

● Wing shape identified 97–100% of female snowberry maggot flies.

● Using wing shape, apple maggot flies are more likely to be misidentified as snowberry maggot flies than are snowberry maggot flies to be misidentified as apple maggot flies.

● Wing shape analysis correctly identified 100% of flies whose identities were questionable based on ovipositor lengths and therefore is a useful method for pest detection and management.

● Different populations of apple maggot flies may have slightly different wing shapes, but apple maggot flies almost always separate out from snowberry maggot flies.

● Microsatellite genetic markers suggest that significant differences may exist between flies reared from apple and black hawthorns in Washington, and that host races of the flies exist.

● None of eight loci analyzed possessed a high frequency allele for the Washington/Oregon fly populations at any of the eight microsatellites that was also not found in the East, implying the eastern and western apple maggot populations are fairly closely genetically related.

● Microsatellite genetic markers suggest that significant differences between apple maggot and snowberry maggot flies in Washington may are detectable.

RESULTS AND DISCUSSION

Objective 1. Increase Sample Sizes for Morphometric Diagnostics Between Apple and Snowberry Maggot.

More flies were added to the analyses that were started in 2007, for totals of 140 female apple maggots and 71 snowberry maggot flies. Addition of these flies resulted in more rigorous results than those obtained in 2007, and in more solid conclusions.

Visual inspection indicated that the wing of the female apple maggot fly is longer and tapers more at the end than that of the snowberry maggot fly, which is more rounded at the tip and stubbier in general appearance (Fig. 1). (Wings of male flies are similar.) However, there are variations in wing shape. For analyzing variation in wing shapes, 18 landmarks were added to the wings of the flies (Fig. 1), an increase from the 14 utilized in 2007, and subjected to canonical variates analysis (CVA). CVA separated western WA flies into two groups, one for apple maggot and one for snowberry maggot fly (Fig. 2). Based on wing shape alone, the assignments test from CVA correctly identified 97% of 140 apple maggots, and correctly identified 100% of 71 snowberry maggots (Table 1). (Central WA flies from black hawthorn were not included because of low sample size.)

When CVA was performed on different fly groups based on host fruit and collection area, including from central WA (Fig. 3), there was evidence that wing shapes of some populations of apple maggot flies differed from one another and that wings of the two populations of snowberry maggot also differed. The assignments test from CVA misclassified many apple maggot flies across groups, but 95% of apple maggots were correctly identified to species, and 97% of snowberry maggots were correctly identified to species (Table 2). Addition of flies to an analysis can change the outcome because variables from the different observations are interrelated.

To test the validity of CVA of wing shape, wings of flies from apple and hawthorns that had intermediate ovipositor lengths (and therefore cannot be distinguished as apple or snowberry maggot flies without host information) were analyzed. A program that compares known flies with unknowns was used for analysis. The program classified unknown specimens into one or the other group based on wing shape variables. The 18 apple maggots entered as "unknowns" were all grouped with apple maggots (Fig. 4) and the assignments test correctly identified 100% of them (Table 3). Of the known apple and snowberry maggots, 99% and 100%, respectively, were correctly identified.

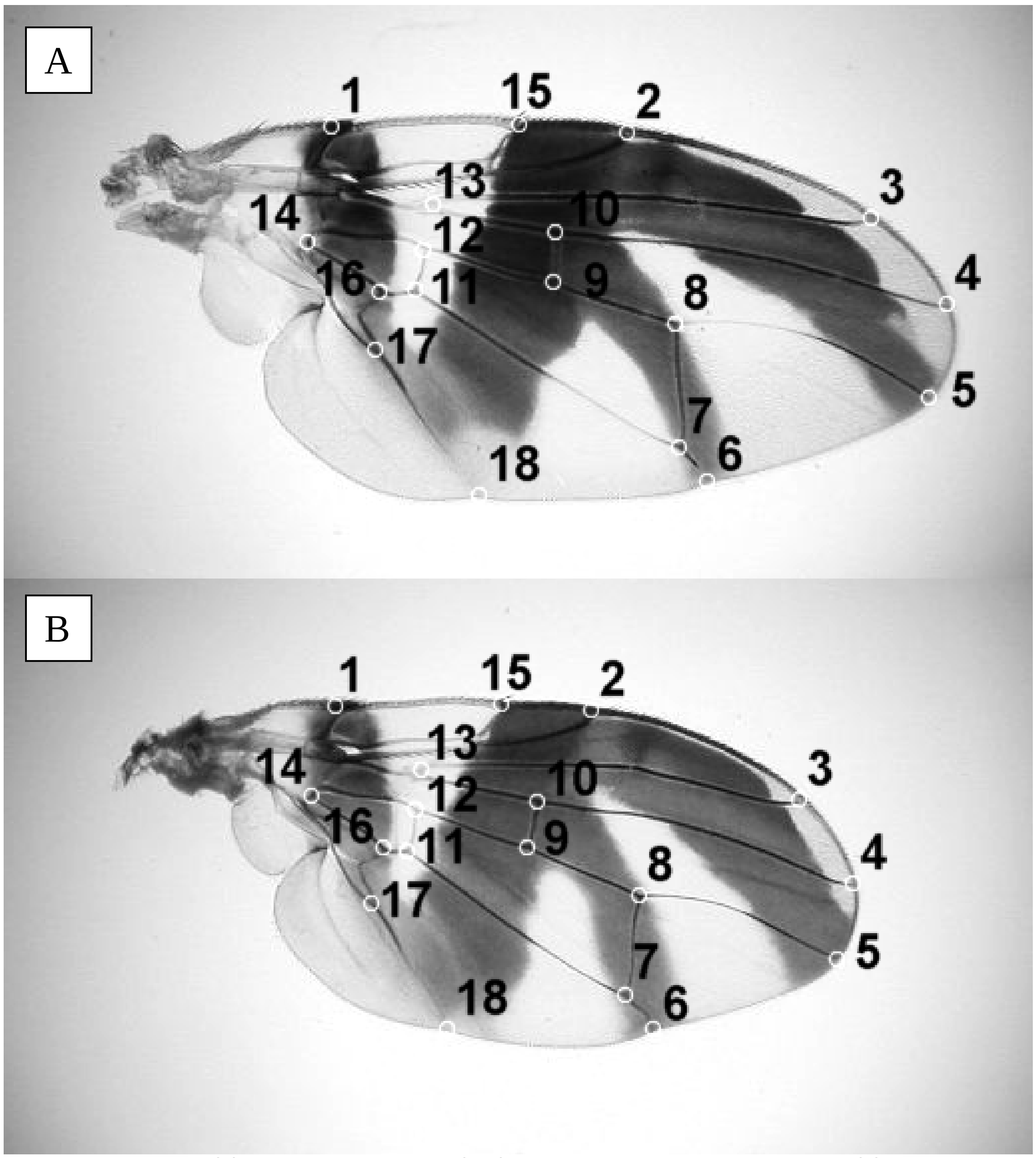

Fig. 1. (A) Wing of female apple maggot fly from black hawthorn; (B) Wing of female snowberry maggot fly from snowberry. Numbers are landmark positions on the wings.

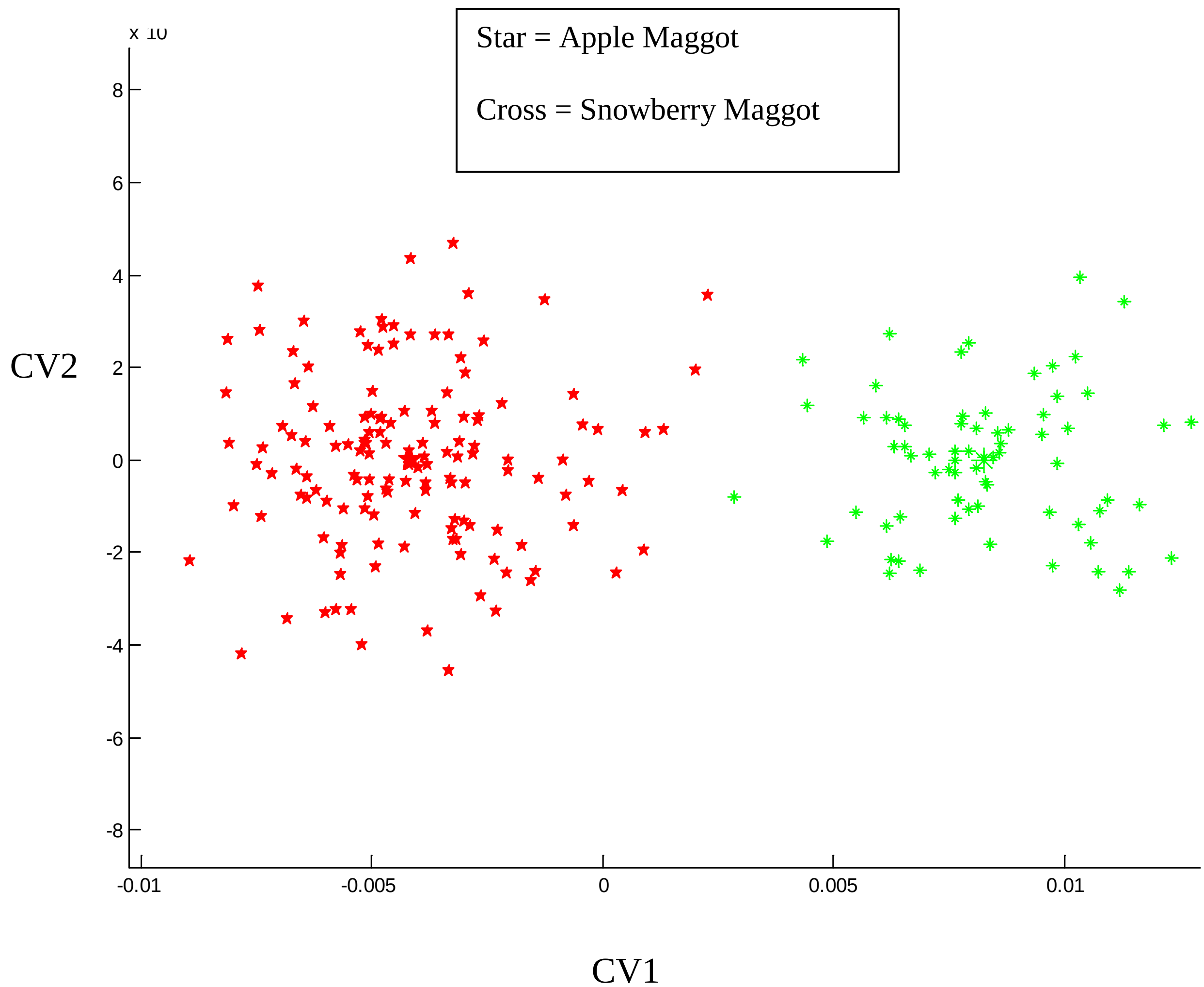

Fig. 2. Separation of female apple maggot and snowberry maggot flies into two groups based on CVA of wing shape. Flies from different hosts and from different sites were combined.

Table 1. Assignments test (numbers, % in parentheses) based on CVA of all WA apple and snowberry maggot flies (jackknifed data). Misclassified flies in bold.

	Classified as:		
Known Species	Apple Maggot	Snowberry Maggot	Totals
Apple Maggot	136 (97)	**4 (3)**	140
Snowberry Maggot	0 (0)	71 (100)	71

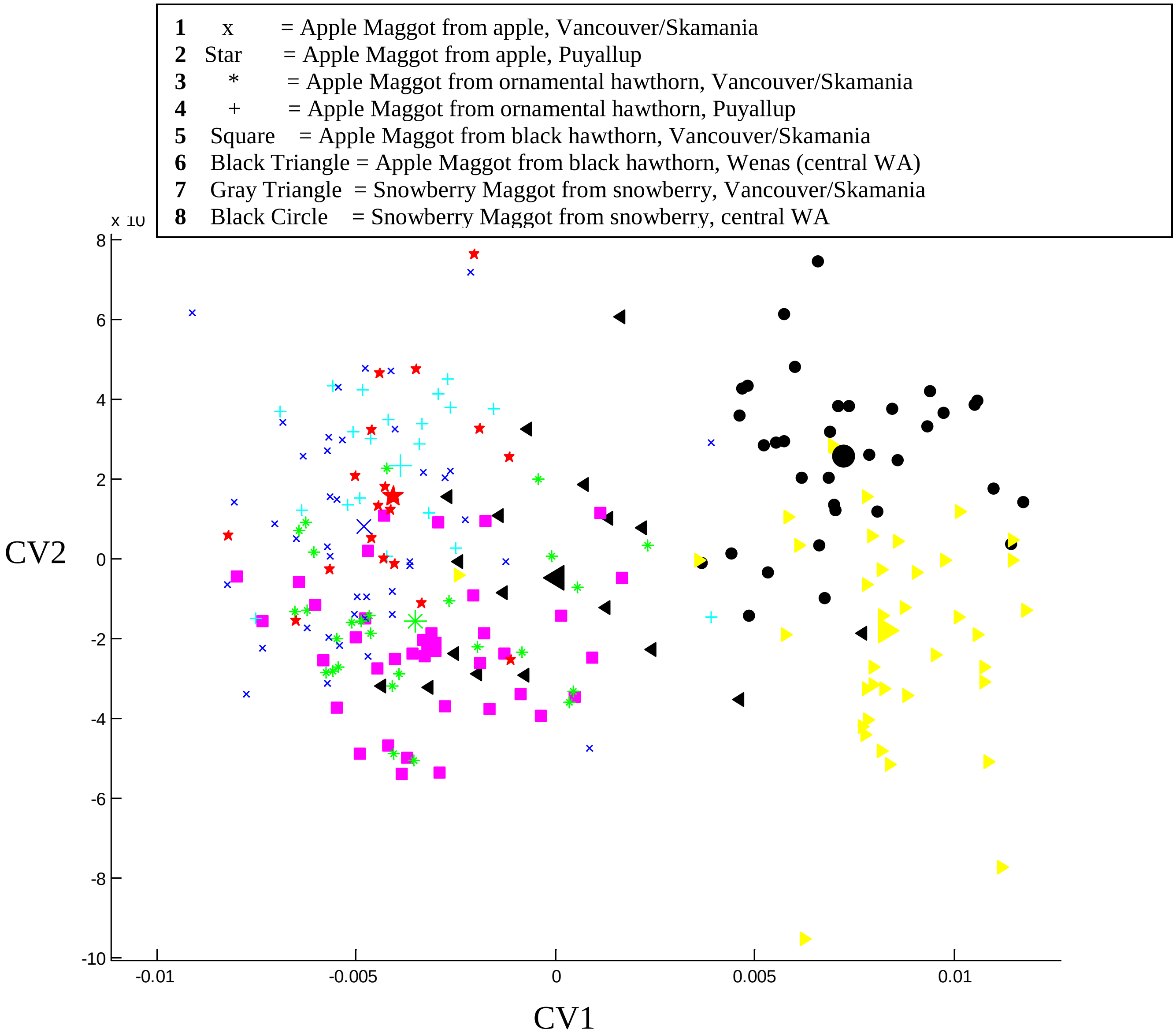

Fig. 3. Separation of female apple maggot and snowberry maggot flies from different host fruit and collection areas in Washington.

Table 2. Assignments test (numbers, % inside parentheses) based on CVA of 8 groups of apple and snowberry maggot flies (jackknifed data). Misclassified flies in bold.

Known Group	Classified as:								
	1, AM	2, AM	3, AM	4, AM	5, AM	6, AM	7, SB	8, SB	Totals
1, AM	16 (38)	5 (12)	4 (10)	7 (17)	6 (14)	3 (7)	**1 (2)**	0	42
2, AM	3 (17)	5 (28)	3 (17)	2 (11)	3 (17)	2 (11)	0	0	18
3, AM	6 (22)	0	5 (18)	3 (11)	10 (37)	2 (7)	0	**1 (4)**	27
4, AM	4 (20)	3 (15)	2 (10)	10 (50)	0	0	0	**1 (5)**	20
5, AM	3 (8)	2 (5)	10 (29)	2 (5)	15 (44)	2 (5)	0	**2 (5)**	34
6, AM	0	2 (11)	1 (6)	1 (6)	1 (6)	10 (55)	**2 (10)**	**1 (6)**	18
7, SB	0	0	0	0	**1 (3)**	**1 (3)**	28 (73)	8 (21)	38
8, SB	0	0	0	0	0	0	9 (26)	25 (74)	34

Numbers of 1–8 same as key in Figure 3; AM, apple maggot; SB, snowberry maggot.

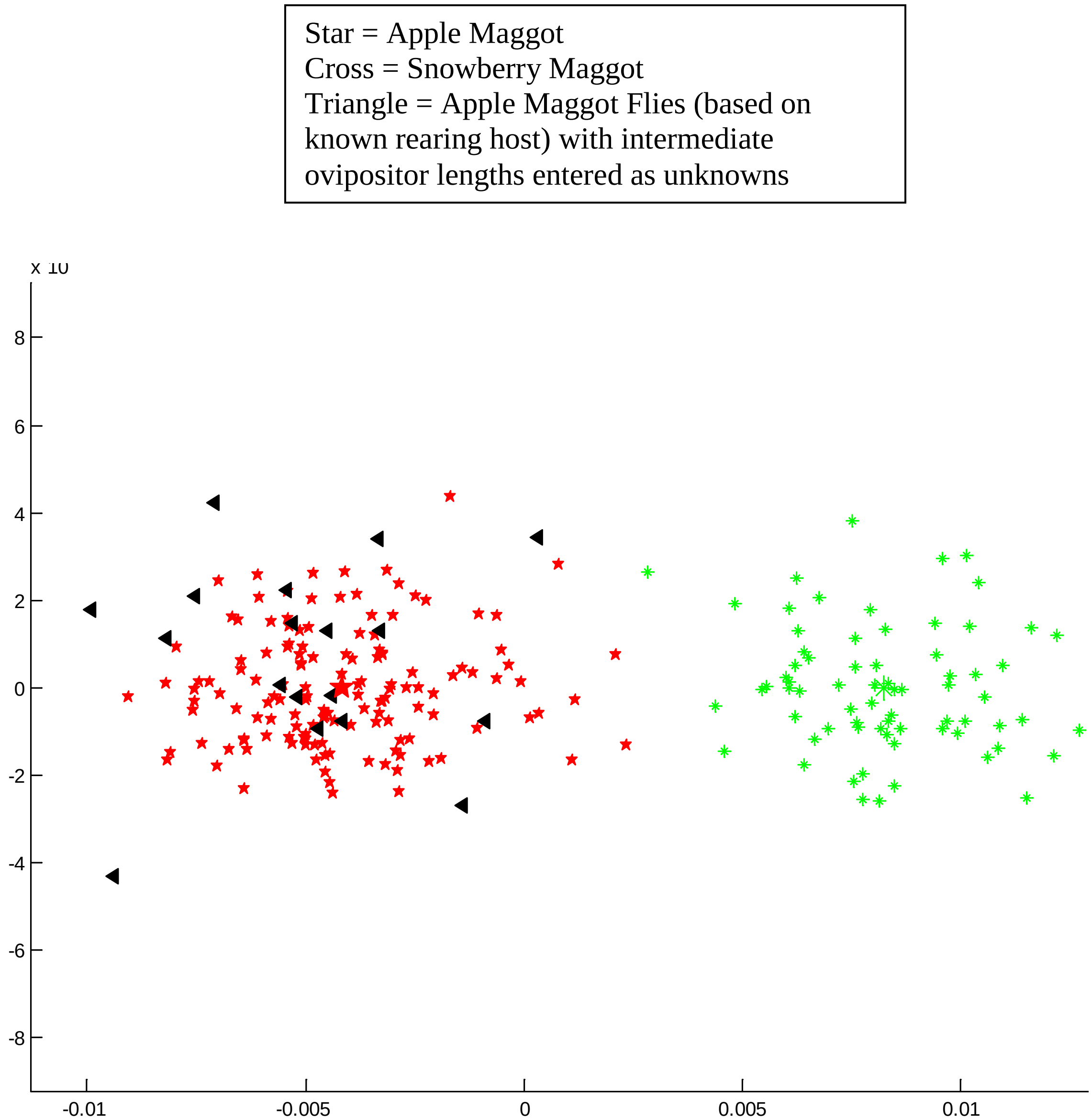

Fig. 4. Classification of 18 female apple maggots (triangles) with intermediate ovipositor lengths into the apple maggot group based on wing shape analysis. Stars, known apple maggot; asterisks, known snowberry maggot.

Table 3. Assignments test (numbers, % inside parentheses) based on CVA of 18 apple maggots called 'unknown flies' (intermediate ovipositor length) into known groups. Misclassified flies in bold.

	Classified as:		
Actual Species	Apple Maggot	Snowberry Maggot	Totals
Apple Maggot	138 (99)	**2 (1)**	140
Snowberry Maggot	0	71 (100)	71
'Unknown Flies'	18 (100)	0	18

Overall results of the wing shape analyses reveal that wing shape is a good character to separate the species. Wings of the two species usually have different shapes, with only about a 3% overlap, which is an improvement over the use of older criteria. In practice, a wing of a questionable fly with intermediate ovipositor length can be digitized with landmarks and entered as an observation in a 'training data set' generated from known flies. The data set can be saved on a computer and used repeatedly for classification purposes. Based on the wing shapes of the other flies, the shape of the unknown is compared and is then placed into one of the known groups by the computer.

Wing shape is good at classifying flies, but a goal should be to eliminate even a 3% probability of misdiagnosis, which might be achieved if shapes of other structures are included in the identification process. Preliminary work in 2008 showed that in addition to wing shape, ovipositor shape may also differ between species. Furthermore, the shape of the male genitalia (specifically the claspers) may be diagnostic of species. Use of a combination of measures, including wing shape, should enable us to positively identify virtually all problematic flies.

Objective 2. Determine if host races of apple maggot exist in Washington using microsatellite variation.

In work partially funded by the WTFRC in 2007 and 2008, we found that microsatellite genetic markers suggest that significant differences may exist between flies reared from apple and hawthorns in Washington, as well as snowberries. We found that all of the 80 PCR primers developed to amplify microsatellite loci for eastern populations of apple maggot, also worked for western apple maggot flies from Washington state. A subset of eight loci has now been scored for approximately 30 flies each collected from two sympatric apple, black hawthorn, ornamental hawthorn and snowberry sites in the Vancouver/Portland area in Happy Valley (Skamania County) and Devine (Clark County) (These eight loci are designated p71, p50, p80, p37, p27, p11, p29, and p60). Six of the eight loci (all except p11 and p29) displayed significant allele frequency differences among the apple and the hawthorn populations ranging on the order of from 10 to 25%; results from four representative loci are shown in **Table 4.** These data are consistent with the existence of host races in the state. Washington flies from the two sites did not show substantial genetic differences from flies from the eastern United States (Illinois, Indiana, and Michigan). None of the eight loci possessed a high frequency allele for the Washington fly populations at any of the eight microsatellites that was also not found in the East. This result implies that the eastern and western apple maggot populations may be fairly closely genetically related. At face value, these current findings are consistent with an introduction of the apple maggot fly from the East to the West. However, more genetic screening from additional loci is needed to confirm the preliminary data.

Table 4. Allele frequencies of four representative microsatellites (gene loci) of two apple maggot populations from apple and black hawthorn and one from snowberry in Washington. Most common allele within a population is in **BOLD**

		Home Valley Population		Devine Site Population		Home Valley
		Apple Maggot		Apple Maggot		Snowberry M.
Locus	Allele No.	Apple	Black Haw	Apple	Black Haw	Snowberry
p29	1	0	0	0	0	0
	2	0	0.0385	0.1429	0.1304	0
	3	0.0227	0	0	0	0
	4	0	0.0769	0.0714	0.0217	0
	5	0	0	0	0	0
	6	0.1818	0.1154	**0.2857**	**0.3696**	0.1250
	7	0	0	0	0	0
	8	0.0227	0.0769	0	0	0
	9	0.1364	0.2308	0.0952	0.0652	0.1250

	10	0	0	0	0.0435	0
	11	**0.3409**	**0.4231**	0.2381	0.1304	0
	12	0.0909	0	0.0476	0.0217	0
	13	0	0	0	0	0
	14	0	0	0	0	0
	15	0.0909	0	0.0238	0.1087	**0.5000**
	16	0.0227	0	0.0238	0	0
	17	0	0	0	0	0
	18	0.0227	0	0.0238	0	0
	19	0.0682	0.0385	0.0476	0.1087	0
	20	0	0	0	0	0.2500
	21	0	0	0	0	0
	22	0	0	0	0	0
Sample Size		22	13	21	23	4
		Home Valley Population		Devine Site Population		Home Valley
		Apple Maggot		Apple Maggot		Snowberry M.
Locus	Allele No.	Apple	Black Haw	Apple	Black Haw	Snowberry
p37	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
	5	0.1053	0.1111	0.1667	0.0690	0
	6	0	0	0	0	0
	7	0	0	0	0.0172	0
	8	0	0.0278	0	0	**0.3889**
	9	0.0789	0.0278	0	0.0172	0
	10	0	0.0278	0	0.0345	0.0556
	11	0	0	0	0	0.1111
	12	0	0	0	0	0
	13	**0.1579**	0.0833	0.1500	**0.2069**	0
	14	0.1053	**0.2778**	**0.2000**	0.1207	0
	15	0	0.0278	0.0333	0.0172	0.3333
	16	0.0263	0	0	0.0690	0
	17	0	0	0	0	0
	18	0	0	0.0167	0	0.0556
	19	0.0526	0.0556	0.1667	0.0862	0
	20	0.0789	0.1389	0.0500	0	0
	21	0	0	0.0167	0	0
	22	0	0.0556	0	0.0172	0
	23	0.0263	0	0	0.0517	0.0556
	24	0.0526	0.0556	0.0500	0.1724	0
	25	**0.1579**	0.0556	0.100	0.0690	0
	26	0	0	0	0	0
	27	0	0	0	0	0
	28	0.0526	0	0	0	0
	29	0.0263	0.0278	0	0	0
	30	0	0	0	0	0
	31	0	0	0	0	0
	32	0	0	0	0	0

	33	0	0	0	0	0
	34	0	0	0	0	0
	35	0	0	0	0	0
	36	0.0526	0.0278	0.0167	0	0
	37	0	0	0.0167	0.0172	0
	38	0.0263	0	0.0167	0.0345	0
	Sample Size	19	18	30	29	9
		Home Valley Population		Devine Site Population		Home Valley
		Apple Maggot		Apple Maggot		Snowberry M.
Locus	Allele No.	Apple	Black Haw	Apple	Black Haw	Snowberry
p60	1	0	0.0278	0	0	-----
	2	0.3529	0.4444	0.4655	**0.6875**	-----
	3	**0.6471**	**0.5278**	**0.5345**	0.3125	-----
	4	0	0	0	0	-----
	5	0	0	0	0	-----
	6	0	0	0	0	-----
	Sample Size	34	18	29	32	0
		Home Valley Population		Devine Site Population		Home Valley
		Apple Maggot		Apple Maggot		Snowberry M.
Locus	Allele No.	Apple	Black Haw	Apple	Black Haw	Snowberry
p71	1	0	0	0	0	0
	2	0	0	0	0	0.0740
	3	0	0	0	0	0
	4	0.0294	0	0	0	0
	5	0.0147	0.0750	0.0938	0.2143	0
	6	0.2500	0.1750	0.1250	0.0893	0.1429
	7	0.2353	0.1500	0.1875	0.2321	0.1429
	8	0.0882	0.0250	0	0	0
	9	**0.3235**	**0.5250**	**0.5625**	**0.4107**	0.0714
	10	0.0441	0	0.0312	0.0536	0.1429
	11	0	0.0500	0	0	**0.3571**
	12	0	0	0	0	0.0714
	13	0.0147	0	0	0	0
	Sample Size	34	20	16	28	7

We also found that all eight of the loci displayed substantial frequency differences between the apple maggot populations attacking apple and hawthorn and snowberry maggots infesting snowberry; representative data are shown on **Table 4.** Generally speaking, common alleles in snowberry flies at each of the eight loci ranging in frequency from 0.25 to 0.5 were rare (~0.03) and in some cases (locus 29) may be absent from apple maggot populations. In addition, for the loci 27 and 29 we found high frequency alleles in apple maggot (~0.30) apparently not present in snowberry maggot. Additional genetic surveys from more sites are needed to confirm these findings, however, they suggest that it may be possible to score field collected flies for several microsatellite loci and statistically distinguish apple from snowberry flies with high accuracy.

Based on the encouraging genetic results, we concentrated our effort in the summer and fall of 2008 on collecting an extensive number of sympatric fly populations on the four major host plants throughout the states of Washington and Oregon. The major hub of collecting was centered in the Vancouver/Portland area where the apple maggot was believed to have initially been introduced in the 1970s. Extending from Vancouver/Portland, we have now collected a total of 16 sites with multiple host plants in three transects running north to Seattle, south to Eugene and east through the Columbia river gorge. These samples are currently being reared in the Washington State University Experimental facility in Vancouver. Our plan is to utilize a portion of the samples for genetic analysis, a portion for morphological analysis, and a portion for controlled rearing to assess the populations for possible host and regional differences in their adult eclosion times.

Significance to the Industry and Potential Economic Benefits

Methods that can be used to accurately distinguish apple and snowberry maggot flies are crucial to the apple industry because identifications are the basis for quarantine-related measures such as certification trapping (mass trapping around orchards) imposed by the WSDA, as well as insecticide spraying and tree removal conducted by county pest boards. These all incur costs, which are wasted if snowberry maggot flies are misidentified as apple maggot flies. Correct identification will determine where apple maggot flies are located, which will result in more effective control measures to prevent their spread into new regions and commercial orchards. Inaction because apple maggot flies are identified as a snowberry maggot flies could result in economic losses because there is no tolerance for any larvae in fruit and shipments from an infested orchard likely would be banned. In addition, in the future, export markets may require more rigid identification methods, in part for political gain, in addition to protection of the market's agricultural industry. These markets have access to older literature showing the difficulty in separating apple maggot and snowberry maggot flies, and to this point there is no way of identifying all flies with 100% accuracy. Preparation for this by generating rigid scientific data and identification methods now will be useful if this happens.

Identification of hawthorn host races may support arguments that certain populations of flies are low threats to the apple industry because these populations prefer hawthorns to apples.

Flies that cannot be easily distinguished can be sent to an agency such as WSDA that potentially will have a data base with shape data from known flies. Data from unknown flies can be entered into a computer program that will classify the fly. Processing flies and the use of the program can be conducted by competent personnel with minimal training and expense.

EXECUTIVE SUMMARY

The apple maggot fly, endemic to eastern North America, is now breeding in central Washington near major apple-growing areas. In 2008, flies were detected at new sites in Okanogan, Kittitas, Yakima, Franklin, Benton, and Walla Walla Counties. There is a zero tolerance for larvae of apple maggot in apples, and to prevent further spread of flies within apple-growing areas, local regulatory agencies rely on early detection and immediate control programs. Apple maggot flies caught near apple orchards pose a quarantine problem for apple export to California as well as virtually all of our export markets abroad. A major problem with detection programs, however, is that flies caught on traps cannot always be identified to species, so quarantines or control measures potentially may not be justified.

Apple maggot fly is almost indistinguishable from the snowberry maggot fly, a native species that attacks snowberry fruit and is caught on the same traps as are apple maggots, usually in much higher numbers. Morphological criteria used in the past to discriminate the two species are continuous and show overlap in both female and male flies. In particular, ovipositor length is used to separate females one from the other species, as apple maggots usually have longer ovipositors than snowberry maggots. Work funded by the WTFRC in 2007 showed that wing shape analysis is potentially an effective method for separating the two species independent of the other morphological data. A project was conducted in 2008 to (1) determine if wing shape is diagnostic of apple and snowberry maggot flies, with emphasis on increasing sample sizes for analysis, and to (1) determine if host races of apple maggot exist in Washington using microsatellite variation. Results showed that wing shape analysis identified female apple maggot flies with 97% accuracy, it misidentified 3% of apple maggot as snowberry maggots, and that it identified 97-100% of female snowberry maggot flies. Apple maggot flies are more likely to be misidentified as snowberry maggots than are snowberry maggot flies to be misidentified as apple maggot flies. Importantly, wing shape analysis correctly identified 100% of flies whose identities were questionable based on ovipositor lengths and therefore is a useful method for pest detection and management.

DNA analysis using microsatellite genetic markers suggest that significant differences may exist between flies reared from apple and hawthorns in Washington, as well as snowberries. We found that all of the 80 PCR primers developed to amplify microsatellite loci for eastern populations of apple maggot, also worked for western apple maggot flies from Washington state. A subset of eight loci has now been scored for flies collected from two sympatric apple, black hawthorn, ornamental hawthorn and snowberry sites in the Vancouver/Portland area. Six of the eight loci displayed significant allele frequency differences among the apple and the hawthorn populations. These data are consistent with the existence of host races in the state. Flies from the two sites did not show substantial genetic differences from flies from the eastern United States. None of the eight loci possessed a high frequency allele for the Washington fly populations at any of the eight microsatellites that was also not found in the East. This result implies that the eastern and western flies may be fairly closely genetically related, suggesting apple maggot fly in the West was introduced from the East. However, more genetic screening from additional loci is needed to confirm the preliminary data. We also found that all eight of the loci also displayed substantial frequency differences between the apple maggot flies infesting apple and hawthorn and snowberry flies infesting snowberry.

More studies are needed to complete the morphometric and DNA diagnostics work for apple and snowberry flies. A future direction is to eliminate even a 3% probability of misdiagnosis using wing shape, which can probably be achieved if shapes of other structures are included in the identification process. Ovipositor shape and male genitalia shape need to be examined in addition to wing shape. Using a combination of measures should enable us to positively identify virtually all problematic flies. The genetic survey of flies needs to be continued and completed to assess the potential genetic source (introduced vs. native) for apple and hawthorn flies in Washington. They will also substantiate whether a subset of microsatellites can diagnostically distinguish apple maggot from snowberry maggot.