*Journal of Economic Entomology,* 110(6), 2017, 2599–2608
doi: 10.1093/jee/tox248
Advance Access Publication Date: 6 October 2017
Research Article



# Genetic Evidence for the Introduction of *Rhagoletis pomonella* (Diptera: Tephritidae) into the Northwestern United States

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Subject Editor: Brian Rector

Received 4 June 2017; Editorial decision 11 August 2017

## Abstract

The apple maggot fly, *Rhagoletis pomonella* Walsh (Diptera: Tephritidae), is a serious quarantine pest in the apple-growing regions of central Washington and Oregon. The fly is believed to have been introduced into the Pacific Northwest via the transport of larval-infested apples near Portland, Oregon, within the last 40 yr. However, *R. pomonella* also attacks native black hawthorn, *Crataegus douglasii* Lindley (Rosales: Rosaceae), and introduced ornamental hawthorn, *Crataegus monogyna* Jacquin, in the region. It is, therefore, possible that *R. pomonella* was not introduced but has always been present on black hawthorn. If true, then the fly may have independently shifted from hawthorn onto apple in the Pacific Northwest within the last 40 yr after apples were introduced. Here, we test the introduction hypothesis through a microsatellite genetic survey of 10 *R. pomonella* sites in Washington and 5 in the eastern United States, as well as a comparison to patterns of genetic variation between populations of *Rhagoletis cingulata* Loew and *Rhagoletis indifferens* Curran, two sister species of cherry-infesting flies known to be native to the eastern and western United States, respectively. We report results based on genetic distance networks, patterns of allelic variation, and estimated times of population divergence that are consistent with the introduction hypothesis for *R. pomonella*. The results have important implications for *R. pomonella* management, suggesting that black hawthorn-infesting flies near commercial apple-growing regions of central Washington may harbor sufficient variation to utilize apple as an alternate host, urging careful monitoring, and possible removal of hawthorn trees near orchards.

**Key words:** apple maggot fly, cherry fruit fly, microsatellite, invasive pest, Washington State

Species can expand their ranges and become invasive by moving into new territories or environments (Lodge et al. 2006, Excoffier et al. 2009). With respect to harmful insect pests, there are two general types of biological invasions. The first involves the introduction of a pest species into a new geographic region. In this case, the insect did not necessarily evolve any new ecological adaptation to the region of introduction, as the pest may have been introduced, for example, via human mediated transport of an infested crop. The second type of invasion involves the shifting of an insect from what may be an unimportant plant onto a new economically valuable host (i.e., a host plant range expansion). In this latter case, the host shift may often be accompanied by ecological adaptation to differing features of the new crop host, such as variation in its phenology, chemistry, or morphology

(Bush 1993, Filchak et al. 2000, Berlocher and Feder 2002, Dres and Mallet 2002). Sometimes the resulting adaptations can generate a degree of reproductive isolation resulting in the formation of new ecotypes or host races of the pest on the commercial crop (Martel et al. 2003).

In both introductions and host shifts, pest insects take advantage of new opportunities provided to them across the spatial and ecological landscape. Moreover, both types of invasions have serious consequences and are of grave concern for pest insect management. In the case of introduction, it is important to identify the original sources and transport vectors (often human) of the harmful pest to try and mitigate its spread (Lodge et al. 2006). In the case of host range expansion, it is important to identify insect populations that pose the greatest risk for host shifting to limit their contact and potential to attack commercially valuable crops (Bush 1993).

The apple maggot fly, *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae), may provide an example of both types of biological invasion for a pest insect. The fly is native to the eastern United States, where its ancestral host plant is hawthorn (*Crataegus* spp.) (Bush 1966). Some time in the mid-1800s, however, the fly shifted and formed a new derived host race on introduced domesticated apple (*Malus domestica* Borkhausen, Rosales: Rosaceae), becoming a major pest of commercial apples in the process.

More recently, it is thought that *R. pomonella* was introduced to the Pacific Northwest region of the United States via apples infested with larvae (AliNiazee and Westcott 1986, Hood et al. 2013). The first report of an established apple infestation was made in a homeowner's yard in the Portland, OR area, in 1979 (AliNiazee and Penrose 1981). A single adult was collected on a yellow sticky board trap near Rowena, OR, in the Columbia River Gorge of Wasco County in 1951. However, no fly was subsequently found in the Rowena area until 1984 (AliNiazee and Westcott 1986), suggesting that the lone specimen may represent either an earlier introduction that did not establish or a misidentification of the native snowberry-infesting fly, *Rhagoletis zephyria* Snow, that is morphologically similar to *R. pomonella* (Yee et al. 2009, 2011). In the 1980s, *R. pomonella* subsequently spread north and south from Portland on the western side of the Cascade Mountains and into the Columbia River Gorge and other passages through the Cascades to encroach on the commercial apple-growing region of central WA (see arrows in Fig. 1A), where it is now a quarantine pest threatening the state's $2.39 billion annual apple industry (Mertz et al. 2016). In central WA, there is a zero-tolerance infestation policy for larvae in apples exported to foreign markets and for domestic consumption (WSDA 2001, Yee et al. 2012). If apple maggots are trapped within ½ mile of an orchard, the orchard is considered 'threatened' and requires field inspection at inconvenience to growers. If larvae are found in the vicinity, then the apples cannot be fresh marketed and a wide surrounding area is placed under quarantine, affecting growers' access to export markets because apples must undergo a prolonged cold treatment. Computer simulations have indicated that slowing the fly's spread by just 10% can save $8 million a year in costs to growers; without more effective control, all apple-producing areas may be infested in <30 yr (Zhao et al. 2007).

The conventional introduction story is complicated, however, because *R. pomonella* also attacks the native black hawthorn, *Crataegus douglasii* Lindley, and the introduced ornamental English hawthorn, *Crataegus monogyna* Jacquin in the Pacific Northwest (Yee and Goughnour 2008). It is, therefore, possible that *R. pomonella* was not introduced but has always been present in the Pacific Northwest on black hawthorn. If true, then the fly may have independently shifted from hawthorn onto apple in the Pacific Northwest within the last 40 yr after apples were earlier introduced by settlers.

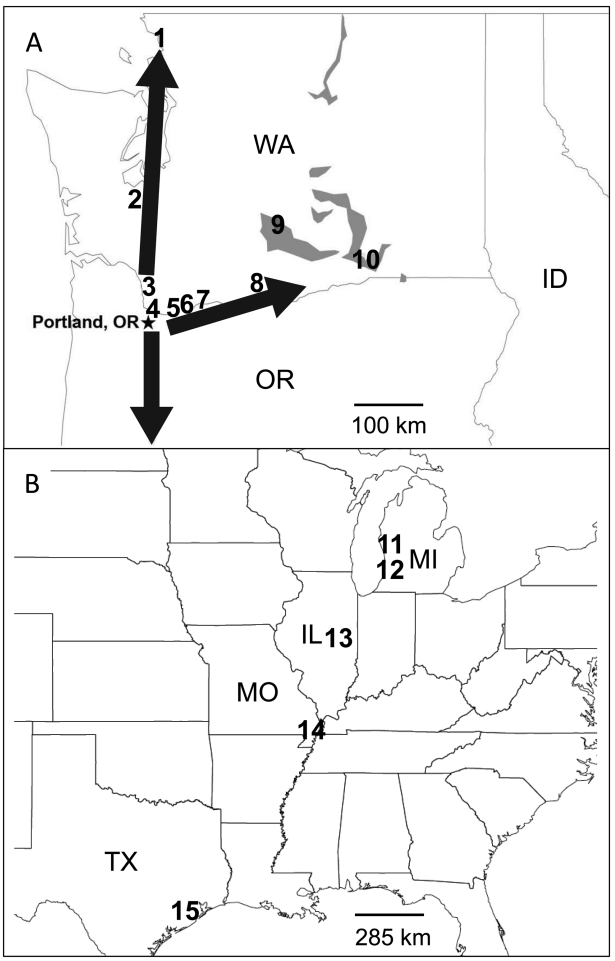


**Fig. 1.** Map of collection sites for *R. pomonella* in WA (panel A of figure) and the eastern United States (panel B). SeeTable 1 for numerical site designations and descriptions. Arrows in panel A denote spread of *R. pomonella* north and south along the western side of the Cascade Mountains and eastward into the Columbia River gorge following its putative introduction into Portland, OR. Black hawthorn-infesting populations of the fly have now encroached on the commercial apple growing region of central WA (see shaded area in panel A).

Resolving the source of *R. pomonella* in the Pacific Northwest is important because of its potential consequences for fly management. If *R. pomonella* is native, then black hawthorn flies may only occasionally explore apple as a novel host and hawthorn flies in the vicinity of apple orchards may represent a more modest risk to growers. Native populations would also suggest that the fly has always been resident, albeit at low population densities, on the fringes of many commercial apple-growing areas. Thus, *R. pomonella* has not so much dispersed or been spread through the Pacific Northwest from an initial focal point in Portland, but rather become more apparent by increasingly infesting apple through time. In contrast, if apple-infesting flies were introduced and recently shifted to black and ornamental hawthorns, these hawthorn fly populations may retain genetic variation for apple odor preference, allowing for frequent use of apple as an alternate host. Under this scenario, hawthorn populations pose a serious risk to apple growers and hawthorn trees should likely be removed from near commercial orchards. Moreover, if the fly is introduced, the movement of fruit

should be tightly monitored and controlled, as this is the most likely avenue that the fly would traverse longer distances to presently noninfested areas.

Current knowledge of the geographic distribution of *R. pomonella* in the western United States is consistent with the introduction hypothesis. A field survey of black hawthorns by Hood et al. (2013) failed to detect *R. pomonella* in the Pacific Northwest aside from areas where the fly was already known to occur. Recently, *R. pomonella* has been found infesting black hawthorn at an isolated site in Troy, Montana (Yee et al. 2015). However, all of the flies from Troy came from just one of 24 trees surveyed in the study and were detected in only 1 of the 5 yr that these black hawthorns were sampled, implying both the rarity and likely introduced nature of the infestation at the site. Moreover, *R. pomonella* was first reported to attack black hawthorn in central WA in 2003 (Yee and Goughnour 2008, Yee et al. 2012). If the fly was native on black hawthorn and did not recently disperse or become introduced into central WA, then it should have been detected earlier.

Here, in an attempt to genetically resolve the source and history of *R. pomonella* in the Pacific Northwest, we genotyped apple, black hawthorn, and ornamental hawthorn-infesting flies from WA using microsatellite markers, as well as mitochondrial DNA (mtDNA), and compared these populations to those in the putative native portion of *R. pomonella*'s range in the eastern United States. If *R. pomonella* was recently introduced, then we predict, following general population genetics invasion theory (Dlugosch and Parker 2008), that western compared with eastern fly populations should show 1) reduced overall genetic variation, 2) reduced genetic diversity with geographic distance from Portland, OR, the purported site of the initial invasion, 3) few unique alleles that, when present, will be found at low frequencies and in only a handful of populations, and 4) a coalescence time on the order of 40 yr. The latter finding would be consistent with the first report of apple flies in the Pacific Northwest in 1979. In contrast, if the native hypothesis is correct, then *R. pomonella* in the Pacific Northwest should be comparable in allelic richness to flies in the East, provided western and eastern populations are of roughly equivalent size. In addition, western flies should contain many, widely distributed alleles unique to the Pacific Northwest and have a coalescence time on the order of at least several thousand years, in line with the last period of glaciation. In this regard, the high mutation rate of microsatellites coupled with the extensive allelic diversity detected in previous surveys of eastern *R. pomonella* populations (Michel et al. 2010; Powell et al. 2013, 2014) make microsatellites potentially very sensitive for detecting the type of demographic genetic footprints that would distinguish the introduction versus native hypotheses.

To further distinguish between the introduction and native hypotheses for the apple maggot fly, we compared patterns of microsatellite and mtDNA variation in eastern versus western *R. pomonella* populations with those for the eastern cherry fruit fly, *Rhagoletis cingulata* Loew, and the western cherry fruit fly, *Rhagoletis indifferens* Curran. These latter two flies are sister taxa to one another in the *R. cingulata* sibling species group. *Rhagoletis cingulata* and *R. indifferens* are known to be endemic to the eastern and western United States (Bush 1966), respectively, and have a combined range very similar to *R. pomonella*'s current range. Consequently, comparison of the pattern of microsatellite differentiation between populations of *R. cingulata* in the eastern United States with *R. indifferens* in the western United States provides a baseline for assessing what may be expected for *R. pomonella* if the fly was also native to the Pacific Northwest, assuming a similar biogeographic history for apple maggot and cherry fruit flies.

## Materials and Methods

### Fly Collection

*Rhagoletis pomonella* flies were collected as larvae in infested fruit from July through September of 2009 to 2012 at 10 different sites in WA (see Table 1 and Fig. 1A for description and map of WA collecting localities). Adult flies were reared from field-collected fruit using standard *Rhagoletis* husbandry methods (Neilson and McAllan 1965), as discussed elsewhere (Feder and Bush 1989; Feder et al. 1990, 1993). Adult flies were kept frozen and stored at −80°C prior to genetic analysis. Similarly, *R. indifferens* flies were collected as larvae from infested bitter cherry fruit, *Prunus emarginata* (Dougl. ex Hook.) Eaton (Rosales: Rosaceae), sampled from June through September in 2004 from one site in British Columbia, Canada (Salmon Arm, BC), and in 2010 in the United States from two sites in WA (Woodland and Ronald), one site in OR (Mt. Hood), and one site in MT (Flathead Lake). These five sites encompass a large portion of the geographic range of *R. indifferens* in western North America (Bush 1966). *Rhagoletis cingulata* flies were collected as larvae from infested black cherry fruit, *Prunus serotina* Ehrh. from August through September in 2012 from one site in IN (Granger), one site in NJ (Cranberry), and one site in FL (Gainesville), United States. These three sites encompass a large portion of the geographic range of *R. cingulata* in the eastern United States (Bush 1966). All cherry fruit fly samples included here are those collected and genotyped in St. Jean (2015).

### Microsatellite Genotyping of Flies

DNA was isolated and purified from head or whole body fly tissue using PUREGENE extraction kits (Gentra Systems, Minneapolis, MN). Purified DNAs were transferred to 96-well plates for microsatellite polymerase chain reaction (PCR) amplification and genotyping. *Rhagoletis pomonella* specimens were genotyped for a total of 28 loci characterized from an enriched GT-dinucleotide repeat *R. pomonella* library (Velez et al. 2006). These 28 microsatellite loci were designated with the prefix letter 'P' to indicate that they were developed for *R. pomonella* followed by a suffix number indicating the order in which they were originally characterized (see Supp. Table 1, [online only] ). The 28 microsatellites analyzed were chosen because they displayed no systematic statistical significance for heterozygote deficiency from Hardy–Weinberg equilibrium due to null alleles, as determined using MICRO-CHECKER (Van Oosterhout et al. 2004), although there was an overall trend for observed heterozygosity ($H_o$) to be lower than expected heterozygosity ($H_e$) across all the loci (Supp. Tables 2 and 3 [online only]). Total genomic DNA was PCR amplified using locus specific primers for 38 cycles (1 cycle = 94°C for 20 s, 55°C for 15 s, 72°C for 30 s), followed by a final incubation for 10 min at 72°C. Genotyping was performed on a Beckman-Coulter CEQ8000 (Indianapolis, IN) including size standards in each gel lane and, in a subset of runs, a subsample of three to five specimens from the eastern U.S. population of *R. pomonella* from Grant, MI to ensure that alleles were aligned and comparably scored among runs. Microsatellite alleles were sized using the Fragment Analysis software provided by Beckman-Coulter. Microsatellite data for 22 of the 28 microsatellites scored in the Pacific Northwest were also available for eastern populations of *R. pomonella* from previous studies (Michel et al. 2007, 2010; Powell et al. 2013, 2014) to provide a genetic comparison from the native range of the fly to assess the introduction hypothesis (the six loci that were only scored in the Pacific Northwest were P12, P19, P36, P55, P64, and P68). Data were compiled for these 22 microsatellites for eight host-associated populations of eastern flies representing five

**Table 1.** Collection sites for *R. pomonella*, *R. indifferens*, and *R. cingulata*

| Site # | Site | State | Host | n | Lat. | Long. | # Loci |
|---|---|---|---|---|---|---|---|
| *Rhagoletis pomonella* | | | | | | | |
| 1 | Bellingham | WA | AB | 37 | 48.74 | −122.48 | 28 |
| 2 | Tumwater | WA | B | 36 | 46.87 | −122.76 | 27 |
| 3 | Vancouver | WA | ABO | 139 | 45.73 | −122.63 | 28 |
| 4 | Vancouver | WA | ABO | 200 | 45.63 | −122.60 | 26–28* |
| 5 | Skamania | WA | ABO | 189 | 45.60 | −122.11 | 28 |
| 6 | Beacon Rock | WA | AB | 95 | 45.65 | −122.01 | 28 |
| 7 | Home Valley | WA | B | 48 | 45.71 | −121.78 | 28 |
| 8 | Klickitat | WA | B | 18 | 45.89 | −120.71 | 25 |
| 9 | Yakima | WA | B | 9 | 46.61 | −120.50 | 28 |
| 10 | Burbank | WA | B | 9 | 46.20 | −119.01 | 28 |
| 1–10 | West total | | | 780 | | | |
| 11 | Grant | MI | AD | 90 | 43.34 | −85.81 | 22 |
| 12 | Fennville | MI | AD | 183 | 42.59 | −86.10 | 20–21* |
| 13 | Urbana | IL | AD | 94 | 40.12 | −88.21 | 21–22* |
| 14 | New Madrid | MO | D | 41 | 36.59 | −89.53 | 22 |
| 15 | Brazos | TX | G | 94 | 29.38 | −95.60 | 22 |
| 11–15 | East total | | | 502 | | | |
| *Rhagoletis indifferens* | | | | | | | |
| 1C | Salmon Arm | BC, CA | C | 19 | 50.92 | −119.36 | 21 |
| 2C | Woodland | WA | C | 43 | 45.94 | −122.68 | 21 |
| 3C | Mt. Hood | OR | C | 16 | 45.54 | −121.62 | 21 |
| 4C | Ronald | WA | C | 48 | 47.24 | −121.04 | 21 |
| 5C | Flathead Lake | MT | C | 11 | 48.09 | −114.23 | 21 |
| 1C–5C | West total | | | 137 | | | |
| *Rhagoletis cingulata* | | | | | | | |
| 6C | Granger | IN | C | 20 | 41.76 | −86.20 | 21 |
| 7C | Cape May | NJ | C | 16 | 39.04 | −74.90 | 21 |
| 8C | Live Oak | FL | C | 14 | 30.37 | −83.24 | 21 |
| 6C–8C | East total | | | 50 | | | |

Note: Hosts = infested host fruit collected at sites (A = apple; B = black hawthorn; O = ornamental hawthorn; D = downy hawthorn, *C. mollis* Scheele; G = green hawthorn, *C. viridis*; C = cherry). Sample size (*n* individuals; see Supp. Table 4 [online only] for exact numbers of individuals genotyped per population per locus), latitude (Lat.), longitude (Long.), and number of microsatellite loci amplified (# Loci) for sites are also given. Data for eastern *R. pomonella* populations (11–15) were compiled from previous studies (Michel et al. 2007, 2010, Powell et al. 2013, 2014), and data for *R. indifferens* and *R. cingulata* were from St. Jean (2015).

*Where listed as a range, the number of microsatellite loci amplified varied among host races (Supp. Table 4 [online only]).

different collecting sites spanning the latitudinal range of *R. pomonella* in the eastern United States (see Table 1 and Fig. 1B for description and a map of the sites). Raw fragment trace outputs from the eastern populations were manually evaluated against the data for western populations and the subsample of rerun flies from Grant, MI, to confirm the accuracy and consistency of allele scoring for *R. pomonella* between the two regions.

Data for a total of 21 microsatellite loci for the cherry-infesting populations of *R. cingulata* flies from the eastern United States and *R. indifferens* from the western United States were compiled from St. Jean (2015). Nine of the 28 locus-specific primer pairs for *R. pomonella* microsatellites were also PCR amplified and found to be polymorphic in *R. cingulata* and *R. indifferens* (P4, P27, P36, P37, P45, P50, P54, P71, and P80) by St. Jean (2015) and those data were included in the current analysis. Twelve additional microsatellite loci were also scored by St. Jean (2015) using primers developed for *R. indifferens* by Maxwell et al. (2009) and those data incorporated into this analysis to characterize nuclear differentiation between eastern and western cherry flies. These 12 loci (WCFF7, WCFF24, WCFF31, WCFF57, WCFF61B, WCFF067, WCFF083, WCFF084A, WCFF86A, WCFF93, WCFF105, WCFF111) were designated with the prefix 'WCFF' to indicate that they were developed for western cherry fruit flies followed by the suffix number originally designated by Maxwell et al. (2009).

## mtDNA Sequencing of Flies

In addition to microsatellites, genetic divergence between eastern and western populations of *R. pomonella* was assessed for mtDNA based on a 431-bp fragment including partial Cytochrome Oxidase I (COI), full tRNA-Leu, and partial COII mtDNA genes (GenBank KT221480–KT221486). These seven haplotypes represented all unique sequences generated from five individuals collected from Skamania, WA, four from Staples, MN, one from Brazos Bend, TX, one from Iowa City, IA, five from Grant, MI, two from Biglerville, PA, two from Geneva, NY, and one from Amherst, MA. The Skamania, WA and Grant, MI sites were the same locations for which flies were also scored for microsatellites. Taken together, the mtDNA data for the seven eastern sites encompass the majority of the longitudinal and latitudinal range of *R. pomonella* in the eastern United States. The same 431-bp mtDNA fragment was analyzed for a subset of three *R. indifferens* specimens sequenced from Vancouver, WA, and 12 from around Flathead Lake, MT (GenBank KT221488), as well as two *R. cingulata* specimens from Granger, IN, three from Cranberry, NJ, and one from Gainesville, FL (GenBank KT221487).

### Data Analysis

A maximum parsimony gene tree was constructed for *R. pomonella*, *R. indifferens*, and *R. cingulata* mtDNA haplotypes using PAUP*b8 (Swofford 2000). For the maximum parsimony mtDNA analysis, deletions were treated as a fifth base pair, with indels of identical length and position recoded to count as single mutational steps. An unrooted neighbor-joining network for all *R. pomonella* sites and individuals (Table 1, Supp. Table 4 [online only]) based on overall Nei's genetic distances (Nei 1972) was constructed using the 22 loci that were scored in common for both eastern and western flies using PowerMarker v3.25 (Liu and Mus 2005). When constructing the network for *R. pomonella*, populations 9 and 10 from central WA, where relatively few flies were genotyped ($n$ = 9 for both sites), were combined to increase the sample size for that region. In a few cases, certain microsatellites failed to properly amplify in a given population (Supp. Table 4 [online only]). In these cases, the locus was not included when calculating overall Nei's genetic distances from the nonamplifying population to all other populations. A separate neighbor-joining network was constructed based on the microsatellite data for *R. cingulata* and *R. indifferens*. Bootstrap support values for nodes in the networks were calculated based on 10,000 replicates across loci.

Allelic richness, corrected for sample size, was estimated in ADZE 1.0 (Szpiech et al. 2008). For the eastern versus western population comparison for *R. pomonella*, only the 22 microsatellites that were scored in both regions were used. Pooling all individuals from eastern and western populations, respectively, rarefaction analysis allowed sampling of up to 606 alleles for richness estimates. The relationship between allelic richness and geographic distance from Portland, OR, for the 10 populations surveyed in WA was assessed using 23 of 28 microsatellites scored in the West and was tested for statistical significance to fit a binomial regression using the program R (R Core Team 2014). The five loci that were removed from the analysis had > 66% missing data for at least one population. Eliminating these five microsatellites resulted in richness estimates based on a maximum of 12 alleles per locus sampled across all populations. Genetic differentiation ($F_{ST}$) values were calculated in Genepop (Raymond and Rousset 1995).

Divergence time between eastern and western U.S. populations of *R. pomonella* was estimated using the Markov Chain Monte Carlo (MCMC) sampling algorithm in the program IMa2p (Hey and Nielsen 2004, Hey 2010, Sethuraman and Hey 2016). Of the 22 microsatellites scored in common between eastern and western populations of *R. pomonella*, eight loci were used in this analysis (P05, P09, P27, P29, P60, P71, P73, and P80); those microsatellites that deviated from the stepwise mutation model (their allele lengths did not incrementally increase by 2 bp) were removed. In addition, we retained only one locus per high linkage disequilibrium (LD) group (LD estimates from Michel et al. 2010) to meet assumptions of the Isolation with Migration (IM) model. Posterior distributions for divergence time were first generated using uninform priors for ancestral population size (θ), migration rates between populations ($m_1$ and $m_2$), and time since divergence ($t$) in generations, which for *R. pomonella* equals the number of years since separation, as these flies are all univoltine. To obtain an upper bound estimate for divergence time, $m_1$ and $m_2$ were both set to 0 under the assumption that if *R. pomonella* was not native to the Pacific Northwest, then only a single introduction event occurred. To streamline the process, the eastern and western *R. pomonella* population pairs most closely genetically related in the neighbor-joining network were used to estimate divergence time based on a step-wise mutation model for microsatellite loci with a mutation rate of $1 \times 10^{-5}$ to $1 \times 10^{-3}$ per meiosis. Triplicate MCMC simulations were run for at least $5 \times 10^6$ iterations after an initial burn-in of $1 \times 10^7$ iterations, using 200 chains and heating parameters ha = 0.99 and hb = 0.4. Results are reported from three pooled runs, at a mutation rate of $1 \times 10^{-3}$, which all converged to similar posterior distributions. If, in fact, *R. pomonella* in the Pacific Northwest were native and not introduced, using the most closely genetically related populations would provide a lower bound estimate for the time of geographic separation between eastern and western populations.

## Results

The maximum parsimony mtDNA gene tree resolved for *R. pomonella*, *R. indifferens*, and *R. cingulata* had a total of 32 substitutions within a 431-bp mtDNA fragment (Fig. 2). The gene tree showed little sequence divergence between *R. pomonella* from the eastern versus western United States, with a maximum of 0.69% nucleotide divergence between regions, compared to 0.46% within WA. Indeed, the most common haplotype at the St. Cloud, WA site, present in three of the five individuals sequenced, was identical to haplotypes found across much of *R. pomonella*'s range in the eastern United

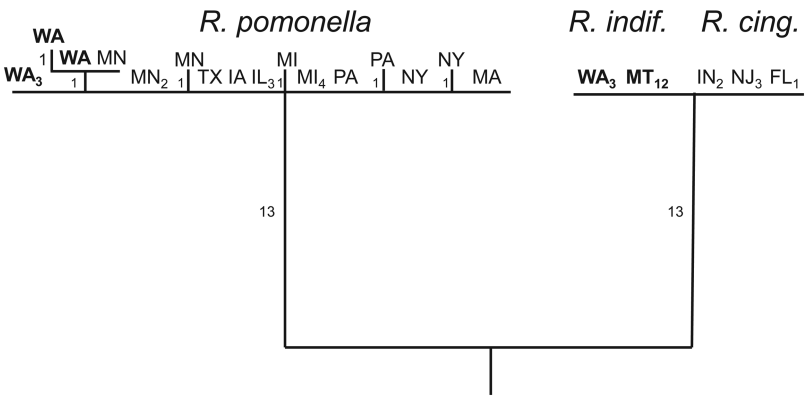


**Fig. 2.** Maximum parsimony gene tree for haplotypes of a 431 bp mtDNA fragment for *R. pomonella*, *R. cingulata*, and *R. indifferens* flies across North America. Sites are described in the Materials and Methods and are designated in the figure according to state abbreviation in the United States (WA = Washington; MN = Minnesota; TX = Texas; IA = Iowa; MI = Michigan; PA = Pennsylvania; NY = New York; MA = Massachusetts; IN = Indiana; NJ = New Jersey; FL = Florida). Subscript on site designations indicates the number of identical mtDNA haplotypes sequenced from the location. Also shown are the numbers of nucleotide substitutions along each branch of the tree. Bootstrap support (10,000 replicates) was 100% for both the node defining *R. pomonella* and that for the two cherry fly species.

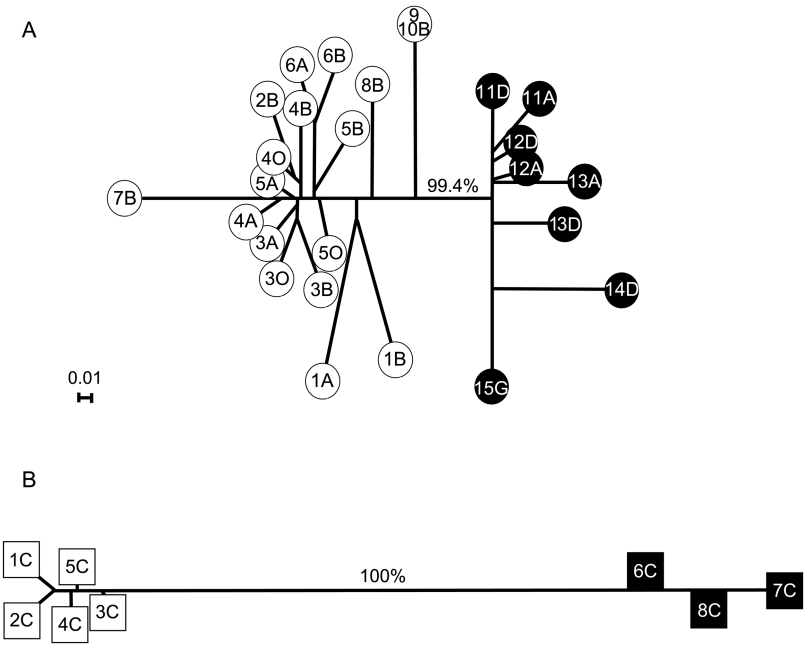


**Fig. 3.** Neighbor joining networks with bootstrap support values (10,000 replicates) for A) western populations of *R. pomonella* in WA (unfilled circles) and in the eastern USA (filled circles) based on Nei's overall genetic distances for 22 microsatellite loci. Populations are designated by their site numbers and host plant sampled (A = apple; B = black hawthorn; O = ornamental hawthorn, D = downy hawthorn) and samples sizes are as given in Table 1; and B) western populations of *R. indifferens* (unfilled squares) eastern populations of *R. cingulata* (filled squares) based on Nei's overall genetic distances for 21 microsatellite loci. Site designations are 1C = British Columbia, Canada (Salmon Arm), 2C = Woodland, WA; 3C = Mt. Hood, OR; 4C = Ronald, WA, 5C= Flat Head Lake Biological Station, MT; 6C = Granger, IN; 7C = Cape May, NJ; 8C = Live Oak, FL; where C denotes cherry-infesting fly population.

States, including MN, IA, TX, MI, PA, NY, and MA (Fig. 2). Similarly, *R. indifferens* and *R. cingulata* native to the western and eastern United States, respectively, showed no mtDNA divergence (Fig. 2).

The neighbor-joining network based on Nei's genetic distances calculated between *R. pomonella* populations for 22 microsatellites grouped western and eastern populations of *R. pomonella* as distinct clusters from one another with high (99.4%) bootstrap support (Fig. 3A). The network suggested that *R. pomonella* flies in the Pacific Northwest were most closely related to flies from Illinois, and the length of the branch separating the clusters of eastern and western United States flies in the network was not long compared with those among populations within the two regions, indicating little divergence between eastern and western populations. This was reflected in the shallow divergence time estimated by IMa2p between western and eastern *R. pomonella* populations, with the highest posterior probability at 29.5 yr and a 95% Bayesian credibility interval from 7.5 to 112.5 yr (Fig. 4).

In comparison to eastern and western populations of *R. pomonella*, eastern populations of *R. cingulata* and western populations of *R. indifferens* were much more genetically distinct from one another (Fig. 3B). Indeed, the genetic distance between the nodes of the closest pair of populations in the neighbor-joining network separating eastern and western cherry fruit flies (0.345; Fig. 3B) and mean $F_{ST}$ value (0.178 ± 0.024 SE; Fig. 5) were an order of magnitude greater than those for eastern and western populations of *R. pomonella* (0.05 and 0.016 ± 0.002, respectively).

In total 52 alleles from the 22 microsatellites scored in common for *R. pomonella* in the eastern and western United States samples were found to be unique for at least 1 of the 10 study sites in WA and not found in any of the 8 sites surveyed in the eastern United States (see Supp. Table 4 [online only] for allele frequency data). However, none of these unique alleles were present across all 10 western *R. pomonella* sites at an average frequency within populations

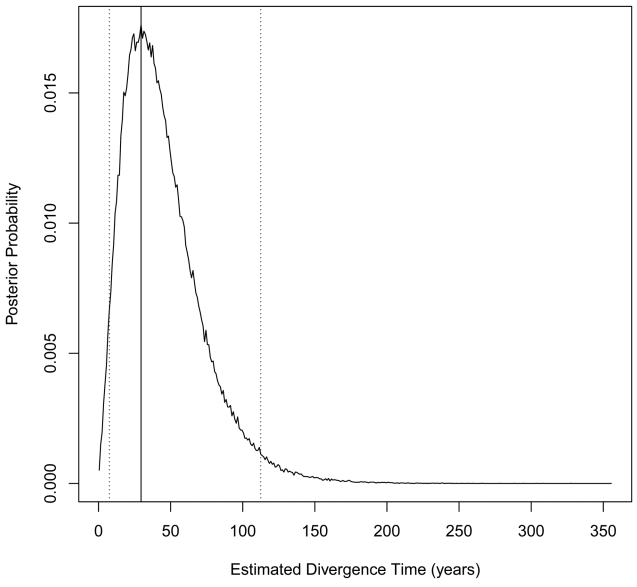


**Fig. 4.** Posterior probability distribution for estimated divergence time between WA and eastern U.S. populations of *R. pomonella* based on the MCMC sampling algorithm in IMa2p (see Material and Methods for details of estimation). The divergence time with the highest posterior probability was estimated to be 29.5 yr, with the mean of the probability distribution being 44.9 yr. Dotted lines represent the Bayesian credibility interval from 7.5 to 112.5 yr containing 95% of the posterior probability distribution centered around 29.5 yr.

greater than 0.05 and most were found at only 1 or a few of the 10 sites. However, allele 231 for locus P46, allele 216 for locus P54, and allele 295 for locus P16 all had average frequencies >0.05 across the multiple western populations in which they were found but were

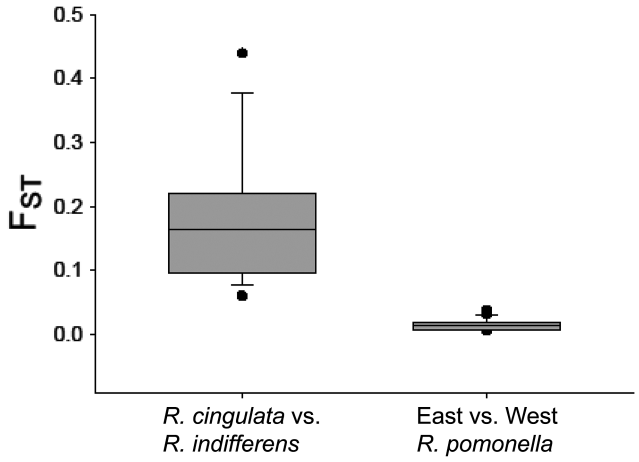


**Fig. 5.** Boxplots of mean FST values ± twice the standard error for *R. cingulata* in eastern versus *R. indifferens* in the western North America and for *R. pomonella* in the eastern versus western USA. Filled circles represent individual loci with FST values greater than twice the standard error.

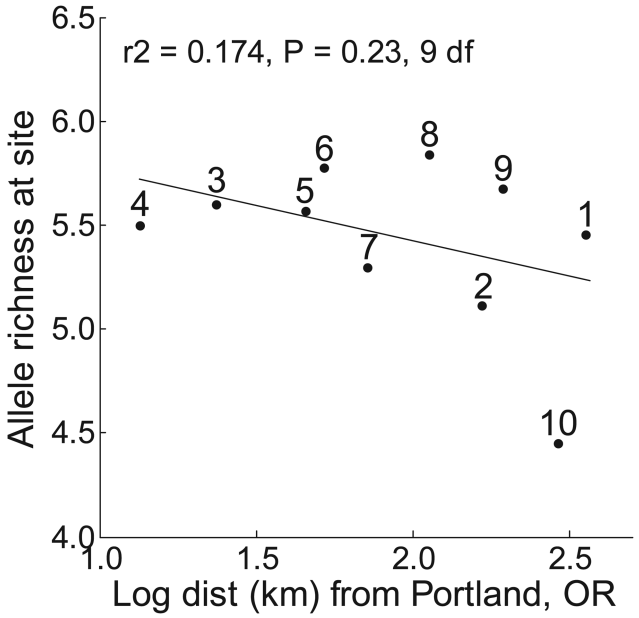


**Fig. 6.** Relationship of mean allelic richness for microsatellites at 10 sites in WA plotted against the logarithm of the geographic distance of the sites (in km) from downtown Portland, OR. Allelic richness was estimated for the 23 microsatellite loci having a sample size of at least 6 individuals at every site.

generally absent from sites in central WA (Supp. Table 4 [online only]). In comparison, 196 alleles were unique to eastern *R. pomonella* populations, approximately a fourfold increase (Supp. Table 4 [online only]). Also, the mean allelic richness for the 22 microsatellite loci scored in common between eastern and western populations after correcting for sample size was significantly higher in the eastern United States (25.8 ± 2.59) than in WA (19.0 ± 1.99 SE; $t = -2.071$; df = 39; $P = 0.045$; Welch Two Sample *t*-test). Allelic richness was also slightly lower for the western distributed *R. indifferens* (4.0 ± 0.45 SE, $g = 94$) than for eastern populations of *R. cingulata* (7.1 ± 0.91; $t = -3.019$; df = 29; $P = 0.0005$; Welch Two Sample *t*-test).

The pattern of genetic variation within *R. pomonella* was not, however, completely uniform across WA (Fig. 6). There was a trend, although not significant, for allelic richness at *R. pomonella* sites to decline with their geographic distance from the putative site of introduction, Portland, OR ($R^2 = 0.174$; df = 9; $P = 0.23$; Fig. 6). However, the trend was mainly driven by the easternmost Burbank site in WA (site 10 in Fig. 6). In the absence of the Burbank site, the relationship between geographic distance from Portland, OR, and allele richness was greatly reduced to $R^2 = 0.026$.

## Discussion

The results from the genetic survey of microsatellite loci for 10 sites in WA support the hypothesis that *R. pomonella* was recently introduced into the Pacific Northwest. Genetic diversity is lower for populations in WA than in the eastern United States. There are fewer total alleles, fewer unique alleles, and reduced overall genetic variation in western fly populations, consistent with the occurrence of a bottleneck and reduced population size accompanying the founding of *R. pomonella* in the Pacific Northwest, as predicted by invasion theory (Dlugosch and Parker 2008). Reduced genetic polymorphism could be explained by fly populations in WA being native on black hawthorn and as old as those in the East but having historically lower effective population sizes due to less suitable habitat (lower hawthorn host plant population density and reduced quality) in the region. In this regard, it could be possible that eastern and western populations have been continuously connected, for example, by a conduit of migration along the Missouri River or waterways through the Plain States of the United States. However, the number of unique alleles is low in WA and those present tended not to be widely distributed, but rather are in low frequency within populations and found at just one or a few sites. Given the generally high mutation rate of microsatellites, such a pattern reflects a recent origin of these unique variants and would not be expected if western fly populations were native and long established. As predicted by the introduction hypothesis, many more unique microsatellite alleles were found and present in higher frequencies in the eastern United States than in WA. Moreover, the estimated separation time of 29.5 yr and the relatively short branch length between eastern and western *R. pomonella* in the genetic distance network argue against the existence of a long-standing native population on black hawthorn in the Pacific Northwest. Instead, the genetic data support a recent introduction, concurring with the first confirmed report of the fly in the area of Portland, OR, in 1979 (AliNiazee and Penrose 1981), ~30 yr before specimens were sampled in the current study. However, there was not strong evidence that populations in the Portland, OR, area displayed increased allelic variation or that variation decayed as the fly spread through OR and WA.

Support for the introduction hypothesis for *R. pomonella* was further strengthened by genetic comparisons with *R. indifferens* and *R. cingulata*. The two cherry-infesting species are allopatrically isolated sister taxa native to the western and eastern United States, respectively. Like *R. pomonella* from eastern and western North America, *R. indifferens* and *R. cingulata* show little mtDNA divergence, suggesting that the two cherry flies have not been separated for an extended period of time. However, *R. indifferens* and *R. cingulata* display much greater microsatellite differentiation than eastern and western *R. pomonella*, with each of 21 microsatellite loci scored for cherry flies possessing at least one allele unique to *R. cingulata* or *R. indifferens*, often at relatively high frequency, and two loci alternately fixed for completely different sets of alleles between eastern and western species of cherry flies (St. Jean 2015). Thus, FST and genetic distances in the neighbor joining network were an order of magnitude greater between *R. cingulata* and *R. indifferens* than

between eastern and western populations of the apple maggot fly. As a result, divergence time estimates between eastern and western cherry flies for the microsatellites based on IMa2p ranged from several hundred to ~20,000 yr, depending upon the value of the mutation rate assumed in the analysis ($10^{-3}$ to $10^{-5}$). If *R. pomonella* were native to the western United States, then one might, therefore, expect to see a similar magnitude of microsatellite divergence and greater divergence time than 29.5 yr for the apple maggot fly. The lack of any high-frequency unique alleles in western *R. pomonella* and the comparatively modest level of divergence from eastern populations is, therefore, most consistent with a recent introduction of the fly into the Pacific Northwest.

Although it would appear probable that *R. pomonella* was introduced into the Pacific Northwest from the eastern United States, several questions remain. One issue concerns the source locality of the introduction. The genetic distance network implies that flies from IL are most closely related to *R. pomonella* in WA. However, additional sampling and greater genetic resolution, for example, based on high-throughput DNA genotyping-by-sequencing, are needed to verify the source of flies in the Pacific Northwest and confirm that they originated from just a single introduction, as it is conceivable that infested fruit from several eastern United States sources contributed to the establishment of the western population.

A second question centers on why apple-infesting populations in WA differ in their olfactory behavior from eastern flies (Cha et al. 2012, Linn et al. 2012). Specifically, apple-origin flies tested from multiple sites throughout WA in flight tunnel assays and analyzed in a field trapping study in Woodland, WA, were attracted to a slightly different blend of apple fruit volatile compounds compared with their counterparts in the eastern United States (Linn et al. 2012, Sim et al. 2012). Western apple flies require the addition of hexyl acetate and hexyl propionate to the standard five-component eastern apple blend to achieve a similar level of behavioral response as eastern apple flies do to the eastern apple blend (Cha et al. 2012, Linn et al. 2012).

One possible explanation for the behavioral difference may involve a founder event associated with the initial introduction of *R. pomonella* into the Pacific Northwest, as inferred from the loss of microsatellite variability. In general, populations of eastern flies display similar levels of attraction to the standard five-component blend with or without the addition of hexyl acetate and hexyl propionate. However, a portion of the eastern fly population does show increased attraction to the apple fruit volatile blend with hexyl acetate and hexyl propionate present. It is, therefore, possible that an initial founding population of apple-infesting flies in the Pacific Northwest contained a disproportionately high percentage of such flies by chance, accounting for the behavioral difference between the eastern and western United States.

Alternatively, the behavioral difference could be due to apple-infesting flies in WA being derived from flies that originally infested ornamental hawthorn. In this regard, although it is likely that *R. pomonella* in the Pacific Northwest was introduced, this does not mean that the source of the introduction was larval-infested apple fruit. The ornamental English hawthorn is also non-native to the Pacific Northwest, having been a popular horticultural introduction following the First World War (Sallabanks 1993). It is, therefore, conceivable that *R. pomonella* was introduced to the Pacific Northwest along with ornamental hawthorn from somewhere in the eastern United States at this time and subsequently shifted to apple, accounting for the olfactory difference observed for western flies. The current microsatellite data cannot distinguish between an apple versus ornamental hawthorn source for *R. pomonella* in the Pacific Northwest; this may prove to be a difficult question to resolve genetically. However, an important clue may come from the composition of the parasitoid wasp community attacking *R. pomonella* in WA.

In the eastern United States, *R. pomonella* infesting apples and hawthorns are attacked by several genera of braconid parasitoid wasps (Feder 1995; Forbes et al. 2009, 2010; Hood et al. 2015). Fly populations infesting ornamental hawthorn and black hawthorn in WA are also attacked by several braconid parasitoid genera (Gut and Brenner 1994). However, apple flies in the region are not parasitized (Forbes et al. 2009, G.R. Hood, pers. obs.), the only exception being a single published account of a parasitoid specimen reared from *R. pomonella* pupae collected from apple in the Willamette Valley farther south in OR (AliNiazee 1985). Therefore, it is possible that the general lack of apple fly parasites in WA is due to the original founding population of apple-infesting flies being devoid of parasitoids. Indeed, the level of parasitism in the eastern apple fly race is significantly lower than that in hawthorn due to the physical protection (i.e., enemy free space) that the larger-sized apple fruit provides fly larvae from attack by natural enemies (Feder 1995). Under this scenario, wasps attacking black and ornamental hawthorn fly populations in the Pacific Northwest would represent host shifts of native parasitoids from other *Rhagoletis* species, such as *R. zephyria*, in the region. However, if *R. pomonella* was introduced via larval-infested ornamental hawthorn (or for that matter any eastern hawthorn), a portion of the parasitoid community currently attacking hawthorn flies in the Pacific Northwest would be expected to be non-native and introduced from the eastern United States, as well. Further genetic study of the western *R. pomonella* parasitoid community could help to resolve this issue.

In conclusion, genetic analyses of microsatellites support the conventionally accepted hypothesis that *R. pomonella* was introduced into the Pacific Northwest from the eastern United States relatively recently, perhaps from the more southern range of the apple fly race in IL. Reduced genetic variation for flies in WA and the pattern of allelic richness are consistent with at least a modest population bottleneck associated with such an invasion. However, it is not definitive that larval-infested apple fruit was the vector for the initial introduction or is the locality of the source population in the eastern United States certain. It is still conceivable that *R. pomonella* was introduced to the Pacific Northwest via non-native hawthorn (or possible from both apple and hawthorn). Regardless of its source, there are currently three different host-associated populations of *R. pomonella* in the Pacific Northwest attacking apple, ornamental hawthorn, and black hawthorn. As the fly is not native to the region, this implies that up to two host shifts have occurred in the Pacific Northwest within the last 40 yr, potentially providing novel examples for the study of contemporary host race formation and ecological speciation in action (Forbes et al. 2017), perhaps even more rapid than the *R. pomonella* host shift in the southern or eastern United States (Feder et al. 1988, 1994; Feder and Bush 1989; Filchak et al. 2000; Michel et al. 2010; Powell et al. 2014). Moreover, it would seem that black hawthorn-infesting flies most likely originated via a shift from apple, given their relatively recent documentation in central WA (Yee and Goughnour 2008, Yee et al. 2012) and near absence across the remainder of the native black hawthorn range east of the Cascade Mountains (Hood et al. 2013). If this is true, then black hawthorn fly populations are expected to harbor significant variation to utilize apple as an alternative host. Indeed, despite any host shift-driven genetic bottlenecks, a small but substantial proportion of black hawthorn flies may still be attracted to apple odors (Linn et al. 2012). Black hawthorn now represents the major host infested by *R. pomonella* in areas near commercial apple-growing regions of

central WA (Yee 2008; Yee and Goughnour 2008; Yee et al. 2012, 2014). As such, these populations should be monitored carefully and perhaps black hawthorn trees removed from near apple orchards as a means for controlling the spread of *R. pomonella* and limiting its economic threat.

## Acknowledgments

We thank the Clark County Washington 78th Street Heritage Farm, the Washington State University Research and Extension Unit, Vancouver, Washington, Blair Wolfley, Doug Stienbarger, Terry Porter, and Kathleen Rogers for their support and assistance on the project. This work was supported in part by grants to J.L.F. from the NSF and the USDA, and to W.L.Y. by the Washington Tree Fruit Research Commission, Washington State Commission on Pesticide Registration, and USDA-FAS.

## Supplementary Data

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

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