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## Phenological Attributes and Phylogenetic Relationships of *Rhagoletis juniperina* Marcovitch (Diptera: Tephritidae) in the Great Lakes Region

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### Abstract

*Rhagoletis juniperina* Marcovitch (Diptera: Tephritidae) infests Eastern Red Cedar (*Juniperus virginiana* L.) and other North American junipers. While several *Rhagoletis* species are of interest as orchard crop pests (apple maggot, blueberry maggot, cherry fruit fly) and as models for studying speciation (*R. pomonella* Walsh species group), *R. juniperina* is of interest because it may tie together evolutionarily the Nearctic and Palearctic *Rhagoletis* fauna. One goal of this study was to test two competing hypotheses first proposed by Bush (1966): i) that *R. juniperina* is more closely related to the Nearctic dogwood-infesting *R. tabellaria* (Fitch), to which it is morphologically similar; or ii) that *R. juniperina* is more closely related to the Eurasian juniper-infesting *R. flavigenualis* Hering. To study *R. juniperina*, which is rarely collected, we first established a local study site by collecting juniper berries from several sites in the Lansing, MI vicinity in fall 2010, finding a heavily-infested juniper tree on the Michigan State University campus. Preliminary mitochondrial COII sequences of reared pupae matched (99.8%) the *R. juniperina* COII sequence in GenBank, allowing tentative identification of these flies as *R. juniperina*. Subsequently, the morphology of adults reared from these pupae the following spring and summer confirmed this diagnosis. Phenological attributes of the Farm Lane Bridge population were determined via weekly fruit collections in fall 2011 and 2012, and “peak” larval infestation was found to occur during the first part of October, while mean post-diapause eclosion time was found to be approximately 103 days. *Rhagoletis juniperina* adults were also reared from infested junipers found in Wisconsin and North Carolina, indicating that the geographic range of *R. juniperina* on *J. virginiana* is broader than previously thought. Hymenopteran parasitoids of *R. juniperina* were also observed; both the egg parasitoid, *Uteles juniperi* (Fischer) (Hymenoptera: Braconidae), and a new pupal parasitoid (*Coptera* n. sp.) (Hymenoptera: Diapriidae) were reared from fruit and pupae, respectively, collected at the MSU campus site. Subsequent phylogenetic analyses based on mitochondrial COI sequences did not resolve the relationships of *R. juniperina* and *R. pomonella* or flies in the *Rhagoletis tabellaria* species group. The sole *R. flavigenualis* individual in our sample was placed sister to an unresolved trichotomy of three clades containing these Nearctic taxa. The analysis also revealed within-species haplotype variability in *R. juniperina*, with a 3.8% nucleotide sequence difference observed between COI sequences of the flies from MI, WI, and NC compared to the Ontario *R. juniperina* sequences in the Barcode of Life database.

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*Rhagoletis juniperina* Marcovitch (Diptera: Tephritidae) is one of 25 described North American *Rhagoletis* species, and one of 65 described *Rhagoletis* species worldwide (Bush 1966, Smith and Bush 2000). The primary host plant of *R. juniperina* in the Great Lakes region is the Eastern Red Cedar (*Juniperus virginiana* L.), which is broadly distributed across the eastern US and extends into southern Ontario. Collections of *R. juniperina* in the eastern US and Canada have been reported from Massachusetts, New York, Ontario, Illinois, Iowa and Texas (Bush 1966, Foote et al. 1993, Jackson et al. 2011), but the fly has only been reared from infested *J. virginiana* fruit in Massachusetts, New York, and Illinois (Bush 1966, Foote et al. 1993, Berlocher, unpublished). *Rhagoletis juniperina* has also been reported from outside the geographic range of *J. virginiana* in Manitoba and several western US states (Bush 1966, Foote et al. 1993), leading to speculation that the host range is greater than its current distribution. However, the only known *R. juniperina* specimen reared from a host apart from *J. virginiana* was a single male reared from *J. monosperma* (Engelm) Sarg. from New Mexico (Bush 1966).

*Rhagoletis juniperina* presents an interesting set of taxonomic, phylogenetic systematic, and evolutionary questions. Bush (1966) pointed out that *Juniperus* spp. were probably part of the Holarctic Arcto-Tertiary geoflora, and that insects associated with juniper hosts should follow similar distributional patterns. Hering (1958) reared *R. flavigenualis* from juniper in Turkey, and the wing pattern of these flies was similar to the pattern observed in *R. juniperina*. Thus, Bush (1966) hypothesized that *R. juniperina*'s closest relatives may be Palearctic, or conversely, that *R. juniperina* might be more closely allied with members of the *R. tabellaria* (Fitch) group, which show morphological similarities to *R. juniperina* and infest shrubby dogwoods (*Cornus* spp.) across North America. Bush (1966) originally placed *R. juniperina* within the *tabellaria* species group. Molecular markers have been used to test these competing hypotheses, and indeed, *R. flavigenualis* Hering disrupted the monophyly of the Nearctic *Rhagoletis* species groups in a phylogenetic study using mitochondrial COII sequences (Smith et al. 2005/6), a result that has been corroborated using DNA sequences from the nuclear CAD locus (Hulbert et al., unpublished observations).

Despite the broad distribution of its potential hosts, *R. juniperina* is rarely collected, and even more rarely reared, from juniper host fruit. *Rhagoletis juniperina* is not an economically important pest species, and the abundance and widespread distribution of its juniper hosts complicates finding infested fruit. Many basic biological attributes of *R. juniperina* remain poorly characterized. *Rhagoletis juniperina* have a typical *Rhagoletis* life history, with females laying their eggs in the fruit of juniper trees in late summer – early autumn. Eggs hatch, go through three larval instars, and when the fruit fall to the ground, 3<sup>rd</sup> instar larvae emerge from the fruit and burrow to a depth of 1–5 cm in the soil, where they pupate, enter diapause, and overwinter (Bush 1992). Diapause breaks as the ground warms in the spring and summer of the following year, and adult flies emerge later in the season, in synchrony with ripening host juniper fruit.

The phenological characteristics of *R. juniperina*, such as timing of adult activity, timing of host fruit availability, and post-diapause eclosion time have not been characterized, nor have rates and peak periods of larval infestation and peak periods of host fruit infestation. Thus, our primary objective in this study was to establish one or more study populations of *R. juniperina* and use these to determine these basic biological attributes. In addition, the braconid parasitoid wasp, *Utetes juniperi* (Fischer) has been reported infesting *R. juniperina* (Wharton and Marsh 1978), and Forbes et al. (2012) reported a new *Coptera* species from *R. juniperina*. Thus, a second objective of this study was to characterize the wasp guild associated with *R. juniperina* and determine rates of parasitism. Finally, a third goal was to determine the mitochondrial DNA

relationships of *R. juniperina* to *Rhagoletis* flies in the *tabellaria* and *pomonella* Walsh species groups. Determination of these relationships potentially can shed light on the history of host associations in *Rhagoletis* as they diverged and radiated into North America.

## Methods

**Field Survey and Insect Collections.** In our initial survey, fruit (juniper berries) were collected from Eastern Red Cedar (*Juniperus virginiana*) trees at seven locations in the East Lansing area between 22 September and 27 October 2010. Trees were selected based on the amount of fruit available for collection, as well as by proximity to other trees from which fruit were collected. Collections in subsequent years were made from Eastern Red Cedar trees at two localities in the East Lansing, MI area, and at single localities in Avoca, WI and Durham, NC.

**Insect Rearing: Determination of Timing and Rates of Larval Infestation.** Timing of peak larval infestation was determined by rearing pupae from juniper fruit collected weekly at the Farm Lane Bridge site from the first of September until mid-November in both 2011 and 2012, with collections being made on the same calendar date in each year. Fruit collected from each tree was laid over moist vermiculite and held at ambient temperature (23-28°C) in a 9" × 13" growers' flat for 28 days. This mimicked the natural process of falling to the ground and allowed the larvae to emerge and pupate in the vermiculite just as they would in soil. The vermiculite was sifted and the pupae, as well as the fruit, were counted to determine larval infestation rates.

**Insect Rearing: Post-diapause Ecdysis Times.** Except for a subset of pupae that was frozen immediately for DNA isolation, all isolated pupae were transferred to vermiculite-filled Petri dishes (100 × 15 mm) and held for 16 weeks in a refrigerator at 6-7°C to simulate over-wintering. After 16 weeks, the Petri plates were placed at 21-24°C under fluorescent lights. Flies and parasitoid wasps were collected from Petri plates every few days as they emerged and placed in cages for 48h to allow exoskeletons to cure prior to freezing them (flies) at -20 °C or preserving them in 95% EtOH (wasps). Emergence curves were generated for all collections by plotting cumulative percent emergence as a function of number of days since the Petri plates were removed from the refrigerator (post-diapause ecdysis time; PDET). The PDET was calculated as the day by which 50% of adults had emerged.

**Mitochondrial DNA PCR and Sequencing.** Mitochondrial COII gene fragments were PCR-amplified using the primers George (C1-J-2792; 5'- ATA CCT CGA CGT TAT TCA GA - 3') and Eva (TK-N-3722; 5'- GAG ACC ATT ACT TGC TTT CAG TCA TCT - 3') developed by Bogdanowicz et al. (1993). PCR was carried out in a total reaction volume of 25 µL containing GeneScript *Taq* polymerase (0.5 µL; 2.5U), 0.5 mM Mg<sup>2+</sup>, 0.2 mM dNTP mix (0.05 mM each), 0.5 µM of both forward and reverse primers, and 50 - 100 ng of template DNA. Reactions were run on an iCycler (BioRad Laboratories, Hercules, CA) using the following temperature profile: 5 min at 95 °C, 35 cycles of 2 min at 94 °C, 90 sec at 52 °C, 2 min at 72 °C; and 7 min at 72 °C for a final extension cycle. The barcode region of the mitochondrial COI gene was amplified using the primers LepF1 (5'- ATA CCT CGA CGT TAT TCA GA - 3') and LepR1 (5'- GAG ACC ATT ACT TGC TTT CAG TCA TCT - 3') developed by Smith et al. (2007) using the same PCR conditions described above. PCR products were purified to remove salts and excess primers using QIAquick PCR Purification Kits (Qiagen Sciences, Germantown, MD). Sequences of both strands were carried out at the MSU Research Technology Support Facility via BigDye Terminator Sequencing using the PCR primers and an Applied Biosystems 3730xl DNA Analyzer (Foster City, CA). Forward and reverse sequences were obtained as .ab1 files and edited using the computer program FinchTV (Geospiza, Inc.,

Seattle, WA). Paired sequences were then used to create consensus sequences for each individual fly. New sequences were submitted to GenBank (Accession #'s KP100550 – KP100561).

**Molecular Variation and Phylogenetic Analysis.** DNA sequences were aligned using the computer program Muscle (Edgar 2004) as implemented in MEGA5.2.2 (Tamura et al. 2011). To assist with the identification of pupae from the Farm Lane Bridge site, putatively identified as *R. juniperina*, DNA sequences of the mitochondrial COII gene were obtained. This allowed direct comparison with known sequences from *R. juniperina* (U53243) and *R. pomonella* (U53229.2). Subsequently, COI sequences were used in phylogenetic analyses to study the relationships of the *R. juniperina* flies from Farm Lane Bridge, MI, Durham, NC and Avoca, WI, to the four Barcode of Life Database (BOLD) sequences reported from *R. juniperina* on the Bruce Peninsula, in Ontario (EU484529 - EU484532). In addition, BOLD sequences from *R. tabellaria* and *R. pomonella* were included in the alignment, as were our own COI sequences from *R. tabellaria*, *R. electromorpha* Berlocher, *R. pomonella* and *R. flavigenualis*, which form part of a larger study on *Rhagoletis* species relationships (Hulbert et al. unpublished). Mitochondrial COI sequences from *R. striatella* Wulp and *R. basiola* (Osten Sacken) served as outgroups. The resulting 633 nucleotide COI alignment was deposited in TreeBase (www.treebase.org) in Study S16845. Phylogenetic analysis was carried out using MrBayes 3.1.2 (Ronquist et al. 2012) with 1,000,000 generations, with trees sampled every 200 generations, discarding the first 20% of these as a burnin. Two independent runs of four chains each were run, with convergence considered to have occurred when the average standard deviation of split frequencies between runs was < 0.02. The best-fit DNA substitution model for this set of sequences was determined to be GTR + I + G using ModelTest (Posada and Crandall 1998) in conjunction with PAUP 4.0b10 (Swofford 2000).

## Results

**Occurrence of *R. juniperina*.** Of seven locations surveyed in 2010 in the Lansing area, two locations yielded pupae that we tentatively identified as *R. juniperina*. The Farm Lane Bridge (FLB) site on the MSU campus yielded over 100 pupae with infestation rates in the 2010 collections ranging from 0.5-26.4% (Table 1), while the Okemos Meijer location yielded only two pupae. Mitochondrial COII gene sequences obtained from four representative pupae collected from the Farm Lane Bridge site were 99.8% similar (549/550 nucleotide sites) to the *R. juniperina* reference sequence in GenBank (U53243; Smith and Bush 1997). On the other hand, these COII sequences had 27 fixed differences from the *R. pomonella* reference sequence (26 transitions, 1 transversion; 21-3<sup>rd</sup> codon position substitutions, 6-1<sup>st</sup> position substitutions, 1 non-synonymous). We concluded that the flies infesting the Farm Lane Bridge juniper were indeed *R. juniperina* based on these mitochondrial COII sequences, the wing pattern similarity of adults reared from these pupae to *R. juniperina* (Bush 1966), and the identity of the host plant (*J. virginiana*) from which the pupae were collected and adult flies were reared. This collection appears to be a new state record for Michigan.

In fall 2011 and 2012, additional field sites with infested junipers were located in East Lansing, MI (Burcham Drive and Bessey Hall), in Durham, NC (Loco), and near Avoca, WI. Adults reared from these collections also had characteristic *R. juniperina* wing banding patterns. Further, mitochondrial COI sequences obtained from three individuals from the Farm Lane Bridge site, and from single individuals from the Durham, NC and Avoca, WI sites, were identical to each other with the exception of four singleton nucleotide substitutions, all synonymous 3<sup>rd</sup> positions, at nucleotide positions 114, 177, 246 and 513 in the 633 base pair alignment. Thus, the flies reared from eastern red cedar at the

Table 1. *Rhagoletis juniperina* collections made in Michigan, North Carolina and Wisconsin in 2010-2013.

State	Locality (County)	Date	Collector*	# fruit	# pupae (% infestation)	adult flies reared	parasitoids reared#
MI	Meijer (Ingham)	22 Sep 2010	JS	1300	2 (0.2)	1	
	Farm Lane Bridge (Ingham)	4 Oct 2010	MF	970	93 (9.6)	7	7
	Farm Lane Bridge (Ingham)	6 Oct 2010	JS	2800	689 (24.6)	96	24
	Farm Lane Bridge (Ingham)	20 Oct 2010	MF/JS	2000	88 (4.4)	2	1
	Farm Lane Bridge (Ingham)	28 Sept 2011	MF	750	16 (2.1)	1	
	Farm Lane Bridge (Ingham)	5 Oct 2011	MF	670	50 (7.5)	1	
	Farm Lane Bridge (Ingham)	12 Oct 2011	MF	950	86 (9.1)	1	
	Farm Lane Bridge (Ingham)	19 Oct 2011	MF	750	12 (1.6)	0	
	Farm Lane Bridge (Ingham)	26 Oct 2011	MF	900	18 (2.0)	0	
	Farm Lane Bridge (Ingham)	2 Nov 2011	JS/MF/WA	1800	31 (1.7)	6	
	Farm Lane Bridge (Ingham)	9 Nov 2011	MF/JS	1000	1 (0.1)	0	
	Farm Lane Bridge (Ingham)	21 Sep 2012	MF	600	13 (2.2)	0	
	Farm Lane Bridge (Ingham)	28 Sep 2012	MF	600	30 (5.0)	0	
	Farm Lane Bridge (Ingham)	5 Oct 2012	MF/WA/JS	1700	252 (14.8)	14	9
	Farm Lane Bridge (Ingham)	12 Oct 2012	MF/WA	930	232 (24.9)	64	16
	Farm Lane Bridge (Ingham)	19 Oct 2012	MF	750	147 (9.6)	62	8
	Farm Lane Bridge (Ingham)	26 Oct 2012	MF	800	24 (3.0)	0	
	Farm Lane Bridge (Ingham)	2 Nov 2012	MF/WA	900	8 (0.9)	0	
	Farm Lane Bridge (Ingham)	9 Nov 2012	MF/WA	650	6 (0.9)	0	
	Farm Lane Bridge (Ingham)	11 Oct 2013	MF/SB	1600	307 (19.2)	nd	
NC	Bessey Hall (Ingham)	12 Oct 2011	MF	1300	35 (2.7)	7	
	Bessey Hall (Ingham)	19 Oct 2011	MF	825	47 (5.7)	1	
	Burcham Drive (Ingham)	26 Oct 2011	SS/JS	2100	120 (5.7)	47	17
	Durham Loco1 (Durham)	5 Nov 2011	JS/BW/JL/LL	1450	55 (3.8)	1	
	Durham Loco3 (Durham)	5 Nov 2011	JS/BW	950	12 (1.3)	3	
WI	Avoca Pleasant Hill (Iowa)	11 Oct 2012	MS/TS	1670	22 (1.3)	5	

\* MF, Megan Frayer; JS, Jim Smith; WA, William Armstrong; SB, Sydney Barosko; SS, Serdar Satar; BW, Brad Williamson; JL, Jon Logsdon; LL, Laurie Lebo; MS, Mike Smith; TS, Ted Smith

nd – not determined

# All parasitoids reared from infested seed cones were *U. juniperi*

sites in North Carolina and Wisconsin were determined to be *R. juniperina*, and both of these collections appear to represent new state records, which increases the distribution of this fly (Fig. 1).

**Timing of Infestation and Infestation Rates.** Weekly collections of juniper berries at the Farm Lane Bridge site in both 2011 and 2012 indicated that peak larval infestation occurred during the first half of October, with the peak both years occurring in the Oct. 12 collection (Fig. 2). While infestation rates were considerably lower in 2011 than in 2012 (see below), the temporal pattern was the same. No pupae were reared from fruit collected prior to Sept. 21<sup>st</sup> or after Nov. 9<sup>th</sup> either year.

The Farm Lane Bridge juniper site had a 24.6% larval infestation rate on Oct. 6, 2010, with subsequent peak rates of 9.1% on Oct. 12, 2011 and 24.9% on Oct. 12, 2012. (The low peak and overall infestation rates observed in 2011 likely resulted as an artifact of fruit and larval desiccation during the rearing process.) Infestation rates at other collection sites were consistent with the pattern observed at Farm Lane Bridge. The Meijer collection in 2010 had an infestation rate of 0.2% on 22 September, the Burcham site had a 5.7% infestation rate on Oct. 26, 2011, and the Durham, NC site was infested at 1.3 - 3.8% on 5 November 2011. The anomaly within the dataset was the Avoca, WI collection, in which the collection made on 11 October 2012 was only infested at 1.3%. However, the fruit from Wisconsin were stored in a drink cooler for 72h prior to being shipped to our lab in Michigan and placed on vermiculite, which may partially explain the low infestation rate.

**Adult Emergence and Post-diapause Ecdysis Timing.** Of the 821 pupae from the 2011 collection that were overwintered, 106 adult flies emerged in the lab between 22 May and 8 August 2012 (overall emergence rate of 12.9%). Similarly, the 2012 collection yielded 631 pupae from which 140 adult flies emerged between 3 May and 15 August 2013 (overall emergence rate of 22.2%). Emergence curves were generated and PDET calculated for the fly collections made at Farm Lane Bridge in 2010 and 2012, and at Burcham Drive in 2011 (Fig. 3). PDET for these three fly collections was found to be an average of 103 days. This compares with 76 days for *R. zephyria* Snow (Riverwalk 2011; n = 25), 64 days for *R. pomonella* (Clinical Center Apple 2011; n = 21), and 55 days for *R. cingulata* (Loew) (NW Festival 2010; n = 37), reared under similar conditions in our lab from collections made in 2010 - 2012.

**Parasitic Hymenoptera.** The braconid parasitoid, *U. juniperi* emerged from pupae reared from junipers collected at Farm Lane Bridge in 2010 and 2012, and Burcham Drive in 2011. Rates of parasitism ranged from 3.7% (based on % of pupae infested) at Farm Lane Bridge in 2010 to 14.1% at Burcham Drive in 2011. Wasp emergence, as measured by PDET, lagged behind fly emergence by an average of 27 days in all three of these collections (Fig. 3), with PDET of 103 days (Farm Lane Bridge 2012), 108 days (Burcham Drive 2011), and 113 days (Farm Lane Bridge 2010), respectively.

We also reared a new species of *Coptera* (Hymenoptera: Diapriidae) from pupae that were isolated from soil beneath the Eastern Red Cedar tree at Farm Lane Bridge in June 2011 (Forbes et al. 2012). From 204 pupae, we reared 58 *R. juniperina* (28.4%), 30 *U. juniperi* (14.7%), and 6 *Coptera* n.sp. (2.9%). The new *Coptera* wasp species will be described in a separate publication.

**Phylogenetic Relationships.** A phylogenetic analysis was carried out based on the mitochondrial barcode region of COI (Hebert et al. 2003) to study the relationships of the *R. juniperina* we collected from MI, WI, and NC to *Rhagoletis* flies in the *tabellaria* and *pomonella* species groups. Originally placed in the *tabellaria* group (Bush 1966), subsequent studies listed *R. juniperina* as unplaced within any species group (Smith et al. 2005/6). We also included in our analysis COI sequences from four individuals in the Barcode



Figure 1. Updated geographic distribution map for *R. juniperina* (adapted from Foote et al. 1993). Open circles are locations of *R. juniperina* from Foote et al. (1993), closed circles are two known, but unpublished, localities, and open triangles are locations surveyed in this study. Sites in Manitoba and the western US are outside the range of Eastern Red Cedar.

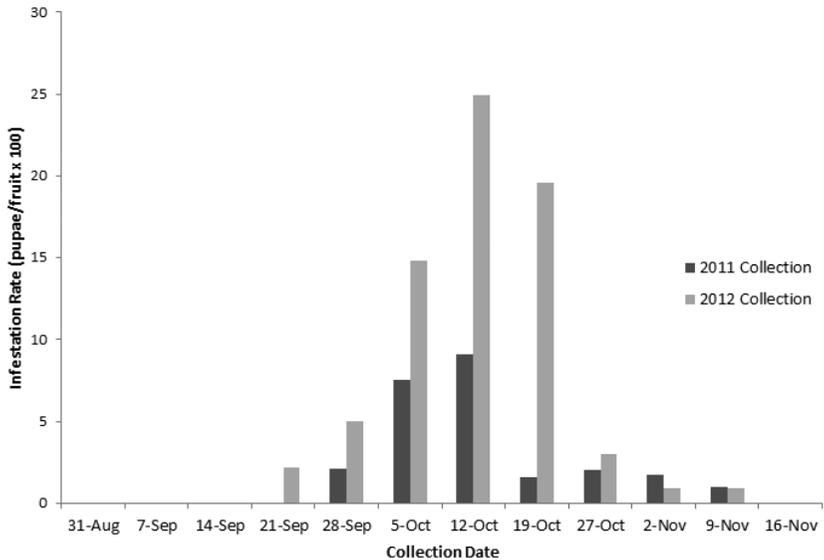


Figure 2. Time course of larval infestation of juniper at the Farm Lane Bridge site in 2011 and 2012. Fruit was collected on every date shown at 1-week intervals from 31 August through 16 November in both years. Pupae were reared from collected fruit and counted. y-axis values are expressed as the percentage of the fruit that were infested. Peak infestation was found to be in the first two weeks of October.

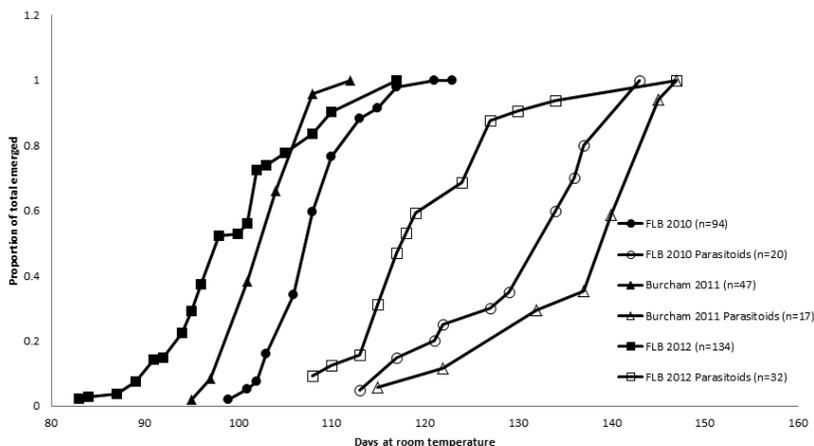


Figure 3. Emergence curves of *R. juniperina* adult flies and *Uletes juniperi* wasp parasitoids for 2010-2012 collections. The 2010 and 2012 emergence curves represent pooled data from all Farm Lane Bridge collections those seasons. The PDET of the flies is approximately 103 days, with parasitoid emergence following by approximately 25 days.

of Life Database (BOLD) identified as *R. juniperina* (Jackson et al. 2011), as well as a single sequence available from the Palearctic *R. flavigenualis*, which infests juniper in the Old World.

The MI, WI and NC *R. juniperina* COI sequences together formed a clade that was a sister group to the BOLD *R. juniperina* COI sequences (Fig. 3). The COI sequences from the MI, WI and NC *R. juniperina* differed from the BOLD *R. juniperina* COI sequences by an average of 3.8% (24 differences over 633 nucleotides), with 4-1<sup>st</sup> codon position substitutions and 20-3<sup>rd</sup> codon position substitutions, all of which were synonymous. The relationship of the *R. juniperina* clade to the *tabellaria* group and the *pomonella* group was not resolved by the phylogenetic analysis of the COI barcode region. The Palearctic juniper-infesting *R. flavigenualis* was placed sister to these three clades in the analysis.

## Discussion

**How common is *R. juniperina*?** *Rhagoletis juniperina* may be neither rare nor uncommon in the eastern United States. While the fly has been described as being rare (Foote et al. 1993), our limited field survey and collections made in 2010 – 2013 indicate that *R. juniperina* may actually be both common and abundant within the range of the Eastern Red Cedar, *J. virginiana*, which serves as its primary host in eastern North America. We reared adult *R. juniperina* from infested *J. virginiana* fruits from three sites in the East Lansing, Michigan area and from single sites in both Wisconsin and North Carolina. All of these collections appear to represent new state records. *Rhagoletis juniperina*, originally described from flies reared from *J. virginiana* in Ithaca, New York (Marcovitch 1915), was subsequently reared from *J. virginiana* fruit collected in Lexington, Massachusetts by Bush (1966). Presumably due to its lack of economic importance, *R. juniperina* is not often collected, a fact that is complicated by the presence of many potential juniper hosts within its geographic range, in particular horticultural varieties. Of the collections that we made, only *J. virginiana* was infested, with no flies emerging from potential juniper hosts that were more horticultural with respect to fruit size and habit.

A more extensive field survey may reveal that *R. juniperina* is both widespread and common on its native host in eastern North America. *Juniperus virginiana* was very widespread and common until the turn of the 20<sup>th</sup> century, when the eastern forests were harvested for this tree, primarily for its use to make pencils (Peattie 1948).

*Rhagoletis juniperina* has been reported from junipers outside the range of *J. virginiana* in the southwestern US, California, and the Pacific Northwest (Foote et al. 1993). Hosts in these areas include *J. monosperma* (Arizona; Bush 1966) and *J. occidentalis* ssp. *australis* in the Sierra Nevada. These hosts are relatively abundant and the extent of their infestation with *R. juniperina* is unknown. Similarly, the relationships of the flies infesting these western hosts to the flies infesting *J. virginiana* in the eastern US are unknown.

***Rhagoletis juniperina* Phenology.** We determined larval infestation rates, the timing of larval infestations, and the post-diapause eclosion times of adult *R. juniperina* collected at two localities in the vicinity of the Michigan State University campus in East Lansing. Peak larval infestation in MI occurred during the 1<sup>st</sup> and 2<sup>nd</sup> weeks of October in both 2011 and 2012. Thus, host fruit availability occurs relatively late in the season for *R. juniperina*, and the window during which fruit are available for infestation appears to be rather short. Consistent with this finding is the fact that PDET is long for *R. juniperina*, approximately 103 days, which is consistent with the late seasonal availability of host fruit for larval infestation. Data for our comparison taxa, *R. zephyria* (76 days), *R. pomonella* (apple, 64 days), and *R. cingulata* (55 days) were similar to published values; Forbes et al. (2009) reported a PDET of 68.8 days for *R. pomonella* (apple). One shortcoming of this current study, however, is the lack of characterization of adult flight activity of *R. juniperina* in the vicinity of its host plants. This is the subject of a future study.

**Hymenopteran Parasitoids.** Both *Uteles* and *Coptera* hymenopteran wasps were reared from *R. juniperina* flies from Farm Lane Bridge and presumably represent egg/larval and pupal parasitoids, respectively. *Rhagoletis juniperina* serves as host for *U. juniperi* (presumably an egg parasitoid) and *Coptera* species typically parasitize *Rhagoletis* pupae. Prokopy and Webster (1978) demonstrated that *U. canaliculatus* (Gahan) oviposits in the host eggs. Nonetheless, the developmental stage that is used by *U. juniperi* remains to be determined. Lags in wasp emergence are well known in *Rhagoletis* species, with braconids typically emerging 30-40 days after their fly hosts (e.g., Forbes et al. 2009).

***Rhagoletis* phylogenetics.** DNA sequences of mitochondrial COI did not allow us to make inferences about the relationships of *R. juniperina* to flies in the *pomonella* and *tabellaria* species groups of the genus *Rhagoletis*. Phylogenetic analysis of the COI region of *R. juniperina* from Michigan in the context of *pomonella* and *tabellaria* species group exemplars analyzed here failed to provide resolution of these three major clades (Fig. 4). In the COI tree, *R. flavigenualis* was placed basal to *R. juniperina*, *R. tabellaria* and *R. pomonella*, indicating divergence from *R. juniperina* prior to the divergence of the *tabellaria* and *pomonella* species groups. Interestingly, while the Palearctic juniper-infesting *R. flavigenualis* is sister to *R. juniperina*, *R. tabellaria* and *R. pomonella* in the COI tree, it is placed sister to *R. juniperina* when nuclear CAD and 28S genes are examined (Hulbert et al., unpublished observations).

The COI DNA data indicate that *R. juniperina* collected on the Bruce Peninsula in Ontario (Jackson et al. 2011) by Malaise trapping in late July 2003 may potentially be a different species. Although our *R. juniperina* samples matched the COII *R. juniperina* sequence in GenBank, they formed a separate group from the COI sequences obtained from the BOLD. While the mitochondrial barcode regions (COI) from the four representative *R. juniperina* flies from Michigan, Wisconsin, and North Carolina collections were similar to each other, the COI

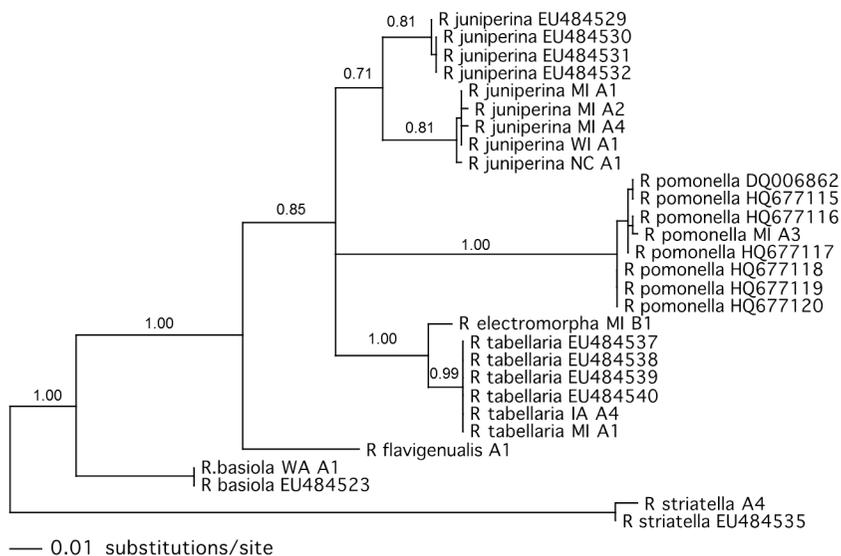


Figure 4. Phylogenetic relationships of *R. juniperina*, *R. tabellaria*, *R. pomonella*, and *R. flavigenualis*. Bayesian analysis of mitochondrial COI sequences from our *Rhagoletis* collections and as well as sequences from the BOLD was performed using MrBayes, with the GTR+I+G evolutionary model, and 1,000,000 generations of MCMC. Branches show posterior probabilities.

sequences for Bruce Peninsula flies differed from these by 3.8% (p-distance). In addition, the flies from Bruce peninsula in Ontario were collected as adults in July. *Rhagoletis juniperina* adult activity in July would appear to be too early for flies with a peak larval infestation time in October, unless the adults were extraordinarily long-lived in the field. Estimates of adult longevity in the field for *Rhagoletis* species are typically 30-40 days (Boller and Prokopy 1976). Thus, either *R. juniperina* consists of two widely divergent mitochondrial COI haplotypes, or the *R. juniperina*-like flies collected in July 2003 on the shores of Lake Huron may be using an as yet unidentified juniper species (perhaps *J. communis* or *J. horizontalis*) as its larval host, and may represent a new *Rhagoletis* species. Sorting out this situation may also help to explain the collection of *R. juniperina* in Manitoba (Foote et al. 1993), which is well outside the range of *J. virginiana*.

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