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Divergence and evolution of reproductive barriers among three allopatric populations of *Rhagoletis cingulata* across eastern North America and Mexico

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Abstract

Geography is often a key factor facilitating population divergence and speciation. In this regard, the geographic distributions of flies in the genus *Rhagoletis* (Diptera: Tephritidae) in temperate North America have been affected by cycles of Pleistocene glaciation and interglacial periods. Fluctuations in climatic conditions may have had their most dramatic effects on geographically isolating *Rhagoletis* flies in the central highland region of Mexico. During past periods of allopatry, a degree of post-zygotic reproductive isolation appears to have evolved between hawthorn-infesting populations of *Rhagoletis pomonella* (Walsh) in the central Eje Volcanico Trans Mexicano (EVTM) and those from the Sierra Madre Oriental Mountains (SMO) of Mexico, as well as hawthorn flies from the eastern USA. Here, we investigate the generality of this finding in the genus *Rhagoletis* by testing for reproductive isolation among populations of *Rhagoletis cingulata* (Loew) (Diptera: Tephritidae) collected from infested domesticated sweet cherry (*Prunus avium* L.) in the USA and black cherry [*Prunus serotina* Ehrh. (both Rosaceae)] from the SMO and EVTM. We report evidence for marked post-mating reproductive isolation among certain *R. cingulata* populations. The high levels of reproductive isolation were observed between *R. cingulata* flies from populations in the USA and SMO differed from the pattern seen for *R. pomonella*, primarily involving the EVTM. In addition, egg hatch was significantly reduced for crosses between SMO males and EVTM females, but not greatly in the opposite direction. We discuss potential causes for the different patterns of post-mating reproductive isolation among *Rhagoletis* flies.

Introduction

Speciation is the evolutionary splitting of one group of interbreeding populations into two or more reproductively isolated groups. For organisms with sexual reproduction, a key issue in the study of speciation involves understanding how genetically based reproductive barriers to gene flow evolve to separate populations (Mayr, 1963; Schluter, 2001; Coyne & Orr, 2004). Reproductive barriers are typically classified as either pre- or post-mating (Mayr, 1963; Coyne & Orr, 2004), depending upon whether they restrict gene flow before vs. after mating occurs. Establishing the timing and relative importance of pre- vs. post-mating reproductive barriers can provide insights into the history and relative importance of different factors and processes causing population divergence (Coyne & Orr, 1989, 1997). In particular, the evolution of intrinsic post-mating isolation due to genomic conflict often requires geographic isolation as a forerunner for population divergence and speciation (cf. Seehausen et al., 2014). In the absence of gene flow, populations can independently evolve genetic differences due to natural selection or genetic drift that are compatible in their respective

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genomic backgrounds, but cause inviability or sterility in hybrids, termed Bateson–Dobzhansky–Muller incompatibilities (Coyne & Orr, 2004).

A powerful approach to study factors contributing to speciation is to compare groups of distantly related taxa that inhabit the same set of environments (Egan et al., 2013) or geographic regions (Bermingham & Moritz, 1998; Avise, 2009). Using this approach, similarities among groups may provide insights into general mechanisms facilitating speciation, whereas differences may reveal factors important to divergence for some, but not all taxa. Here, we investigate patterns of reproductive isolation among populations of *Rhagoletis cingulata* (Loew) (Diptera: Tephritidae), which infest fruits of many black cherry [*Prunus serotina* Ehrh. (Rosaceae)] from the central Eje Volcanico Trans Mexicano (EVTM) and the Sierra Madre Oriental Mountains (SMO) of Mexico, as well as flies from the eastern USA (US).

The *R. cingulata* species group exhibits evidence of both sympatric and allopatric divergence (Bush, 1966). In comparison, the well-documented *Rhagoletis pomonella* (Walsh) sibling species group is a model for speciation-with-gene-flow via sympatric host plant shifts (Bush, 1969; Berlocher, 2000; Berlocher & Feder, 2002; Xie et al., 2007, 2008). Various aspects of host plant ecology have been shown to cause divergent selection on *R. pomonella* fly populations, generating reproductive isolation in the face of gene flow (Hall, 1943; Reissig & Smith, 1978; Smith, 1986; Bierbaum & Bush, 1990; Smith et al., 1993; Feder et al., 1993, 1994, 1997; Linn et al., 2003, 2004; Schwarz & McPheron, 2007; Yee & Goughnour, 2011). Consistent with the sympatric hypothesis, non-host-related reproductive isolation is either non-existent or extremely limited among taxa in early stages of divergence, including the apple and hawthorn host races of *R. pomonella* and their immediate sister species infesting flowering dogwood, *Cornus florida* L. (Reissig & Smith, 1978; Smith, 1986). Strong ecological barriers accompanied by modest post-mating isolation exist between *R. pomonella* and the more distantly related sibling species attacking snowberries (*Rhagoletis zephyria* Snow) and blueberries (*Rhagoletis mendax* Curran). Strong post-zygotic isolation is observed only between *R. pomonella* and the basal taxon in the *pomonella* group, *Rhagoletis cornivora* Bush, that attacks the silky dogwood, *Cornus amomum* Mill. Thus, a general pattern is observed in the *pomonella* group in which the evolution of extrinsic ecological barriers to gene flow is followed by increasing degrees of post-mating isolation.

However, recent studies on the *pomonella* species group have revealed an exception to this pattern involving hawthorn-infesting populations of *R. pomonella* in Mexico (Rull et al., 2006). Populations of hawthorn flies in the EVTM region of central Mexico appear to have diverged, in part, from *R. pomonella* populations in Mexico and the USA due to periods of past geographic isolation, resulting from glaciations occurring over the last ca. 1.5 My (Feder et al., 2003, 2005; Michel et al., 2007). One such population attacking late fruiting hawthorns in the EVTM may have contributed in facilitating sympatric host shifts documented in the eastern USA. It has been hypothesized that following secondary contact and gene flow, chromosomal inversions originating in the EVTM population introgressed northward and infused USA populations with genetic variation for diapause life history traits that subsequently aided adaptation to host plants with different fruiting phenologies (Feder et al., 2003, 2005; Xie et al., 2007, 2008). Thus, part of the standing variation underlying the sympatric ecological radiation of several of the *R. pomonella* group species and host races may have had geographic origins.

Mating studies have revealed evidence for non-host-related reproductive isolation at the present time among certain hawthorn-infesting *R. pomonella* populations from Mexico and the USA (Rull et al., 2010). In particular, EVTM populations showed a higher degree of post-zygotic isolation from two other fly populations from Mexico (the SMOs and the Chiapas Highlands) and one from the USA (South Bend, Indiana) than the latter did among themselves. Only slight non-host-related pre-mating isolation was observed, again involving EVTM flies.

Only a few studies have examined reproductive isolation for *Rhagoletis* flies outside the *R. pomonella* species group (Hood et al., 2012; Rull et al., 2012; Tadeo et al., 2013). However, analysis of several morphologically differentiated taxa found almost complete assortative mating between adults in eight of nine species combinations (Hood et al., 2012). The only exception was *R. cingulata* and *Rhagoletis indifferens* Curran, which both belong to the *R. cingulata* species group (Bush, 1966). In contrast to the other taxa in the study of Hood et al. (2012), the two cherry-infesting flies displayed only slight morphological differences (in the presence or absence of a wing spot and shading of the femur; Bush, 1966) and showed only a modest and statistically non-significant reduction in mating frequency relative to intra-specific trials. In the *Rhagoletis suavis* (Loew) species group of morphologically distinct walnut-infesting flies, pre- and post-zygotic isolation between taxa can be pronounced. However, Rull et al. (2012) reported little reproductive isolation in the laboratory in hybrid crosses between *Rhagoletis completa* Cresson and *Rhagoletis zoqui* Bush and that the two species appear to be hybridizing naturally in a Mexican contact zone. *Rhagoletis completa* and *R. zoqui* are

genetically very similar, represent the most recently derived taxa in the *R. suavis* group, and overlap in distribution with *R. cingulata* (Rull et al., 2013). However, there was a greater tendency for *R. completa* females to engage in heterospecific matings than *R. zoqui* females (Tadeo et al., 2013) and a degree of hybrid breakdown was observed in the F2 generation (Rull et al., 2012).

In North America, the *R. cingulata* species group is composed of five species and thought to have originated by a combination of sympatric and allopatric modes of speciation (Bush, 1966). Although *R. cingulata* and *R. indifferens* diverged in geographical isolation, *Rhagoletis osmanthi* Bush and *Rhagoletis chionanthi* Bush may be the result of sympatric speciation (Smith & Bush, 1997). No speciation hypothesis has so far been put forth for *Rhagoletis turpiniae* Hernández-Ortiz, a species that infests *Turpinia insignis* (Kunth) Tulasne in cloud forest areas of Mexico, where they are parapatric with black cherry (*P. serotina*) and bear high mtDNA genetic resemblance for COI sequences to *R. cingulata* (Frey et al., 2013). Two ecologically differentiated populations of *R. cingulata* have been recently discovered in Mexico (Rull et al., 2011). The geographic ranges of these two *R. cingulata* populations are disjunct in Mexico. One isolated population currently is found in the central portion of the country and infests the early-fruiting black cherry *P. serotina* ssp. *capuli*, whereas the second population attacks the later fruiting *P. serotina* ssp. *virens* in the Northeastern Sierra Madre Oriental. Further north in the eastern US and Canada, *R. cingulata* infests wild black cherries and commercial sweet and sour cherries. In the western US, bitter cherry, *Prunus emarginata* (Douglas ex Hook.) D. Dietr., is the native host of *R. indifferens*, with western populations also attacking sweet and sour cherries (Bush, 1966). Determining the degree and pattern of reproductive isolation among cherry flies may therefore: (1) help further resolve the taxonomic status of these populations and reveal the existence of cryptic or incipient species; (2) allow comparisons with other groups of *Rhagoletis* flies, including *R. pomonella*, to test for similar effects of biogeography on population divergence (Bermingham & Moritz, 1998; Avise, 2009); and (3) provide evidence supporting or refuting the different modes of speciation hypothesized to have occurred to generate the various species groups of *Rhagoletis* flies. Here, we report on experiments quantifying differences in eclosion timing and reproductive isolation among three populations of *R. cingulata* across a large portion of the fly's geographic distribution: one site from the northeastern USA (West Virginia), one site from northeastern Mexico (Coahuila) in the SMO, and one site from central Mexico (Tlaxcala) in the EVTM.

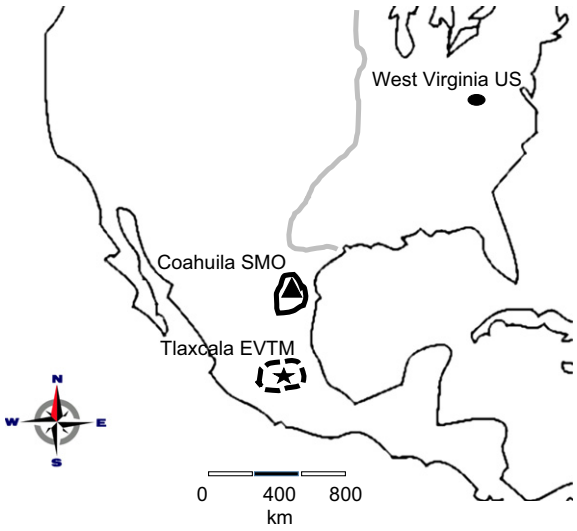

Figure 1 Known distributional ranges of allopatric populations of *Rhagoletis cingulata* from the eastern USA (West Virginia collecting site indicated by oval spot), northeastern Mexico [Sierra Madre Oriental (SMO), Coahuila collecting site indicated by triangle], and central Mexican highlands [Eje Volcanico Trans Mexicano (EVTM), Tlaxcala collecting site indicated by star].

Materials and methods

Source of flies

To obtain *R. cingulata* adults for pre- and post-mating isolation tests, larval infested black cherries were collected from under the canopy of trees at three geographically distant localities. In central Mexico, fruits of *P. serotina* var. *capuli* were collected from May to August in the locality of Huamantla (19.18N, 98.05W) in the state of Tlaxcala on the EVTM. In northeastern Mexico, fruits from *P. serotina* var. *virens* were collected in September in the locality of Los Lirios (25.23N, 100.35W) in the state of Coahuila on the Sierra Madre Oriental (SMO). In the northeastern USA fruit from sweet cherry trees [*Prunus avium* L. (Rosaceae)] were collected in July in Kearnesville, WV, USA (39.23N, 77.59W) (Figure 1). Infested fruit was taken to the laboratory and handled following methods outlined in Rull et al. (2006) to recover pupae. Pupae were placed in 250-ml plastic cups covered with a perforated lid to allow airflow and lined with a 2.5-cm layer of vermiculite that was regularly moistened with a solution of 2 g of benzoate per liter of water to prevent fungal growth.

Diapause regulation

Pupae in cups were left at ambient temperature for ca. 1 month and then transferred to a refrigerator at 5 °C to simulate winter conditions. Pupae from the three populations were left at 5 °C for 14 weeks and then transferred to

an environmentally controlled laboratory at 23 °C, 65% r.h., and a L12:D12 photoperiod. The length of the 'overwintering period' was chosen based on results maximizing adult survivorship for *R. pomonella* US populations by Feder et al. (1997), as no information existed for *R. cingulata* or any Mexican population of *Rhagoletis*. Emergence of adults was recorded on a daily basis.

Longevity

To compare adult lifespan among *R. cingulata* from different populations male–female couples of identical age were placed in cylindrical plastic 2-l cages (25 × 12 × 12 cm) with free access to water and food (a 1:3 mixture of hydrolized protein and sugar). A 1.5-cm-diameter sphere, made with 14.6 g of agar (BDBioxon® Becton Dickinson and Company, Franklin Lakes, NJ, USA) in 400 ml of water and 1.5 ml of green food coloring (McCormick, Sparks, MA, USA) wrapped in Parafilm® (Bemis, Neenah, WI, USA) paper, was hung from the cage ceiling to serve as an egg laying device and mating arena—such devices have previously been used with success in similar studies with other species in the genus *Rhagoletis* (Rull et al., 2010, 2012; Tadeo et al., 2013). In total, 11 cages were set up for each population (33 cages).

Pre-mating isolation

To test for mate discrimination and assortative mating between two allopatric populations of *R. cingulata*, four sexually mature (15–25 days old) flies of each sex from northeastern Mexico (SMO) and four sexually mature flies of each sex from central Mexico (EVTM) (choice) were marked on the back of the thorax with a distinctive dot of vinyl paint according to geographical origin and simultaneously released inside a Plexiglas cage (30 × 30 × 30 cm) with water, food, and eight blueberry fruits hung from the cage ceiling to serve as mating arenas (no cherries were available at the time and blueberries were found to be attractive to flies for mating and egg laying). All copulations and their duration were recorded from 10:00 to 18:00 hours for three consecutive days. The experiment was replicated 4×. Pre-mating isolation tests were performed after the more lengthy and laborious post-mating isolation tests. In addition, USA (US) and Mexican populations responded differently to chilling regimes and an unanticipated substantial number of Mexican flies entered two-season diapause, it was not possible to synchronize adult availability for the three populations. No live US flies were available for these behavioral trials.

Post-mating isolation

To determine whether—and the degree to which—different *R. cingulata* populations are reproductively isolated among themselves, we compared fecundity and fertility for nine no-choice mating combinations of flies: US♂ × US♀, US♂ × SMO♀, SMO♂ × US♀, SMO♂ × SMO♀, EVTM♂ × EVTM♀, EVTM♂ × SMO♀, SMO♂ × EVTM♀, US♂ × EVTM♀, and EVTM♂ × US♀. For each mating combination, seven males and seven females (seven pairs) between 15 and 20 days of age (sexually mature) were placed together inside seven Plexiglass cages (30 × 30 × 30 cm) with free access to water and food. Seven 1.5-cm-diameter agar spheres were hung from the cage ceiling to serve as mating arenas and receive eggs. Due to low fly numbers, for the US♂ × EVTM♀ and EVTM♂ × US♀ combinations only two and three cages, respectively, containing two males and one female each could be set up for fecundity and fertility trials.

Flies were left in cages for 7 days and observed regularly. Fruit guarding, mating, egg laying, and deposition of host marking pheromone activities were observed in cages for all mating combinations. At the end of the 7-day period, agar spheres were replaced by freshly prepared ones to allow egg laying. Thereafter, spheres were replaced every 24 h and eggs removed and counted for seven consecutive days. Eggs deposited in spheres were lined over a dark cloth placed over a piece of cotton in a Petri dish and moistened with a water-benzoate solution (2 g l^{-1} of water). Eggs were incubated at 23 °C and examined under a dissecting microscope after 3, 6, and 9 days to record egg hatch.

Statistical analysis

The length of time in days elapsed from the end of the 14-week simulated winter to emergence of adults was compared among populations and between sexes by means of two-way ANOVAs. Longevity was compared among populations with a Kaplan–Meier survival analysis followed by the Holm–Sidak multiple comparison procedure. Total numbers of copulations were compared among pure and hybrid mating combinations of EVTM and SMO adults using JMATING software. JMATING resamples 10 000 times the observed values of mating pairs to estimate the bootstrap sampling distribution for the estimator (I_{PSI}, YA, and Yule's V). Then the program calculates the bootstrap average and standard deviation as well as the two-tail probability of getting a sexual isolation estimate significantly different from zero (equivalent to random mating) (Carvajal-Rodriguez & Rolan-Alvarez, 2006). Duration of copulations in EVTM × SMO pre-mating isolation tests were compared among mating combinations by means of a repeated measures ANOVA with copulations per mating combination as a factor repeated over three consecutive observational periods. Number of eggs laid and % egg hatch over a 7-day observational period in post-mating

isolation tests were compared among mating combinations by means of nested-factor ANOVAs and Fisher's exact tests. For the EVTM × US combination a GLM one-way ANOVA was used to compare eggs laid per cage and % eclosion. Data were ranked to normalize distributions when necessary using STATISTICA 7 software (StatSoft, Tulsa, OK, USA).

Results

Eclosion time

For the US population (West Virginia), 22 females and 24 males emerged of a total of 120 pupae that were overwintered (38.8% emergence). From the SMO population (Coahuila), 70 females and 43 males emerged from 1 235 pupae (9.1% emergence). From the EVTM population (Tlaxcala), 48 females and 47 males emerged from 445 pupae (21.3% emergence). A two-way ANOVA on ranked emergence data revealed significant differences in mean eclosion time from the end of the overwinter chilling period among populations ($F_{2,253}$ = 199.62, P<0.001), but not between sexes within populations ($F_{1,253}$ = 0.899, P = 0.41). Adults from the SMO population emerged significantly later (mean ± SE = 95.79 ± 1.93 days) than adults from the US (58.38 ± 2.97 days) or the EVTM (55.67 ± 0.93 days) populations (Figure 2).

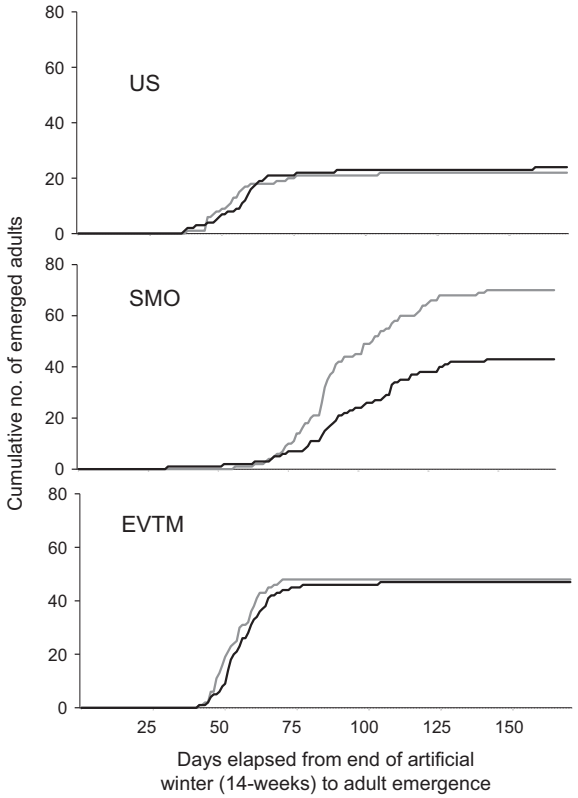

Figure 2 Cumulative emergence curves of *Rhagoletis cingulata* adult female (gray lines) and male (black lines) flies for US (West Virginia), SMO (northeast Mexico), and EVTM (central Mexico) populations.

Longevity

A Kaplan–Meier survival analysis revealed significant differences in longevity among *R. cingulata* populations (Kaplan–Meier Statistic = 27.18, d.f. = 2, P = 0.001). Holm–Sidak multiple comparisons revealed that EVTM adults lived longer (117.27 ± days 8.77) than adults from the SMO population (80.95 ± 7.34 days), which in turn lived longer than the US adults (61.86 ± 5.47 days).

Pre-mating isolation

Partial sexual isolation was detected in behavioral trials between male and female *R. cingulata* from the SMO and EVTM populations. Of a total of 191 observed copulations, 22.0% (n = 42) were between SMO♂ and SMO♀, 18.9% (n = 36) between EVTM♂ and SMO♀, 10.5% (n = 20) between SMO♂ and EVTM♀, and 48.7% (n = 93) between EVTM♂ and EVTM♀. There were more copulations between pure than hybrid couples, resulting in significant incomplete sexual isolation being detected between SMO and EVTM flies (mean I_{PSI} ± SD = 0.404 ± 0.070, P<0.0001; Figure 3). There was no significant difference found in duration of copulation among the different mating combinations ($F_{3,187}$ = 0.840, P = 0.47). SMO♂ × SMO♀ combinations remained in copulation for (mean ± SE =) 29.28 ± 2.76 min, SMO♂ × EVTM♀ for 36.25 ± 6.60 min, EVTM♂ × SMO♀ for 29.13 ± 2.24 min, and EVTM♂ × EVTM♀ for 33.80 ± 1.92 min.

Post-mating isolation: US × SMO

Significant differences in fecundity were detected among the four US × SMO mating combinations (nested-factor ANOVA on ranked data: $F_{3,168}$ = 160.04, P<0.001). Flies from US♂ × US♀ crosses laid more eggs (n = 302 total eggs per cross type) than flies from SMO♂ × SMO♀ crosses (n = 144 eggs), which in turn laid more eggs than flies from SMO♂ × US♀ crosses (n = 66 eggs). Female flies from the US♂ × SMO♀ crosses generated no eggs (Figure 4A). Significant differences were also observed in fertility (i.e., % egg hatch) among US × SMO pure and hybrid mating combinations (nested-factor ANOVA on ranked data: $F_{3,168}$ = 137.42, P<0.001). Eggs oviposited by US♂ × US♀ females exhibited lower hatch rates (43.2%) than eggs deposited by SMO♂ × SMO♀ females (70.2%), whereas for the SMO♂ × US♀ hybrid combination only 4.0% of eggs hatched (Figure 4B). The overall reproduc-

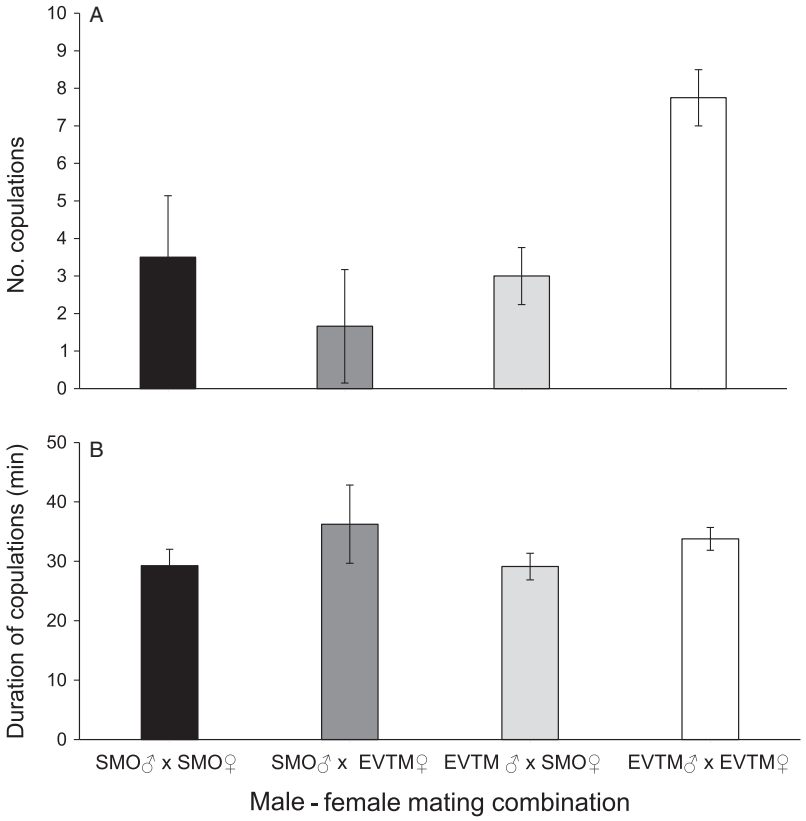

Figure 3 Mean (± SE) (A) number and (B) duration (min) of copulations (total n = 191) per replicate for all possible male–female mating combinations between adult *Rhagoletis cingulata* from the SMO and EVTM populations.

tive output of crosses estimated by multiplying the number of eggs laid by females and the % egg hatch and standardizing relative to 100% for the pure parental US × US cross, were 77.4% for SMO × SMO, 1.9% for SMO♂ × US♀, and 0% for the US♂ × SMO♀crosses.

Post-mating isolation: SMO × EVTM

Significant differences existed among SMO × EVTM crosses in the number of eggs laid by females between pure vs. hybrid mating combinations ($F_{3,168}$ = 37.215, P<0.001). The SMO♂ × SMO♀ crosses produced 144 eggs, the EVTM♂ × SMO♀ crosses produced 129 eggs, the SMO♂ × EVTM♀ crosses produced 366 eggs, and the EVTM♂ × EVTM♀ crosses produced 105 eggs (Figure 5A). Thus, in contrast to the results for US × SMO parings, increased rather than decreased egg laying was seen in the hybrid crosses, at least for SMO♂ × EVTM♀. There were also differences in egg hatch rates among females from the different SMO × EVTM cross-types ($F_{3,168}$ = 5.5375, P = 0.001). The egg hatch rate for SMO♂ × SMO♀ crosses was 70.2%, for EVTM♂ × SMO♀ crosses 58.7%, for SMO♂ × EVTM♀ crosses only 14.4%, and for EVTM♂ × EVTM♀ crosses 70.8% (Figure 5B). The overall reproductive output of SMO × EVTM crosses relative to 100% for the pure parental US × US cross, were 77.4% for SMO × SMO, 57.9% for EVTM♂ × SMO♀, 57.0% for EVTM × EVTM, and 40.2% for SMO♂ × EVTM♀. Thus, in contrast to the results for the US × SMO parings, there was not a dramatic reduction in the net number of offspring reared from hybrid SMO × EVTM crosses, although fewer progeny were obtained from the SMO♂ × EVTM♀ cross than from the other pairings.

Post-mating isolation: US × EVTM

For the EVTM × US comparison, females in the US♂ × US♀ crosses laid more eggs than all other combinations ($F_{3,15}$ = 15.619, P<0.001) (Figure 6A). Flies from US♂ × US♀ crosses laid more eggs (n = 302) than flies from EVTM♂ × EVTM♀ crosses (n = 105 eggs), which in turn laid more eggs than flies from EVTM♂ × US♀ crosses (n = 38 eggs) (only three females) and US♂ × EVTM♀ (n = 15 eggs) (only two females). In addition, differences in egg hatch were detected among cross-types (one-way ANOVA: $F_{3,15}$ = 18.73, P<0.001) (Figure 6B), with the highest mean % egg hatch observed for the EVTM♂ × EVTM♀ cross. The overall reproductive output of US × EVTM crosses relative to 100% for the pure parental US × US cross, were 57.0% for EVTM × EVTM, 57.9% for EVTM♂ × US♀, and 40.2%

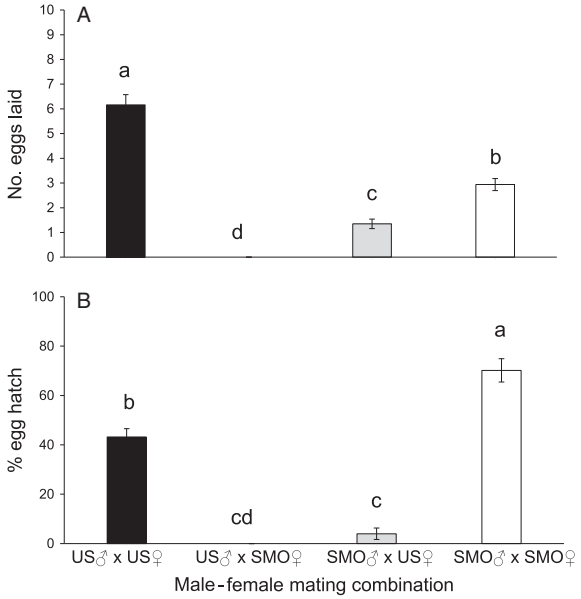

Figure 4 Mean (± SE) (A) number of eggs laid per agar sphere and (B) egg hatch (%) for US × SMO *Rhagoletis cingulata* crosses. Means capped with the same letter do not differ significantly (nested-factor ANOVA on ranked data: P>0.05).

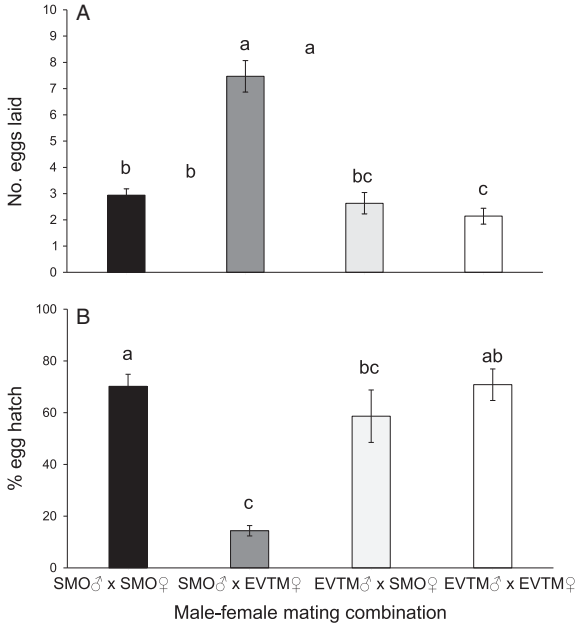

Figure 5 Mean (± SE) (A) number of eggs laid per agar sphere and (B) egg hatch (%) for SMO × EVTM *Rhagoletis cingulata* crosses. Means capped with the same letter do not differ significantly (nested-factor ANOVA on ranked data: P>0.05).

for US♂ × EVTM♀. Thus, like the US × SMO results, hybrid crosses produced a lower net number of offspring, although the reduction compared to pure parental US

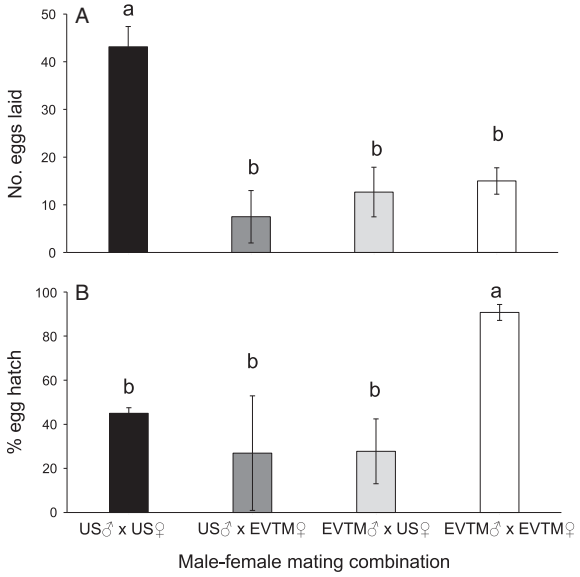

Figure 6 Mean (± SE) (A) number of eggs laid per agar sphere and (B) egg hatch (%) for US × EVTM *Rhagoletis cingulata* crosses. Means capped with the same letter do not differ significantly (GLM one-way ANOVA on ranked data: P>0.05).

and EVTM pairings was not as dramatic as that observed for US × SMO crosses.

Discussion

Our results indicate that US, SMO, and EVTM populations differ in adult eclosion time and longevity, as well as displaying post-zygotic reproductive isolation and, for SMO and EVTM flies, a degree of partial pre-mating isolation. Eclosion and longevity traits may reflect ecological adaptation of US, SMO, and EVTM cherry fly populations to differences in regional environment conditions. Variation in host plant fruiting phenology has been shown to exert strong divergent selection pressures, in general, on *Rhagoletis* populations, including the apple and hawthorn-infesting host races of *R. pomonella* (Feder & Filchak, 2003). *Rhagoletis* flies are univoltine, with flies having one generation per year. Indeed, the 2- to 4-month mean longevities observed for cherry flies in this study are among the longest know for *Rhagoletis*. The life span of *R. pomonella* adults has been estimated at about 1 month in the field (Opp & Prokopy, 1980). As a result, adult eclosion time must match when host fruit ripen on plants to maximize the amount of fruit resources available for *Rhagoletis* courtship and female oviposition to maximize fly fitness. Because *Rhagoletis* host plants differ in when they fruit during the season, there can be strong divergent selection pressures on fly populations to eclose at different

times to match when ripe fruit is present on alternative host plants (Bush, 1966; Feder et al., 1993; Xie et al., 2008). The same can be true among fly populations infesting the same host plant across their geographic range. In this case, latitudinal or local/regional varietal differences can exist in when plants fruit, exerting differential selection pressures among conspecific fly population. This latter situation could explain why *R. cingulata* in the SMO in northeastern Mexico infesting *P. serotina* var. *virens* emerged as adults substantially later than those attacking *P. serotina* var. *capuli* in central Mexico and cherries in West Virginia, USA; *P. serotina* var. *virens* has a significantly later fruiting time than the other black cherries (McVaugh, 1951; Rull et al., 2011).

In a related manner, differences in the host fruiting phenology could also be responsible for the variation observed among populations in adult longevity. In this case, EVTM flies lived longer on average than adults from the SMO and the US. The differences could be due to variation in the fruiting period of host cherries, either reflecting a more limited growing season in the north or differences among host varieties/species sampled in the study. Although the fruiting period of individual trees is relatively short for all the host cherries examined here, there is large variation in fruiting phenology among trees within regions (early-mid and late fruiting individual trees of the same species/subspecies). Most notable, as opposed to native black cherry varieties sampled from Mexico, flies from West Virginia in our study were collected from commercial sweet cherries, a plant with a short fruiting period compared to native black cherries in the same region (Teixeira et al., 2007). However, commercial sweet and wild black cherry host populations in the state of Michigan, USA, do not appear to be genetically differentiated (Smith et al., 2014), and the western cherry fruit fly, *R. indifferens*, a close relative to *R. cingulata* collected from native *P. emarginata* (bitter cherries) in the state of Washington, USA, has been found to live for periods corresponding roughly to half the lifespan of central Mexican populations of *R. cingulata*. Thus, additional work is needed to establish the fruiting windows of other cherry fly hosts to further test the hypothesis that fruiting period strongly selects on adult cherry fly longevity.

Regardless of their cause, the observation of differences in likely highly heritable eclosion and longevity traits—diapause differences have been shown to be strongly genetically based in *R. pomonella* flies (Dambroski & Feder, 2007; Ragland et al., 2011)—implies that life history timing can rapidly evolve and diverge among cherry fly populations. As touched upon above, only minimal genetic differences have been detected to date among cherry fruit fly populations (Frey et al., 2013; Johannesen et al., 2013; Maxwell et al., 2014), suggesting that although allopatric now, US, SMO, and EVTM populations of *R. cingulata* have only relatively recently become isolated from one another. Despite this, EVTM flies took, on average, almost 40 days longer to eclose than those from West Virginia and lived twice as long in the laboratory. Interestingly, US flies were nevertheless more fecund, laying more eggs than females from Mexican populations. The increased fecundity of US females could have been due to their development in sweet cherries, which have been found to yield more fecund *R. indifferens* females than bitter cherries (Yee et al., 2011). Alternatively, discrete fruiting periods in northern latitudes could select for short-lived individuals that invest more heavily in reproduction early in life, a well-known life history trade-off (Partridge et al., 1999; Kirkwood & Austad, 2000), that has been documented for species of *Drosophila* along altitudinal gradients (Norry et al., 2006). However, Moraiti et al. (2012) have found that *R. cerasi* flies from populations exploiting cherries in the highlands of Greece were longer lived and more fecund than adults from areas with shorter fruiting periods along the Greek coast and in Germany. Further work is therefore needed to verify a relationship between fecundity, life span, and host fruiting period among cherry fly populations. Such experiments should control for fly size and host origin (e.g., by rearing flies from all populations in the same host and comparing life history traits among F1 generation individuals of similar size).

Pre-mating isolation among *Rhagoletis cingulata* populations

Pre-mating isolation can often be strong among *Rhagoletis* flies outside of the *R. pomonella* sibling species group. For example, Hood et al. (2012) examined sexual isolation between pairs of nine morphologically differentiated species of *Rhagoletis*. The only instance where a substantial number of hybrid matings occurred was between US populations of the eastern cherry fly, *R. cingulata*, and the western cherry fly, *R. indifferens*, with partial sexual isolation observed between these two species (I_{PSI} = 0.27). We also found partial pre-mating isolation between *R. cingulata* populations from the SMO and EVTM (I_{PSI} = 0.43). The current geographic isolation of SMO and US *R. cingulata* populations in North America and their close genetic relationship based on mtDNA (Frey et al., 2013) implies that the pre-mating isolation likely evolved relatively recently following the separation of the populations associated with Pleistocene glacial-interglacial climatic cycles. As the populations do not presently overlap to form a hybrid zone, it would seem unlikely that the pre-mating isolation arose due to reinforcement favoring increased sexual isolation to avoid the production of unfit hybrids.

Post-mating isolation among *Rhagoletis cingulata* populations

We also found evidence for post-mating isolation among US, SMO, and EVTM populations of *R. cingulata*. Most notably, hybrid crosses between US and SMO flies yielded either no eggs or substantial reductions in egg hatch, depending upon the direction of the cross. Post-mating isolation was also detected between US and EVTM flies, although to a lesser extent than the SMO. The strength of the post-mating isolation between Mexican and US cherry flies was somewhat surprising given the implication from mtDNA of a fairly recent separation between these same populations (Frey et al., 2013). Post-mating isolation due to Bateson–Dobzhansky–Muller (D-M) incompatibilities are generally thought to accumulate gradually through time as mutations differentially establish between allopatric populations that are compatible within parental gene pools but cause genomic conflict (negative pleiotropic fitness consequences) when brought together in genomes of mixed hybrid ancestry (Coyne & Orr, 2004). Indeed, allopatric populations of hawthorn-infesting flies in the EVTM also display post-zygotic isolation with other *R. pomonella*, but the degree of reproductive isolation is less than the levels observed in this study of *R. cingulata*. However, US and EVTM populations of hawthorn flies show much more pronounced mtDNA differentiation than cherry flies from the same regions, suggesting an older time of divergence for *R. pomonella* than for *R. cingulata* dating back over 1.5 Mya (Feder et al., 2003, 2005; Xie et al., 2007, 2008). The high rate that post-mating isolation has arisen in *R. cingulata*, therefore, suggests that other factors than just the evolution of D-M compatibilities may contribute to the reproductive isolation between US and Mexican populations.

One potential cause for the apparently rapid evolution of post-mating isolation in *R. cingulata* could be that the SMO flies were reared from black cherries and the US population originated from sweet cherry. It is possible that some host-related adaptation (e.g., the observed difference in eclosion timing) or aspect of the host plant environment could generate post-zygotic incompatibility. However, this explanation seems unlikely for several reasons. First, sweet and black cherry-infesting populations of *R. cingulata* co-occur in the eastern USA and there is no evidence for any reproductive isolation or genetic differentiation between them (Smith et al., 2014). The lack of differentiation among flies exploiting different plant species/varieties has also been documented for *R. indifferens* (Maxwell et al., 2014). Second, the black cherry attacking EVTM population also had an early mean eclosion time like the sweet cherry-infesting US population, but did not display a dramatic decrease in net reproductive output in crosses with late-eclosing SMO flies, as US flies did.

A second possibility is that Mexican and US populations possess different communities of endosymbiotic bacteria (e.g., different *Wolbachia* strains) that cause cytoplasmic incompatibilities between flies. *Rhagoletis* do harbor *Wolbachia* and different strains have been documented in the fly (Schuler et al., 2009, 2013). Such an explanation could account for the rapid appearance of post-zygotic isolation among certain *R. cingulata* populations. However, further work is needed to verify that *Wolbachia* contributes to reproductive isolation in *Rhagoletis* and, if so, that this can occur rapidly.

A final possibility is that conflicting meiotic drive systems are acting in *R. cingulata* from the US and SMO, and in hybrids of mixed ancestry, and that these competing systems are causing problems during gamete formation, fertilization, and/or zygote development, resulting in decreased egg production and hatching. As with cytoplasmic incompatibility, genomic conflict caused by meiotic drive is a hypothesis requiring further testing.

Conclusion

We have documented the existence of life history, partial pre-mating, and extensive post-zygotic reproductive isolation between certain populations of cherry-infesting *R. cingulata* from the USA and Mexico. Based on mtDNA differentiation, these differences can be inferred to have arisen rapidly between cherry fly populations, potentially associated with ancestral *R. cingulata* taking refuge and becoming isolated in Mexico during Pleistocene glaciations and interglacial periods. Although the biogeography of *R. cingulata* shows similarities with that of hawthorn-infesting populations of *R. pomonella*, including the existence of currently separated populations of flies in the US, SMO, and EVTM, the timing of geographic isolation and the degree and pace that post-mating reproductive isolation has evolved appears to differ. Thus, *R. pomonella* and *R. cingulata* may have initially become geographically isolated and primarily evolved their differences during subsequent glacial cycles. Moreover, the cause(s) for post-mating isolation may differ between these flies (possibly standard D-M incompatibilities for *R. pomonella* vs. *Wolbachia* or meiotic drive for *R. cingulata*). These hypotheses require additional testing but imply that similar biogeography at the current time can mask differences in the history and mechanisms underlying the evolution of reproductive isolation and the radiation of taxonomic groups of *Rhagoletis* flies.

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