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Limited genetic evidence for host plant-related differentiation in the Western cherry fruit fly, *Rhagoletis indifferens*

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Abstract

The shift of the fruit fly *Rhagoletis pomonella* (Walsh) in the mid-1800s from downy hawthorn, *Crataegus mollis* (Torrey & Asa Gray) Scheele, to introduced domesticated apple, *Malus domestica* (Borkhausen), in the eastern USA is a model for ecological divergence with gene flow. A similar system may exist in the northwestern USA and British Columbia, Canada, where *Rhagoletis indifferens* Curran (Diptera: Tephritidae) attacks the native bitter cherry *Prunus emarginata* (Douglas ex Hooker) Eaton (Rosaceae). Populations of *R. indifferens* have shifted and became economic pests on domesticated sweet cherry, *Prunus avium* (L.) L., shortly after sweet cherries were introduced to the region in the mid-1800s. The fruiting phenologies of the two cherries differ in a similar manner as apples and hawthorns, with domesticated sweet cherries typically ripening in June and July, and bitter cherries in July and August. Here we report, however, little evidence for genetic differentiation between bitter vs. sweet cherry populations of *R. indifferens* or for pronounced genetic associations between allele frequencies and adult eclosion time, as has been documented for apple and downy hawthorn flies. The current findings support a previous more geographically limited survey of *R. indifferens* in the province of British Columbia, Canada, and an analysis of its sister species, *R. cingulata*, in the state of Michigan, USA, implying a lack of host-related differentiation for flies infesting different cherry host plants. Possible causes for why host races are readily genetically detected for *R. pomonella* but not for *R. indifferens* are discussed.

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Introduction

Ecological speciation occurs as populations adapt to differing habitats or environments and become reproductively isolated communities (Schluter, 2009). A central tenet of ecological speciation is that phenotypes or traits conferring higher fitness in one habitat result in lower performance or survivorship in alternate habitats (Bush, 1969, 1993; Schluter, 2001). Thus, migrants have traits ill-

suited to non-natal environments and/or hybrids of mixed ancestry possess phenotypes reducing fitness in parental habitats, generating ecologically based reproductive isolation (Nosil, 2012).

Testing the ecological speciation hypothesis can be complicated because many factors, including habitat-related fitness tradeoffs, often contribute to the reduction in gene flow between populations (Mayr, 1963; Coyne & Orr, 2004). As such, no speciation event from beginning to end is likely to be wholly attributable to the evolution of ecologically based reproductive isolation but may be due to the combined effects of several factors, including divergent ecology (Mayr, 1963). Nevertheless, it can still be important to distinguish whether the key impetus for population divergence was ecologically based natural selection, as opposed to genetic drift occurring independently in geographically separated demes, for example. In this case, inferences may be drawn as to how often and quickly, in the absence of divergent ecology, we might expect to see new taxa arise. If infrequently, then although ecology may not be the only factor contributing to the evolution of reproductive isolation during speciation, differential adaptation to alternate habitats early in the divergence process may be a key signpost of incipient speciation for many organisms.

Several groups of organisms have contributed to our understanding of the role of ecological adaptation in population divergence including insects (Egan & Funk, 2009), fish (Rogers & Bernatchez, 2007), birds (Sorenson et al., 2003), and plants (Schemske & Bradshaw, 1999). One group of particular significance is phytophagous (plant eating) insects, due to their great diversity and often close association between feeding ecology and systems of mating (Medina, 2017). The host specificity of phytophagous insects has been argued to make them generally more amenable than other organisms to adaptive diversification (Berlocher & Feder, 2002; Dres & Mallet, 2002; Dickey & Medina, 2012). Indeed, speciation-with-gene flow was first proposed in regards to sympatric host shifting for phytophagous insects (Walsh, 1864).

Fruit flies in the genus *Rhagoletis* (Diptera: Tephritidae) are a model for understanding ecological speciation via host plant shifting (Bush, 1966, 1969; Feder et al., 1988; Berlocher & Feder, 2002) with the apple maggot fly, *Rhagoletis pomonella* (Walsh), perhaps the best-known example. In the mid-1800s, flies from the ancestral host downy hawthorn, *Crataegus mollis* (Torrey & Asa Gray) Scheele, shifted to and formed a new host race on introduced, domesticated apple, *Malus domestica* (Borkhausen), in the eastern USA (Walsh, 1867; Feder et al., 1988; Filchak et al., 2000; Berlocher & Feder, 2002).

One key host plant-related ecological adaptation differentiating the apple and hawthorn-infesting host races of *R. pomonella* is the timing of adult eclosion (Feder et al., 1993; Dambroski & Feder, 2007). Given that *R. pomonella* is short-lived (ca. 28 days), overwinters in a facultative pupal diapause, and is univoltine (Dean & Chapman, 1973), the fly must synchronize its eclosion phenology with the timing of fruit maturation of its host plant. Apple varieties most conducive for larval development and survivorship fruit 3–4 weeks before downy hawthorn. As a result, apple-origin flies are genetically programmed to eclose 2–3 weeks earlier than hawthorn flies (Feder et al., 1993, 1994). This heritable developmental difference generates allochronic pre-zygotic, as well as ecologically based post-zygotic, reproductive isolation between the host races (Feder et al., 1993, 1994; Filchak et al., 2000). Genetic differentiation between apple and hawthorn flies is characterized by allele frequency differentiation at many allozyme, microsatellite, cDNA, and ddRAD-seq single nucleotide polymorphisms superimposed on strong latitudinal effects on loci associated with diapause life-history timing (Feder et al., 1988; McPheron et al., 1988; Feder & Bush, 1989; Michel et al., 2010; Egan et al., 2015; Ragland et al., 2017). The correlation of markers displaying allele frequency differences between apple and hawthorn flies with diapause variation links host-related adaptation in the system with ecologically based reproductive isolation and population divergence.

A similar system may exist in the northwestern USA and Canada. The western cherry fruit fly, *Rhagoletis indifferens* Curran, is native to the bitter cherry, *Prunus emarginata* (Douglas ex Hooker) Eaton (Rosaceae), in the northwestern USA and British Columbia, Canada (Frick et al., 1954; Raine & Andison, 1958; Bush, 1966). Populations of *R. indifferens* established and became economic pests on introduced domesticated sweet cherry, *Prunus avium* (L.) L., around 100 years ago (Wilson & Lovett, 1913) after it was introduced to the northwestern USA in the mid-1800s (McClintock, 1967). Although sweet cherry was also introduced to British Columbia, Canada, around this time, *R. indifferens* was not observed on bitter or sweet cherry in British Columbia prior to 1968 (Madsen, 1970). The phenologies of the two host plants differ, with sweet cherries typically ripening in June and July and bitter cherries in July and August (Esser, 1995; Long et al., 2007; Yee et al., 2015). Correspondingly, adult flies usually appear earlier in sweet than in bitter cherry trees (Yee et al., 2015), suggesting fly populations on the two hosts could have different eclosion times that are genetically controlled. Thus, synchronization and potential allochronic isolation between bitter and sweet cherry-infesting populations of *R. indifferens* in the western USA may offer a similar case

of host race formation as the classic story of *R. pomonella* in the eastern USA. Indeed, the natural history of *R. indifferens* in sweet cherry is similar to that of other *Rhagoletis* flies. Females oviposit in cherries, where eggs hatch and larvae feed for 2–4 weeks. After fruit abscises from trees, third instars leave the fruit, burrow into the soil, form puparia, pupate, and overwinter. Cherry fly pupae undergo a near obligatory diapause, which is maintained at high temperatures and broken after prolonged chilling (Frick et al., 1954; Van Kirk & AliNiazee, 1982). There is one major generation a year, with less than 8% of pupae diapausing for more than 1 year after prolonged chilling (150–210 days) at 0–4.4°C (Frick et al., 1954).

The objectives of this study were to determine whether (1) *R. indifferens* infesting sweet vs. bitter cherry in the northwestern USA and British Columbia, Canada (hereafter referred to collectively as the Pacific Northwest) genetically differ from each other for microsatellites, and (2) any of the genetic differences observed between bitter and sweet cherry flies are associated with diapause life-history timing (eclosion time), as is the case between the apple and hawthorn-infesting host races of *R. pomonella* (Feder et al., 1997; Filchak, et al., 2000; Michel et al., 2010; Egan et al., 2015; Ragland et al., 2017).

Materials and methods

Study sites and specimens

A previous survey of microsatellite variation for *R. indifferens* in British Columbia, Canada, reported evidence for geographic differentiation among cherry-infesting fly populations on spatial scales exceeding 20 km, but not among flies attacking different host species of cherries, including *P. emarginata* and *P. avium* (Maxwell et al., 2014). However, the samples of *R. indifferens* from British Columbia analyzed in Maxwell et al. (2014) were collected from Salmon Arm (bitter cherry, site 1 in this study) and Peachland (sweet cherry, site 7 in this study), areas of contrasting precipitation and microclimate, separated by >120 km, making it difficult to draw firm conclusions about host-related genetic differentiation for the fly.

To address the issue of host-related differentiation in this study, we surveyed an additional four collecting areas in the USA, representing five new pairs of *R. indifferens* populations infesting native bitter vs. domesticated sweet cherries across the states of Washington, Oregon, and Montana (Table 1, Figure 1A). An attempt was made to sample flies infesting the two alternative cherry hosts as geographically proximate to one another as possible. However, in the northwestern USA, sweet and bitter cherries thrive at different elevations, with domesticated cherries found more often at lower altitudes in the bottomlands and bitter cherries at higher altitudes in the ponderosa pine ecosystem (Lyons & Merilees, 1995). Nevertheless, the maximum distance between pairs of bitter and sweet cherries at sites in this study was 10 km. In the vicinity of Woodland (WA), sweet cherry flies collected from Caples Road (site 8) were located 1.5 km west from those sampled at the Burgerville restaurant on Lewis River Road (site 9). Corresponding bitter cherry flies from the Lewis River Cemetery (site 3) and Little Kalama River (site 2) were located 5.5 and 8.0 km northeast, respectively, from the site-9 sweet cherry flies. At Hood River (OR), the bitter cherry site at Trout Creek Ridge (site 4) is 10 km south of the sweet cherry site at Indian Creek (site 10). The remaining two pairs of bitter cherry and sweet cherry locations (Ronald, WA: sites 5 and 11; Flathead Lake, MT: sites 6 and 12) are both separated by approximately 1 km. It is

Table 1 List and description of *Rhagoletis indifferens* collecting sites analyzed in the study, including latitude (°N), longitude (°W), host (BC = bitter cherry, SC = sweet cherry), sample size (n = number of flies genotyped), and date collected

Site	Location	Latitude	Longitude	Host	n	Date
1	Salmon Arm, Canada	50°54′54″	119°21′26″	BC	19	17 August 2004
2	L. Kalama Rd, Woodland, WA, USA	45°56′24″	122°40′41″	BC	43	11 August 2011
3	Lewis River, Woodland, WA, USA	45°56′20″	122°38′5″	BC	34	11 August 2011
4	Trout Creek, Hood River, OR, USA	45°32′9″	121°37′14″	BC	16	20 August 2011
5	Ronald, Kittitas County, WA, USA	47°23′50′’	121°02′67′’	BC	48	22 August 2012
6	Flathead Lake, MT, USA	48°05′24″	114°13′48″	BC	11	12 August 2005
7	Peachland, Canada	49°45′16″	119°45′23″	SC	12	12 July 2004
8	Caples Road, Woodland, WA, USA	45°54′10″	122°46′49″	SC	32	11 July 2011
9	Burgerville, Woodland, WA, USA	45°54′28″	122°44′21″	SC	38	10 July 2011
10	Indian Creek, Hood River, OR, USA	45°41′53″	121°30′57″	SC	46	15 July 2011
11	Ronald, Kittitas County, WA, USA	47°14′38″	121° 2′7″	SC	96	15 July 2012
12	Flathead Lake, MT, USA	48°05′24″	114°13′48″	SC	21	12 July 2005

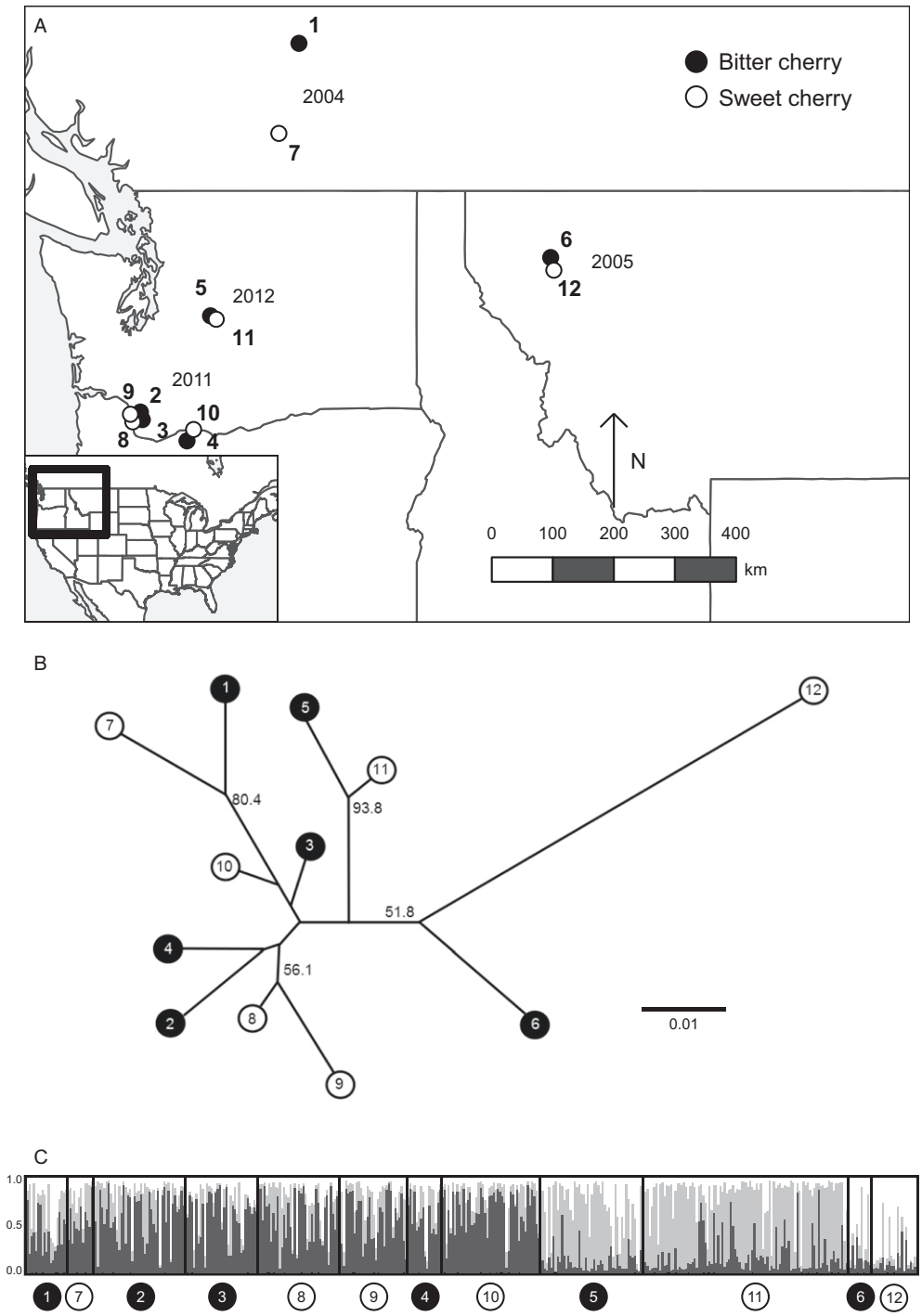

Figure 1 (A) Map of collecting sites for *Rhagoletis indifferens* flies infesting bitter (sites 1–6, black dots) and sweet cherry (sites 7–12, white dots) from across British Columbia, Canada, and the northwestern USA. The collection year is listed near each location. See Table 1 for additional description of study sites. (B) Neighbor-joining genetic distance network for bitter (sites 1–6) and sweet cherry-infesting (sites 7–12) *R. indifferens* populations of flies, based on the 19 microsatellites scored. Also shown are bootstrap support levels for nodes based on 10 000 replicates. A Nei's genetic distance of 0.01 is indicated by the scale bar. (C) STRUCTURE plot for bitter (sites 1–6) and sweet (sites 7–12) cherry-infesting populations of *R. indifferens* based on 16 microsatellites for the 12 populations considered together. The best fit number of subpopulations, evaluated using the methods of Evanno et al. (2005), was K = 3 (Table S2). Each vertical bar represents a sampled individual's probability of assignment to each of the three subpopulations (dark gray, light gray, and white). Vertical black lines separate sampled populations designated below by numbers corresponding to Figure 1A.

important to note that not all sites were sampled in the same year. Populations in Woodland, WA (sites 2, 3, 8, and 9) and Hood River, OR (sites 4 and 10) were all collected in 2011. However, western cherry flies were sampled from British Columbia (sites 1 and 7) in 2004, from Montana (sites 6 and 12) in 2005, and from Ronald, WA (sites 5 and 11) in 2012. These latter locations represent the sites that were most geographically separated from one another in the study. Thus, axes of temporal and spatial sampling covaried in the study, which we elaborate upon further when describing methods for data analysis. Adult flies were reared from field-collected fruit using standard *Rhagoletis* husbandry methods (Neilson & Mcallan, 1965), as described elsewhere (Feder et al., 1989, 1990, 1993). Specimens were kept frozen and were stored at −80°C prior to genetic analysis.

Eclosion time analysis

To test for host-related adult eclosion time differences, sweet and bitter cherries collected from sites 5 and 11 (Ronald, WA) were held at 23–24°C, L16:D8 photoperiod, and 30–40% r.h. during larval emergence from fruit. Cherries were placed on hardware cloth suspended above a plastic tub holding a small amount of soil. Every 2 days, pupae were collected from the tub and placed in moist soil inside sealed 473-ml clear plastic containers. Pupae were held at 20–23°C for 10–12 days and then chilled at 4.7–4.9°C inside temperature cabinets for a period of 4 months. Temperatures were monitored using Hobo Pro v.2 data loggers (Onset Computer, Bourne, MA, USA) throughout the chilling period. Post chilling, the sealed plastic containers with pupae were transferred to and held at L16:D8 photoperiod and 23–24°C in a constant-temperature room. Adult fly eclosion was monitored on a daily basis over a total period of 120 days by placing the plastic containers inside a 0.03-m^3 window screen cage with a cloth sleeve and removing the lids from the containers. Eclosing flies were then captured inside the cage, placed in glass vials, their sex determined, and frozen at −80°C for later genetic analysis.

Microsatellites

DNA was isolated and purified from adult head or from whole body tissue of adult flies for microsatellite analysis using Puregene extraction kits (Gentra Systems, Minneapolis, MN, USA). Purified DNAs were transferred to 96-well plates for microsatellite PCR amplification and genotyping. Flies were genotyped for a total of 19 microsatellite loci. Twelve of the loci (WCFF007, WCFF024, WCFF031, WCFF057, WCFF061B, WCFF067, WCFF083, WCFF084A, WCFF086A, WCFF093, WCFF 105, WCFF111) were developed for *R. indifferens* by Maxwell et al. (2009) and seven loci (P4, P27, P37, P50, P45, P54, P71) were developed originally for *R. pomonella* by Velez et al. (2006), but also cross amplify and are polymorphic for cherry flies. PCR reactions for the two sets of microsatellites were performed according to the protocols described in Maxwell et al. (2009) and Michel et al. (2010), respectively, where details concerning forward and reverse PCR primers used to amplify the loci can also be found. The 19 microsatellites analyzed were chosen because they displayed no systematic evidence for heterozygote deficiency from Hardy-Weinberg equilibrium (HWE) due to null alleles, as determined using Micro-Checker (Van Oosterhout et al., 2004). Genotyping was performed on a Beckman-Coulter CEQ8000 (Brea, CA, USA). Microsatellite alleles were sized using the Fragment Analysis software provided by Beckman-Coulter. Size standards were included in each gel lane to ensure that alleles were aligned and comparably scored among runs.

Genetic analysis of microsatellites

To test for genetic correlations among the 19 microsatellites, linkage disequilibrium (LD) was calculated between pairs of loci using Burrow's composite Δ (Weir, 1979), which does not assume HWE or require phased data, but instead provides a joint metric of intra- and inter-locus disequilibria based solely on genotype frequencies. Thus, Δ is equivalent to the LD parameter D under HWE (Weir, 1979). Linkage disequilibrium values were calculated separately for each of the 12 populations surveyed in the study for each pair of loci and these values were then transformed to a standardized correlation coefficient, as described by Weir (1979). Correlation coefficients were then combined across populations using the method of Fisher (Sokal & Rohlf, 2012) to derive an overall measure tested for significance by a χ^2 test, as described in Weir (1979).

An unrooted neighbor-joining (NJ) network for the 19 microsatellites based on overall Nei's genetic distances (Nei, 1972) between populations was constructed using PowerMarker v.3.25 (Liu & Mus, 2005). Bootstrap support values for nodes in the network were calculated based on 10 000 replicates across loci.

To test for the effects of collection year, geography, and host fruit on genetic differentiation, we performed a redundancy analysis (RDA) in the R package 'vegan' v.2.4-3 (Oksanen et al., 2017; R Core Team, 2017). The RDA was performed on the principle coordinates of the microsatellite allele frequencies, computed in R package 'adegenet' v.2.1-1 (Jombart, 2008; Jombart & Ahmed, 2011). We calculated the distance-based Moran's eigenvector maps (MEM) from latitude and

longitude, to account for geographic distance between the sites, with 'adespatial' v.0.1-1 (Dray et al., 2017). To determine the effect of host fruit alone on genetic variation, we used partial RDA to condition the variation for collection year, MEM, and both collection year and MEM. The models were then compared with an ANOVA with 999 permutations.

Tests for evidence of host-related genetic subdivision for the microsatellites were also performed using the program STRUCTURE v.2.3.4 (Pritchard et al., 2000) among all 12 bitter and sweet cherry sites considered together, as well as between flies attacking the two cherry host species at paired sites. To meet assumptions of the STRUCTURE model, loci in high LD with one another (WCFF084A, WCFF093, and WCFF105; see Results) were removed, leaving 16 microsatellite loci in the data set. For each comparison, 10 replicate runs were conducted under the admixture model with correlated allele frequencies, the number of clusters (K values) ranging from 1–12 (all site comparison) or 1–2 (pairwise site comparisons), 500 000 burn-in iterations, and 1 000 000 data collecting steps. The methods of Evanno et al. (2005) – based on the rate of change in the natural logarithm of the probability of data between successive K values (ΔK) – and of Pritchard et al. (2000) – involving direct comparisons of the ln probabilities of K – were both performed to choose the number of clusters that best fit the data.

In addition, tests were conducted for significant allele frequency differences between host-associated populations at paired sites using a non-parametric, Monte Carlo approach. Microsatellite genotypes were randomly resampled with replacement from the combined data set for a locus at each paired site in numbers corresponding to the sample sizes of bitter and sweet cherry flies scored to determine the probability from 100 000 replicates of generating a Nei's genetic distance value D greater than or equal to the observed value between populations by chance. For the four sites at Woodland (WA), allele frequencies were tested separately between the two bitter cherry sites (2 and 3) against the two sweet cherry sites (8 and 9), their physically nearest paired sites.

Tests for sex-related differences at the sweet cherry site 9 (Woodland, WA) and the bitter and sweet cherry sites 5 and 11, respectively (Ronald, WA), were performed using a similar non-parametric, Monte Carlo approach, as was done for host-related divergence. Unfortunately, due to experimental error, these were the only three sites where the sexes of flies were recorded in sample sheets prior to genetic analysis. To test for sex-related differences, random samples were drawn from the combined data set for males and females at each of the sites 5, 9, and 11 in the numbers corresponding to the sample size for each sex at the site. The probability was then estimated from 100 000 replicates of generating a Nei's genetic distance value D greater than or equal to the observed value between the sexes for a locus at sites 5, 9, and 11 by chance.

Eclosion time (i.e., the number of days of post-winter warming until an adult fly emerged) at sites 5 and 11 (Ronald, WA) was first analyzed in an ANOVA with sex (male or female) and host (bitter or sweet cherry) considered as main effects. We then conducted a one-way ANOVA comparing eclosion time considering genotype (the number of the most common microsatellite allele possessed by a fly for the locus being tested) as the main effect after normalizing the eclosion time date for each fly by its standard deviation from the mean eclosion time for its sex and host at sites 5 and 11. Normalizing eclosion times allowed the data to be combined across sexes and hosts to test for a genetic association. The significance of the two-way and one-way ANOVAs was determined using a non-parametric, Monte Carlo approach. Eclosion times were permutated and randomly assigned with respect to sex, host, or genotypes of flies to determine the proportion of times in 100 000 replicates of generating an F-value greater than or equal to the observed value for the locus. A stepwise multiple regression was also conducted for all of the loci against normalized eclosion times to consider the overall extent that the microsatellites explained variation in *R. indifferens* eclosion time.

Results

Linkage disequilibrium and sex-related differentiation

Microsatellite loci were generally in equilibrium with one another in pairwise composite tests of LD. However, three loci (WCFF084A, WCFF093, and WCFF105) showed significant LD with one another across all 12 populations (WCFF084A vs. WCFF093: $r = 0.45$; WCFF084A vs. WCFF105: $r = 0.82$; WCFF093 vs. WCFF105: $r = 0.59$, all $P<0.0001$). Previously, Maxwell et al. (2014) reported that the three microsatellites WCFF007, WCFF084A, and WCFF093 displayed significant sex-associated allele frequency differences in *R. indifferens* populations surveyed from British Columbia, Canada. However, inspection of the supplementary genotype data provided by Maxwell et al. (2014) indicated that WCFF105, and not WCFF007, should have been reported as being sex-linked, consistent with the findings of this study. Also, in contrast to Maxwell et al. (2014) and despite the presence of significant LD, we found no significant allele frequency difference between males and females for WCFF084A, WCFF093, or WCFF105 at sites 5, 9, or 11 where the sex of flies was determined before genotyping. Indeed, only WCFF111

displayed a significant allele frequency difference between the sexes when sites 5, 9, or 11 where considered together. Moreover, for locus WCF111, the difference between the sexes was only marginally significant (P = 0.042 by Monte Carlo simulation) with the total frequency for the common allele 222 in males being 0.806 (n = 108) compared to 0.732 (n = 58) in females. The results suggest that the association of the microsatellites WCFF084A, WCFF093, and WCFF105 with the sex-determining factor in *R. indifferens* is stronger for flies in British Columbia than in Washington, perhaps due to tighter physical linkage or possibly smaller effective population sizes and drift in Canadian populations situated at the edge of the species' range.

Geographic and host-related differentiation

The neighbor-joining Nei's genetic distance network for all 19 microsatellite loci did not group the six sweet cherry-infesting fly populations (sites 7–12) as being distinct from the six bitter cherry fly populations (sites 1–6) surveyed in the study (Figure 1B). There was a trend, however, for local paired sweet and bitter cherry populations to cluster in the genetic distance network (Figure 1B). The only exceptions to this trend were site 3 (Woodland, WA) and sites 4 and 10 (Hood River, OR). As a result, in a partial RDA, conditioned on collection year, geographic distance (MEM) along with host significantly predicted microsatellite variation ($F_{3,409}$ = 9.87, P = 0.001). However, we caution that collection year also affected microsatellite variation, as a partial RDA including collection year and host, conditioned on MEM, also significantly explained genetic variation ($F_{4,409}$ = 12.857, P = 0.001). Several individual loci did display significant allele frequency differences between sweet vs. bitter cherry fly populations at local paired sites (Table 2). Nevertheless, the level of local host-related divergence was not sufficient for it to be a significant predictor of overall genetic variation in a partial RDA, conditioned on both MEM and collection year ($F_{1,409}$ = 0.6172, P = 0.69).

The general absence of host-related differentiation was also observed in STRUCTURE plots of population divergence. Overall, the best estimate for the number of diverged cherry fly populations in the Pacific Northwest, as indicated by the method Evanno et al. (2005), was K = 3 (Table S2). STRUCTURE analyses indicated that the bitter and sweet cherry populations at sites (Ronald, WA) 5 and 11, respectively, as well as the bitter and sweet cherry populations at sites (Flathead Lake, MT) 6 and 12, respectively, each clustered independently from the remaining eight

Table 2 Microsatellite loci displaying significant host-related allele frequency differences between bitter and sweet cherry *Rhagoletis indifferens* populations at indicated paired collecting sites or a significant association with eclosion time at sites 5 and 11 (Ronald, WA, USA) (non-parametric, Monte Carlo simulations: *0.01<P<0.05, **0.001<P<0.01, ***0.0001<P<0.001, ****P<0.0001). See Table 1 for site designations and Figure 1A for map

	Paired site comparison bitter vs. sweet cherry populations								
Locus	1 vs. 7	2 vs. 8	2 vs. 9	3 vs. 8	3 vs. 9	4 vs. 10	5 vs. 11	6 vs. 12	Eclosion time
WCFF007		****	**	*					***
WCFF024									
WCFF031			***		**		*	*	
WCFF057			*			*			
WCFF061B		***	*	*		*	**	*	
WCFF067									
WCFF083					*				
WCFF084A									
WCFF086A		*				****	*	**	
WCFF093					*		***		
WCFF105	**	*		*				*	
WCFF111		***	**		***		***		*
P4								***	
P27	*								
P37									
P45		**		***	****		*	**	
P50									
P54								*	
P71		**	***			*		*	

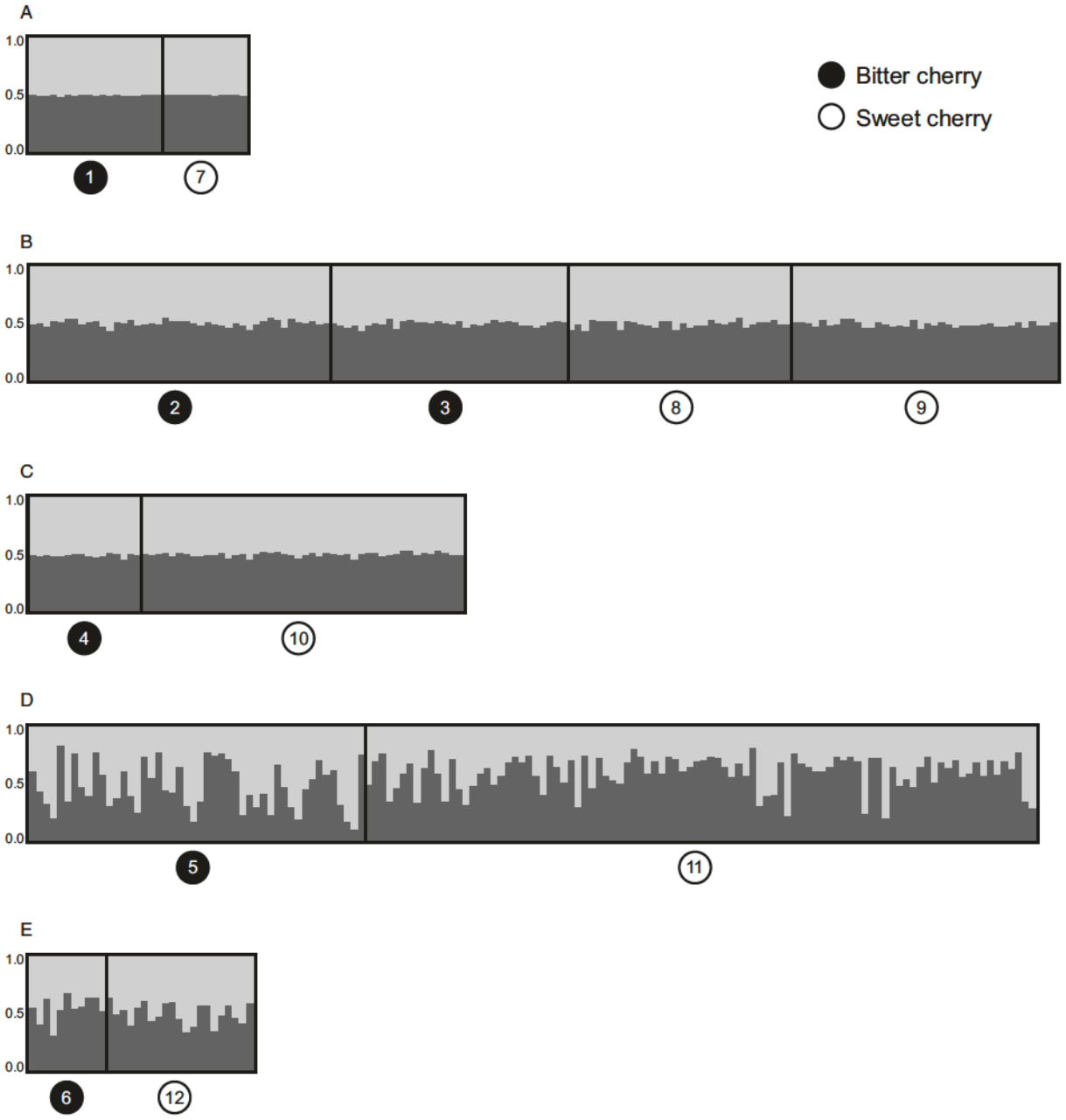

Figure 2 STRUCTURE plots for paired bitter (black dots) and sweet cherry-infesting (white dots) populations of *Rhagoletis indifferens* based on 16 microsatellites, assuming K = 2 subpopulations (i.e., two host-associated populations may exist), for (A) sites 1 (Salmon Arm, Canada) and 7 (Peachland, Canada), (B) sites 2, 3, 8, and 9 (Woodland, WA, USA), (C) sites 4 and 10 (Hood River, OR, USA), (D) sites 5 and 11 (Ronald, WA, USA), and (E) sites 6 and 12 (Flathead Lake, MT, USA). Each vertical bar represents a sampled individual's probability of assignment to each of the two assumed subpopulations (dark gray and light gray). Vertical black lines separate sampled populations designated below by numbers corresponding to Figure 1A. For all comparisons, the best fit (highest ln-likelihood) number of subpopulations was actually K = 1 (Table S3), implying a lack of host-associated differentiation.

populations surveyed in the study, which also included a mix of bitter and sweet cherry sites (Figure 1C). This result is consistent with both the Nei's genetic distance network (Figure 1B) and the significant effect of geographic distance on microsatellite variation (see above), implying a degree of geographic divergence for sites 5 and 11 (Ronald, WA) and sites 6 and 12 (Flat Head Lake, MT). STRUCTURE plots did not

distinguish sweet from bitter cherry subpopulations at any of the paired sampling sites (Figure 2, Table S3), implying random mating and an overall lack of genetic differentiation between populations collected from the two hosts.

Adult eclosion time

Eclosion time displayed a significant relationship with sex ($F = 4.43$, d.f. = 1, $P = 0.037$), but not with host ($F = 0.82$, d.f. = 1, $P = 0.36$), nor with a sex*host interaction ($F = 0.12$, d.f. = 125, $P = 0.70$), at sites 5 and 11 (Ronald, WA). The mean ($\pm$ SE) eclosion time of females at sites 5 and 11 (37.1 $\pm$ 0.36 days, n = 42) was almost 2 days earlier than that for males (38.9 $\pm$ 0.39 days, n = 87). In comparison, the difference between sweet cherry- vs. bitter cherry-infesting flies was less than 1 day: 38.1 $\pm$ 0.31 days (site 11, n = 91) vs. 38.8 $\pm$ 0.67 days (site 5, n = 38). Of the 19 microsatellites, only WCFF007 and WCFF111 displayed significant relationships with eclosion time at sites 5 and 11 (Table 2); these were also the only two significant loci in the stepwise forward multiple regression of microsatellite genotypes vs. eclosion time ($r = 0.36$, d.f. = 128, $P<0.05$).

Discussion

In the last few decades, there has been a growing appreciation of the importance that ecology plays in initiating speciation (Rundle & Nosil, 2005). Traits undergoing differential adaptation to ecologically dissimilar habitats could often serve as key initial barriers to gene flow between nascent species. An important question then is how common the process of ecological speciation is in nature.

Here, we tested for evidence of genetically differentiated host races and a role for divergent ecological selection in the timing of adult eclosion between populations of *R. indifferens* infesting native bitter cherry, *P. emarginata*, and introduced, domesticated sweet cherry, *P. avium*, in British Columbia, Canada, and the northwestern USA. *Rhagoletis* fruit flies in the *R. pomonella* group, a clade related to *R. indifferens*, are a model for ecological speciation with gene flow via host plant shifting. Thus, the shift of *R. indifferens* from the later fruiting bitter cherry to the earlier fruiting and recently introduced sweet cherry provided a test for rapid host race formation in western North America paralleling the shift of *R. pomonella* from hawthorn to apple in the eastern USA (Feder et al., 1988; McPheron et al., 1988).

However, unlike for *R. pomonella*, we found limited evidence for microsatellite genetic differentiation between populations of *R. indifferens* infesting bitter vs. sweet cherries. Certain loci displayed significant host-related allele frequency differences between local paired populations of bitter vs. sweet cherry-infesting flies. However, the differences generally were not global across the range of *R. indifferens*. Moreover, at sites 5 and 11 (Ronald, WA), there was little evidence for host-related differences in eclosion time between bitter vs. sweet cherries following laboratory rearing despite the 2-3-week earlier fruiting phenology of sweet cherries in the field at the site (Yee, 2014). In addition, in comparison to *R. pomonella* (Feder et al., 1997; Michel et al., 2010; Egan et al., 2015; Ragland et al., 2017), there was not as dramatic a relationship between genetic markers displaying host-related divergence and the timing of adult eclosion. There was a significant effect of geographic distance, controlled for collection year, on genetic variation among *R. indifferens* populations across British Columbia and the northwestern USA. The finding of significant geographic differentiation extends the regional pattern detected by Maxwell et al. (2014), for *R. indifferens* in British Columbia, across much of the range of the western cherry fruit fly in North America. The implication is that gene flow between local bitter and sweet cherry populations is greater than that between geographically more distant populations of flies infesting the same host plant. As a result, local populations of bitter and sweet cherry flies, although differing to some degree, overall tended to cluster together on the neighbor-joining Nei's genetic distance network. Smith et al. (2014) also found little host-related microsatellite divergence between native black cherry, *P. serotina*, and domesticated cherry-infesting populations of *R. cingulata* in managed orchards in the state of Michigan.

Why do *R. indifferens* and *R. pomonella* differ despite the apparently similar biology and natural history, including the difference in host fruiting time? One possibility that requires further testing is that *R. indifferens* adults are longer lived than *R. pomonella* in nature, resulting in the opportunity for many *R. indifferens* to attack both sweet and bitter cherry host plants during the field season. It is unlikely that *R. indifferens* have two generations in nature, as rearing experiments indicate that the fly has a near obligate overwintering diapause with few individuals eclosing as non-diapausing adults when exposed to prolonged heating (Yee et al., 2015).

A second related possibility is that diapause duration and termination in *R. indifferens* are more canalized genetically and developmentally and not affected by environmental conditions, as may be the case in *R. pomonella*, where the interaction of host-related genetic differences and the environment can be important in determining when adults eclose (Feder et al., 1997; Filchak et al., 2000). As a consequence, eclosion times for bitter and sweet

cherry fly populations may be more concordant in nature, resulting in minimal allochronic separation, despite the 2–3 weeks difference in host fruiting time.

Third, fidelity for the natal host fruit they feed on as larvae may not be strong for sweet and bitter cherry flies. *Rhagoletis* flies mate exclusively on or near the fruit of their respective host plants (Prokopy et al., 1971, 1972). Thus, host plant choice relates directly to mate choice, with differences in host preference establishing a system of positive assortative mating that translates into pre-zygotic reproductive isolation. For *R. pomonella*, a series of genetically-based behavioral differences exists between the apple and hawthorn host races (Prokopy & Roitberg, 1984), including variation in fruit odor preference (Linn et al., 2003, 2005; Dambroski et al., 2005; Forbes et al., 2005; Forbes & Feder, 2006), that results in differential host choice of apple flies for apple trees and hawthorn flies for hawthorn trees, reducing gene flow between the races to ca. 4% per generation (Feder et al., 1994). Such host preference differences may not exist for *R. indifferens*, resulting in random mating between bitter and sweet cherry-origin flies, unlike the situation for the apple and hawthorn host races of *R. pomonella*.

A fourth possibility, related to the three explanations discussed above, is that *R. indifferens* can oviposit into bitter cherry fruit before they are completely ripe. If true, then the seasonal difference between sweet and bitter cherry fruit availability would be reduced, potentially increasing the rate of gene flow between fly populations attacking the two alternative cherry hosts.

Finally, the genomic architecture of *R. indifferens* may differ from that of *R. pomonella* in ways that make it more difficult to detect host-related genetic differentiation and associations with diapause and adult eclosion time. Most importantly, extensive inversion polymorphism generating significant LD between genetic markers has been found across the *R. pomonella* genome (Feder et al., 1989, 2003; Michel et al., 2010; Egan et al., 2015; Ragland et al., 2017). As a result, it may be easier to detect host-related differences in *R. pomonella*, as many markers may be in LD with the actual targets of divergent ecological selection in the genome. In contrast, chromosomal rearrangements and other features of genomic architecture that reduce recombination and elevate LD may be rarer in *R. indifferens*. Consequently, detecting host-related differences may be more difficult in *R. indifferens*, as the window for finding a marker in LD with the target of selection will be smaller, in centi-Morgans, for the western cherry fruit fly than for the apple maggot fly. Support for this hypothesis comes from the observation that besides the three sex-linked loci WCFF084A, WCFF093, and WCFF105, none of the remaining 16 microsatellites in this study displayed significant LD with one another. Thus, the possibility exists that more extensive screening of the *R. indifferens* genome based on genotyping-by-DNA-sequencing combined with increased sample sizes at paired sites could reveal more significant allele frequency differences and evidence for host-related differentiation between bitter and sweet cherry-infesting flies than we found for the microsatellites.

In conclusion, in contrast to other *Rhagoletis* flies (Feder et al., 1988; Cha et al., 2012; Powell et al., 2012, 2014; Sim et al., 2012), we found little evidence for host races in the western cherry fruit fly, *R. indifferens*. The results could be due to native bitter vs. domesticated sweet cherries not providing strong divergent selection pressures between fly populations, or to the need for more extensive genomic screening to detect host-associated divergence because of a general lack of LD in *R. indifferens*, except for sex-linked markers. Other members of the *R. cingulata* sibling species group related to *R. indifferens* attack different hosts besides cherries, including native olives (*Rhagoletis osmanthi* Bush, host: *Osmanthus americanus* Slight; *Rhagoletis chionanthi* Bush, host: *Chionanthus virginicus* WS Earle) and muttonwood (*Rhagoletis turpinae* Hernandez-Ortiz, host: *Turpinia occidentalis* SwG Don). The two olive-infesting flies are sympatric with the more widely distributed *R. cingulata* in the southeastern USA, whereas *R. turpinae* co-occurs with *R. cingulata* in southern Mexico. It remains to be determined whether these other plants are more conducive to host-related differentiation and initiating ecological speciation in *Rhagoletis* than cherries appear to be. Such studies will provide a more comprehensive understanding of when and why host shifts, and novel environmental opportunities and challenges, generate new biodiversity and when they do not, a central issue in evolutionary biology.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Table S1. Allele frequencies for microsatellite loci analyzed in the study.

Table S2. Mean estimated ln-likelihood, standard deviation, and ΔK (Evanno et al., 2005) calculated across 10 replicates, based on STRUCTURE analyses including all 12 populations for K = 1–12, using 500 000 burn-in iterations followed by 1 000 000 MCMC repetitions under a correlated allele frequencies with admixture model.

Table S3. Mean estimated ln-likelihood and standard deviation across 10 replicates, based on STRUCTURE analysis of paired local fly populations on bitter cherry (BC) and sweet cherry (SC) for K = 1 and 2, using 500 000 burn-in iterations, and 1 000 000 data collecting MCMC repetitions under a correlated allele frequency with admixture model.