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Host specificity and variation in oviposition behaviour of milkweed stem weevils and implications for species divergence

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Abstract. 1. An herbivore's life-history strategy, including optimization of resource use, is constrained by its evolutionary history and ecological factors varying across the landscape.

2. We asked if related and co-distributed herbivore species maintain consistency of host preference and oviposition behaviours along the species' range. We surveyed two putative species of milkweed stem weevils, *Rhyssomatus lineaticollis* and *R. annectens*, which co-occur alongside their hosts, *Asclepias syriaca* and *A. incarnata*.

3. We confirmed the two species status of weevils, supported by differences in morphology and a bilocus gene phylogeny. Furthermore, we found that species divergence recapitulated the weevils current host plant use.

4. We found oviposition variation within and between species. *R. annectens* poked the stem haphazardly or girdled it before oviposition. Meanwhile, *R. lineaticollis* primarily trenched stems in the north, but poked or girdled in the south. Variation in oviposition patterns could be a response to variation in host plant defenses.

5. In nature, weevils strictly oviposited on their respective host plants, while in bioassays, *R. lineaticollis* exhibited strong preference for *A. syriaca* and *R. annectens* fed equally on both host plants.

6. Overall, our results support that milkweed stem weevils are strict specialists but might be undergoing changes in host use. *R. lineaticollis* specializes on *A. syriaca* but has two distinct modes of oviposition. Meanwhile *R. annectens* seems to be more accepting of other hosts. We hypothesize that these weevils might be shifting host use associated with changes in host plant distributions.

Key words. Clinal patterns, host specialization, oviposition strategies, phenotypic variation.

Introduction

With the diversification of flowering plants came the radiation of herbivorous insects, many of which have specialized and are dependent on particular plants to complete their life cycle (McKenna *et al.*, 2009; Wiens *et al.*, 2015). The degree to which insects specialize on host plants is cornerstone to our understanding of insect evolution and ecology (Tilmon, 2008; Nylin *et al.*, 2014; Wiens *et al.*, 2015; Wang *et al.*, 2017). For instance, one of the best-known models for the high diversity of insect herbivores is the coevolutionary escape-and-radiate hypothesis, which suggests that specializing insects adapt to novel plant defences, facilitating host shifts to related species (Ehrlich & Raven, 1964; Thompson, 1999). How an insect specializes on a specific host plant resource is dependent on its evolutionary constraints and spatio-temporal variation of ecological interactions.

Determining the eco-evolutionary processes that lead to insect herbivore specialization requires an understanding of population-level processes. Whether differentiated populations are a single species or a complex of cryptic species exhibiting niche partitioning is a critical starting point to assess specialization (see examples Hebert *et al.*, 2004; Smith *et al.*, 2008). In addition, three fundamental factors affect population level

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specialization: (1) insect behaviour, (2) host plant quality, and (3) host plant abundance (Fox & Morrow, 1981). Adaptive traits involved in recognition and use of host plants (e.g. oviposition behaviours), avoidance of plant defenses, host availability, and plant co-occurrence with the herbivore should all play an important role in limiting host plant use by specialized insects (Fox & Morrow, 1981. McLeish *et al.*, 2007).

A frequent argument for why plant defenses affect the evolution of insect herbivores is the negative correlation between the degree of plant toxicity and the number of insect herbivores that can thrive under such toxicity (Ehrlich & Raven, 1964; Fox & Morrow, 1981; Rasmann & Agrawal, 2011). For example, plants in the genus *Asclepias* produce cardenolides and latex as defensive mechanisms and only a handful of insects (c. 12) are able to feed on them (Agrawal *et al.*, 2008, 2012; Rasmann, 2014; Birnbaum & Abbot, 2018). Thus, specialization and costs of specific adaptations are implied as important influences in the evolution of herbivores (Karageorgi *et al.*, 2019). Variation in these adaptive traits among milkweed insects makes them an excellent system to study plant-herbivore co-evolution and the role that plant defenses play in insect specialization (Price & Wilson, 1979; Van Zandt & Agrawal, 2004; Birnbaum & Abbot, 2018).

In addition, a high level of specialization is also expected for insects that complete their whole life cycle on one host plant (Price, 2003), such as milkweed stem weevils (Coleoptera: Curculionidae: *Rhyssomatus* spp.). Strict specialization (i.e. monophagy, or feeding on a single host plant species) is suspected for milkweed stem weevils, stem borers specialized on milkweed plants. Two closely related putative species, *Rhyssomatus annectens* (Casey, 1895) and *Rhyssomatus lineaticollis* (Say, 1824), are thought to be strict specialists on *Asclepias incarnata* and *Asclepias syriaca*, respectively (Price & Wilson, 1979; St Pierre & Hendrix, 2003). Even though milkweed stem weevils are one of the seasonally earliest herbivores on *Asclepias* plants and can affect the insect community on these plants (Price & Wilson, 1979; Van Zandt & Agrawal, 2004), only anecdotal information is available about the degree of host fidelity and the role of host distribution for the specialization of these weevils.

The species status of these putative species is unclear. They were originally distinguished by only a few external morphological traits that are not entirely discrete (i.e., *R. annectens* should have a smaller prothorax than *R. lineaticollis*, and oblique pronotal rugae) (Casey, 1895). Despite the presumed specialization, there are reports that *R. lineaticollis* occasionally feeds on seedpods of *A. syriaca* (Price & Wilson, 1979; Fordyce & Malcolm, 2000) and, to a lesser degree, on at least four other milkweed species (Price & Wilson, 1979; Chaplin & Walker, 1982; Betz, 1989). Furthermore, *R. annectens* larvae can apparently feed on stems of *A. syriaca* and *A. incarnata* plants (Price & Wilson, 1979), which generate uncertainty as to the validity of *R. lineaticollis* and *R. annectens* as separate species. While *R. annectens* dispersal ability is not clear, *R. lineaticollis* seems to be a poor disperser that relies heavily on very local milkweeds (Betz, 1989; St Pierre & Hendrix, 2003). We addressed whether the milkweed stem weevils (*Rhyssomatus* spp.) are distinct species, strict specialists, if host fidelity is constant, and if there are oviposition behaviour differences across the species range along the east coast of North America.

We first tested if species divergence is linked to host plant specialization and revised the species status of *Rhyssomatus* weevils collected both on *A. syriaca* and *A. incarnata*. Under a scenario of ecological specialization and speciation, plant toxins should impose strong selection on insect herbivores (Mopper *et al.*, 1995; Schluter, 2009; Nyman *et al.*, 2010). Therefore, we expected morphological and phylogenetic divergence between *R. lineaticollis* and *R. annectens* that is consistent with their host plant use. Because milkweeds vary in the level and composition of plant defense within and among species (Agrawal *et al.*, 2008; Woods *et al.*, 2012; Fig. S1), we also expected to see strong host plant fidelity by feeding adults. Using a series of surveys and bioassays, we also assessed variation in oviposition strategies that might be relevant to circumventing host plant defenses (i.e., location of oviposition on the plant or tissue manipulation before oviposition, such as stem poking that might reduce latex in egg chambers). Variation in oviposition strategy is particularly relevant for herbivorous insects that have limited dispersal ability and where larval performance depends entirely on the host plant on which they were oviposited (Akimoto, 1990; Thompson & Pellmyr, 1991; Bonebrake *et al.*, 2010). Given that previous work on milkweed plants found variation of host plant defenses along a latitudinal gradient (Woods *et al.*, 2012), we studied host use and oviposition behaviour along the same geographic gradient in northeastern U.S.A. and Canada. We also expected to observe oviposition differences across the milkweed stem weevils, matching host plant defense variation.

Materials and methods

Study system

We studied milkweed stem weevils (Coleoptera: Curculionidae: *Rhyssomatus* spp.) to determine if different populations of weevils varied in oviposition behaviour and larval diet. Previous studies working with milkweed stem weevils have concentrated in a few populations of *R. lineaticollis*, finding that they are most active in the spring (May–June) and have one or two broods per year (Price & Wilson, 1979; Franson & Willson, 1983; Fordyce & Malcolm, 2000; personal observations). Adult weevils feed mostly on leaves and are thought to assess plant quality and chemistry by chewing and poking the plant (Fordyce & Malcolm, 2000; personal observations). They poke the leaf midribs, stems, and seedpods, allowing the plant latex to potentially be drained from the tissue (Fordyce & Malcolm, 2000; Agrawal & Malcolm, 2002; Agrawal & Van Zandt, 2003). In some cases, the poked holes will serve as oviposition chambers where the eggs will hatch and larvae will develop inside the stem or in the seedpods of milkweed plants. If oviposition scars are visible on the stems, larvae are usually found inside the stem eating the stem pit. Larvae have also been found feeding from seeds inside seedpods (Price & Wilson, 1979; Fordyce & Malcolm, 2000; personal observations). There are no published records of larvae moving from the stem to the seedpods or to other milkweed stems. Our personal observations showed that *Rhyssomatus* eggs usually hatch around day 10 after oviposition. Larvae developed for 3–4 weeks, and pupae reached maturity after 2 weeks. We assumed that adults overwinter in the ground near the crown root of the milkweed stem based on our life cycle observations and capture rates in early spring. Given the very limited dispersal of the juvenile stages, oviposition behaviour by adults should be crucial for growth and survival of the larvae (Poore & Steinberg, 1999; Scheirs *et al.*, 2000).

Weevil collections and survey locations

During the late spring and summer in each year of 2014 to 2017, we visited locations across the northeast region looking for both species of milkweeds and *Rhyssomatus* weevils. While little is known about the distribution of these weevils, *R. lineaticollis* has been reported in 22 states in Central and Eastern U.S.A., and *R. annectens* has been reported in 5 states in Eastern U.S.A. and Texas (O'Brien & Wibmer, 1982). These records indicate that these two putative species might overlap in New York state (O'Brien & Wibmer, 1982). Accordingly, we decided to concentrate our collection and survey efforts in New York state and surrounding regions. We visited most milkweed patches along a predetermined geographic region for 2–3 days at a time (~320 km were driven per day in one direction. 72 days total). We used topographic maps from New York, Pennsylvania, and New Jersey (DeLorme® 2003), as well as Google Maps to select locations with swamps or near water bodies; we also stopped along the way if we saw evidence of a milkweed patch. We checked the plants for weevil damage, and if found, we manually collected weevils for 2–3 h at each patch, as well as recorded the type of oviposition damage observed. Adult weevils were collected for bioassays and transported live to the greenhouse in deli-cups, or stored in 96% ethanol and then in a − 80°C freezer for molecular analyses.

Weevil identity: Morphology

Weevils in the genus *Rhyssomatus* (Coleoptera: Curculionidae) are a taxonomically difficult group, with 17 described species in North America, that needs to be revised (last genus revision by Casey, 1895; Arnett *et al.*, 2002). To determine if host specialization was consistent between the two *Rhyssomatus* species, it was important for us to analyse morphological and genetic differences, as historically, species identity may have been confounded by host plant species. We analysed morphological characters by measuring head length, pronotum length and width, scape length, femur length, tibia length, and elytra length, following Marvaldi and Lanteri (2005), from one to six weevils per location (23 locations, $n = 58$). Since weevil size can also be attributed to plant quality, we also visually assessed qualitative traits of genitalia in six males and six females from four locations (weevils collected from each host plant, one location in the north and one in the south).

We used the seven variables from the morphological dataset to perform a multivariate analysis of variance (MANOVA.; manova in R v3.5.1. See all models in supplementary materials), testing for population differentiation among the weevils collected on different host plants. We also used this approach to test morphological variation on the weevils collected on *A. syriaca* that presented different oviposition behaviours. Finally, we also performed an analysis of variance (ANOVA; FactoMineR package and aov in R v3.5.1.) on weevil total length (i.e. the sum of head, pronotum, and elytra length) to test for morphological differences among weevils that had different host plants and different oviposition behaviours.

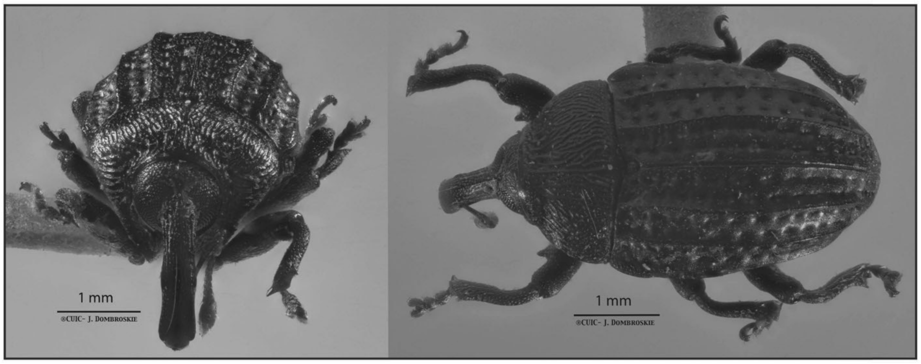
Weevil identity: Molecular analyses

To establish the extent of genetic differences and monophyly between *R. lineaticollis* and *R. annectens*, we extracted genomic DNA from whole bodies or three legs depending on tissue availability from 52 milkweed stem weevils (6 locations with weevils collected from *A. incarnata* and 19 locations with weevils collected from *A. syriaca*) and 3 outlier weevils in the *Rhsyssomatus* genus (*R. pruinosus* ID 1410365-1, *R. rovalis* ID 14010486-1, and *R. palmacollis* ID 14010391-1 collected by Bruno de Medeiros). We disrupted the tissue with liquid nitrogen and used the DNeasy blood & tissue kit (QIAGEN) for DNA extraction. We amplified the mitochondrial gene cytochrome oxidase I (CoxI) using HCO (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) and LCO (5′-GGTCAACAAATCATAAAGATATTGG-3′) primers (Folmer *et al.*, 1994; McKenna *et al.*, 2009); and the nuclear gene arginine kinase (ArgK) using the primers forB2 (5′-GAYTCCGGWATYGGWATCTAYGCTCC-3′) and revB2 (5′-GTATGYTCMCCRCGRGTACCACG-3′)(Dole *et al.*, 2010). The PCR reactions were performed using the following protocols: for CoxI, 95°C for 5 min; 6 cycles of 95°C for 45 s, touchdown 55°C to 49°C for 45 s, and 72°C for 1:45 s; 28 cycles of 95°C for 45 s, 49°C for 45 s, and 72°C for 1:45; 72°C for 5 min; and kept at 10°C. For ArgK, 95°C for 2 min; 34 cycles of 95°C for 1 min, 58°C for 45 s, and 72°C for 1 min; and 72°C for 5 min and kept at 10°C.

After obtaining the CoxI and ArgK gene sequences, we checked all the nucleotide traces for sequence concordance and coded all ambiguous calls using SeqMan codes. The consensus sequences generated were trimmed to 570 bp (CoxI), or 620 bp (ArgK) and aligned (ClustalW) using the DNASTAR software. The sequence data were partitioned and substitution models for the codon positions were generated using PartionFinder (see model partitions in Table S2). We inferred species identity by generating Bayesian trees with the substitution models for each gene with the program MrBayes in the Cipres platform (Miller *et al.*, 2010). The Markov Chain Monte Carlo (MCMC; 4 chains) search ran twice for 10 000 000 generations and sampled every 100th iteration; stability of parameters was confirmed with Tracer v1.6 (Rambaut *et al.*, 2014).

We compared our inferred tree with three topological models to test whether our tree topology was consistent with a priori hypothesis (Fig. 3a, unconstrained model not shown). We hypothesized that specialized weevils formed two separate clades depending on host plant of origin. For the first model (our hypothesis), we constrained weevils that came from *A. syriaca* and *A. incarnata* to different monophyletic clades, and the outgroup to a different third clade. For model 2, we

(A) *Rhyssomatus lineaticollis*



(B) Phenotypes of milkeed stem weevil damage.

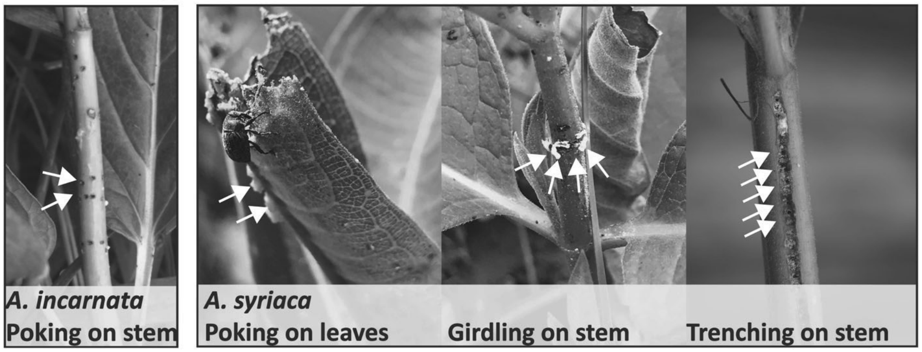


Fig 1. (a) Frontal and dorsal views of *R. lineaticollis*. (b) Oviposition phenotypes (arrows pointing towards poking, girdling, or trenching) observed in the field on *A. incarnata* and *A. syriaca*. (a) *Rhyssomatus lineaticollis*; (b) phenotypes of milkeed stem weevil damage.

only constrained the outgroup to a monophyletic clade. Finally, model 3 had no constrains. We ran all MCMC searches as described above and statistically determined which of the three models best explained our initial tree topology. We compared the log likelihood scores (LnL) using the posterior simulation-based analogue of Akaike's Information Criterion (AICM) with 100 bootstrap replicates.

Weevil and host plant survey

In those locations, where we found weevil damage on *Asclepias* plants, we classified the type of oviposition damage as poking when there was no apparent pattern in the oviposition scars left by weevils after laying their eggs (Fig. 1). The girdling pattern occurred when the weevils poked holes surrounding the apical meristem and other parts of the stem; we found weevils' eggs between the girdled sections (Fig. 1b). This type of manipulation could potentially produce stem death at an early stage of plant development as we observed in the field. Trenching behaviour occurred when a weevil made a longitudinal trench towards the base of the stem and eggs are laid inside this trench (Fig. 1b).

We choose nine patches of *A. syriaca* and five patches of *A. incarnata* during the 2015 and 2017 field seasons to compare variation of damage type within and among populations. We selected patches based on previous presence of weevil damage and strove to cover southern, central, and northern regions in the North East and Mid-Atlantic regions where the two weevils seemed to overlap in range (O'Brien & Marshall, 1986). We recorded frequency and type of oviposition damage per patch. If the patch had more than 130 plants, we recorded information from a 10 m × 10 m plot within the patch. For each patch during 2015, we also chose five sets of plants to measure stem diameter, type of damage (Fig. 1b, trenching and poking only: girdling is considered within poking), and amount of damage (i.e. number of oviposition scars). Each set of plants consisted of one weevil-damaged plant and the nearest undamaged plant that was at least 1 m apart.

For our survey of weevil oviposition behaviour, we only analysed locations where weevil damage was present and divided our data into two different sets. The first one is an analysis of presence/absence of two different types of behaviours: poking (including girdling because this behaviour seems to be an extension of poking and its observation is not reliable in the field), or trenching per patch. The second data set is the frequency of each type of damage for the 14 locations surveyed for damage frequency in 2015 and 2017 (Fig. 4c,d). We ran statistical analyses for the frequency data but the low sample size did not allow for model convergence. Instead, we described these patterns using pie charts and qualitatively compare them to the presence/absence data. We analysed the presence/absence data with a generalized linear mixed model (glmer; lme4 and lmerTest packages in R v3.5.1) with a binomial distribution and type of damage as a response variable (i.e. poking or trenching). Host plant (i.e. plant species where the weevil was collected) was a fixed variable and it was nested within location. We included latitude as an explanatory variable because latex exudation varies by plant species and it is correlated with latitude (Woods *et al.*, 2012; Fig. S1). Latitude was centred to zero by subtracting the latitude value to the mean latitude to prevent lack of convergence in the model (all statistical analyses were performed in R version 3.5.1. See all models in the supplementary material).

Behaviour experiment 1: Host plant acceptance

We tested for weevil host acceptance and type of oviposition behaviour in a transplant experiment in the greenhouse. In 2014, we tested a total of 114 weevils from 20 locations (41 weevils from *A. incarnata* and 73 from *A. syriaca*) throughout the spring and summer in a total of five experiments (an average of 22 weevils per experiment). Each experiment consisted of trial A and B, where one weevil was exposed to both plants in a randomized order. After collecting weevils, we starved the weevils for a day, after which we randomly placed half of the weevils on *A. incarnata* and half on *A. syriaca* plants. Plants were cut from a natural field population on the same day of the experimental trial and were placed in a 50 ml conical tube with water. We labelled the weevils with a combination of three coloured dots on the thorax and/or elytra using a paint pen (Craft Smart®) to identify individuals. We then caged each plant with a mesh sleeve to prevent weevil movement from plant to plant. We determined acceptance of plant and oviposition behaviour by scoring feeding and oviposition marks after 24 and 48 h (trial A). At this point, we removed the weevils, starved them for a day, and switched the treatment plant to the plant species the weevils had not experienced previously (trial B). In other words, if the weevil was initially placed on *A. syriaca*, it was now placed on *A. incarnata* and vice versa for weevils that were initially on *A. incarnata*. We scored their behaviour again at 24 and 48 h.

Behaviour experiment 2: Host plant preference

In 2015, we determined feeding preference of weevils by cutting leaf discs (12 mm) of *A. syriaca* and *A. incarnata* from plants from a natural population in Ithaca, NY. We collected the two youngest fully expanded leaves from over 30 plants. From each leaf tip, we cut two discs (one on each side of the midrib). We tested a total of 123 weevils from 12 locations (20 weevils from *A. incarnata* and 103 from *A. syriaca*). We placed one disc of each plant species in a small plastic cup (30 ml). Both discs sat at the bottom of the cup without touching and we placed one weevil in the centre. After 24 h, we removed the weevil and quantified the amount of tissue consumed in both plants using ImageJ (1.49v).

Behaviour experiments: Statistical analyses

We analysed the data using a generalized mixed effects model (glmer; lme4 and lmerTest packages in R v3.5.1) to determine acceptance and preference of host plant. Specifically, we tested if there is host specialization among the weevils we collected and whether there is an association with oviposition type. Location and weevil ID were random variables. Location was nested within host plant (i.e. the plant species on which the weevil was collected), and weevil ID was nested within treatment plant. Host plant, treatment plant, and their interaction were fixed factors. Herbivory and frequency of girdling were the response variables.

Results

Weevil identity

The two species status was supported by morphological and molecular analyses. Most weevils collected from *A. incarnata* were determined to be *R. annectens* based on smaller body size (i.e. seven morphological measurements), genitalia shape differences (i.e. narrower female and male terminalia, as well as different shapes), and an 'annectens clade' based on both genetic markers (i.e. CoxI and ArgK genes). Similarly, most weevils collected on *A. syriaca* were determined to be *R. lineaticollis*. Based on these results, we continue using the original weevil species names below, *R. annectens* and *R. lineaticollis*, and discuss potential ambiguity in the data.

Weevil identity: Morphology

We found size and genitalia trait differences between *R. annectens* and *R. lineaticollis. Rhyssomatus annectens* (collected on *A. incarnata*) tends to be smaller in size (mean total length ± se: 9.7 mm ± 0.2 mm) than *R. lineaticollis* (collected on *A. syriaca*. Figure 2a; mean total length ± se: 11.9 mm ± 0.7 mm; ANOVA $F_{1,56} = 4.867$, $P < 0.05$). This trend was similar for all the morphological traits we measured (MANOVA, $F_{7,49} = 17.921$, $P << 0.05$), except for elytra length (Fig. S6). Elytra were on average of equal size for the two weevil types (ANOVA, $F_{1,55} = 1.147$, $P \gg 0.05$). Since *R. lineaticollis* showed two distinct oviposition behaviours, we also analysed if there was a body size difference based on this phenotype, but there was no statistical difference (Fig. 2b; ANOVA $F_{1,39} = 0.901$, $P \gg 0.05$).

We also noted qualitative differences in the weevil's internal morphology when comparing individuals from different host plants and locations (Fig. 2. LMAH and SRD independently assessed three males and three females from each location and found consistent results). *Rhyssomatus annectens* has genitalia with subtly distinct morphology (See Figs. 2 and S3–S5). The male terminalia have an aedeagal median lobe that is overall narrower and more broadly curved in lateral view, and the manubrium and spiculum gastrale are narrower compared to those in *R. lineaticollis* (Fig. 2c–e compared to Fig. 2h–j. Also see Fig. S4–S5). Regarding the female terminalia, while there were also slight differences between the two species in the shape of the spermatheca and sternite VIII; the main difference was in tergite VIII, which had a broad, round membranous region in *R. annectens* and an elongate, narrow region in *R. lineaticollis* (Fig. 2f–g,k–l).

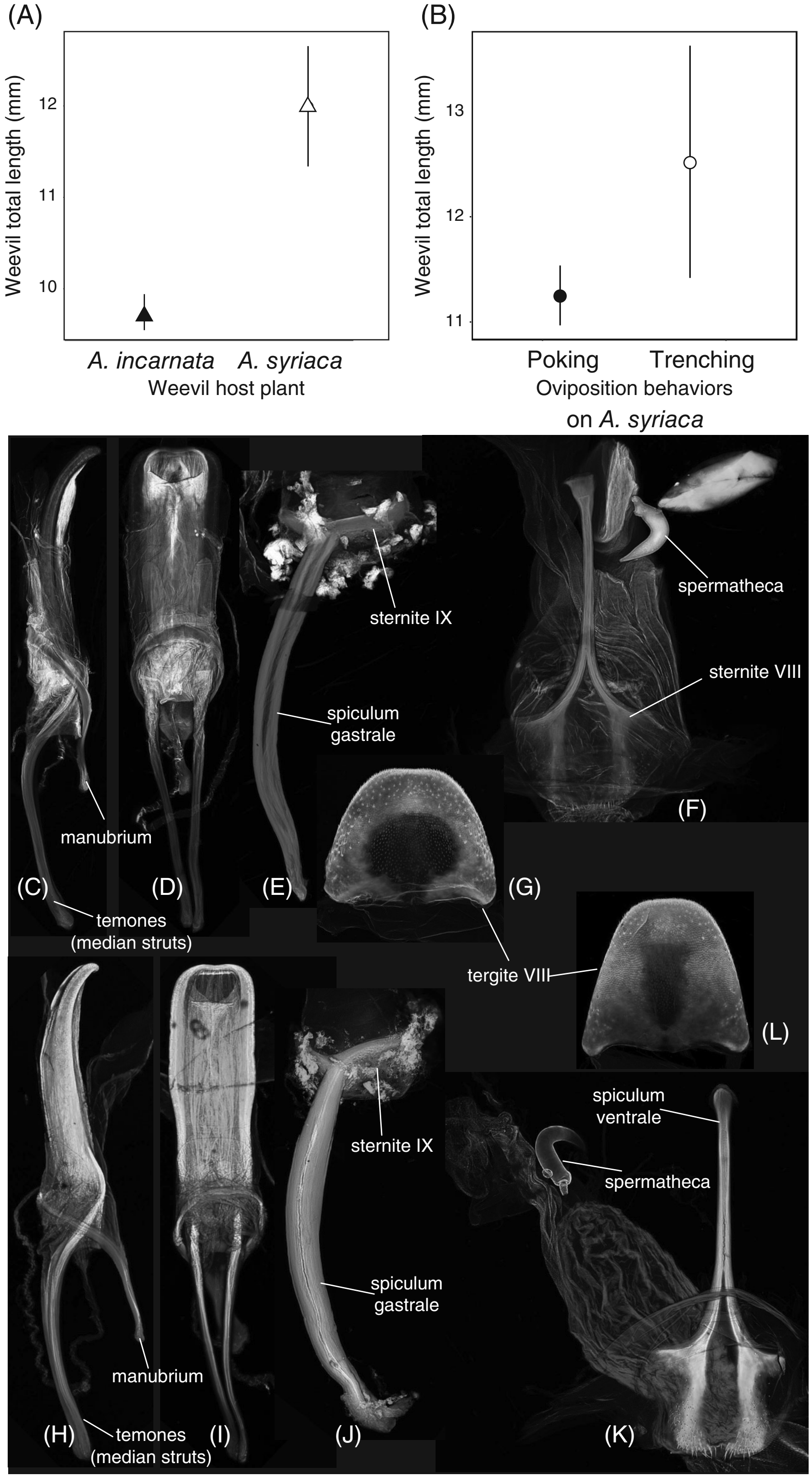


Fig 2. (a) Black triangle shows average total length ± SE of weevils collected on *A. incarnata* and the open triangle shows the average total length ± se of weevils collected on *A. syriaca*. (b) Total length ± se by oviposition behaviour of weevils collected on different patches of *A. syriaca* (black circles: poking, and open circles: trenching). Male and female terminalia of *R. annectens* (c–g) and *R. lineaticollis* (h-l): (c–e,h–j) male terminalia; (f–g,k–l) female terminalia. c,h) Aedeagus, lateral view; (d,i) aedeagus, dorsal view; e,j) sternite IX; f,k) sternite VIII and spermatheca; (g,l) tergite VIII. Notice the more robust spiculum gastrale in the male and narrower membranous region in the middle of tergite VIII in female of *R. lineaticollis*. [Colour figure can be viewed at wileyonlinelibrary.com].

Weevil identity: Molecular analyses

When analysing the unconstrained trees, both molecular markers showed support for at least one clade with *R. annectens* using *A. incarnata* as hosts (Fig. 3b,c). However, the tree topologies using these markers were drastically different, showing incongruence among different clades. The phylogeny obtained with the nuclear marker ArgK showed all *R. annectens* as part of the same clade with 79% bootstrap support. Additionally, there

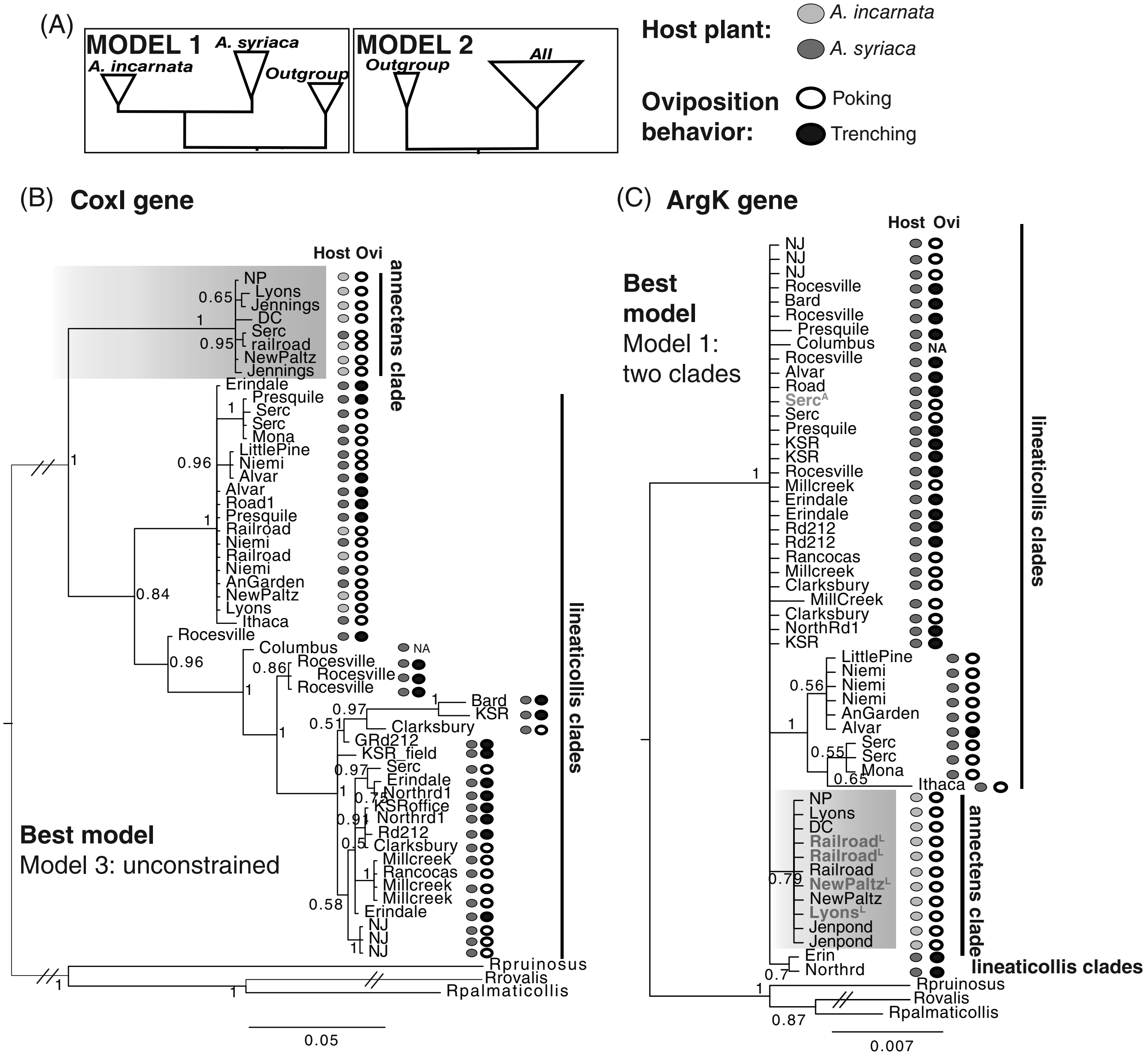


Fig 3. (a) Representation of clade constrains for model 1 and model 2. Bayesian phylogenies for milkweed stem weevils and three outgroup species using (b) CoxI and (c) ArgK sequences. Posterior probabilities are shown above nodes. Tips are labelled by location. Specimens that were placed in a different clade in the CoxI phylogeny are bolded in the ArgK phylogeny (yellow for those previously placed in the annectens (A) clade and teal for those previously placed in the lineaticollis (L) clade). The *R. annectens* clade is highlighted in yellow, while all other individuals are considered *R. lineaticollis* in each phylogeny. Host plants are in yellow (*A. incarnata*) and teal (*A. syriaca*) circles. Oviposition types are represented by a closed circle (trenching) or an open circle (poking or girdling). Two vertical lines crossing a branch show that this branch was shortened for visualization purposes. [Colour figure can be viewed at wileyonlinelibrary.com].

were two other clades with *R. lineaticollis* from different locations collected on *A. syriaca* plants with bootstrap values higher than 70% (Fig. 3c). The model comparison using AICM showed that constraining clades by host plant origin was the model that fit the ArgK tree topology best (Models in Fig. 3a; Model 1: 2 model AICM = 2934.396, and Model 3: unconstrained AICM = 2938.668, see Table S3).

The mitochondrial gene CoxI tree was better supported and resolved a higher number of internal clade relationships than the nuclear gene. Even though an *R. annectens* clade is recovered, 4 out of the 11 individuals collected on *A. incarnata* fall outside this clade. The model analysis did not support the two species hypothesis as clearly as the nuclear gene topology (Model 1: 2 clade AICM = 5212.227 and Model 3: unconstrained AICM = 2911.137). Several other clades in this tree have individuals that shared either the same host plant species or the same location of origin (Fig. 3b). The combined phylogeny also recovers a clade with weevils using *A. incarnata* as host plants,

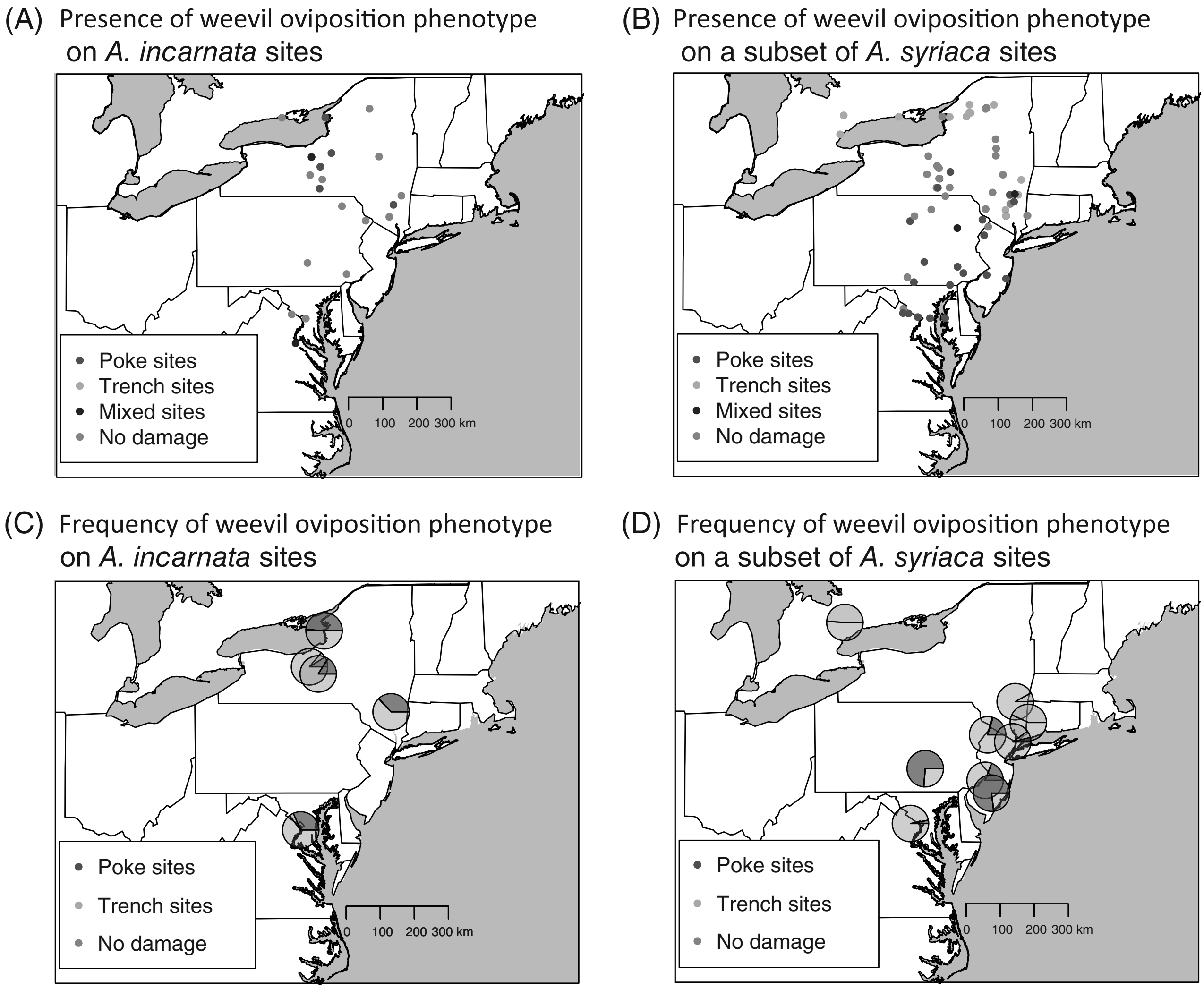


Fig 4. Presence and frequency of weevil damage on (a–c) *A. incarnata* and (b–d) *A. syriaca* plants observed on a latitudinal gradient during spring and summers of 2014 to 2017. No damage refers to locations where weevil damage was not observed, while mixed-site refers to locations where both poking and trenching by weevils was observed. (a) Presence of weevil oviposition phenotype on *A. incarnata* sites. (b) Presence of weevil oviposition phenotype on a subset of *A. syriaca* sites. (c) Frequency of weevil oviposition phenotype on *A. incarnata* sites. (d) Frequency of weevil oviposition phenotype on a subset of *A. syriaca* sites. [Colour figure can be viewed at wileyonlinelibrary.com].

while individuals in other clades share a common geographic origin (Fig. S2).

Weevil and host plant survey

To determine the pattern of host plant use by milkweed stem weevils, we surveyed host plants and weevil-induced damage across the North-East and the Mid-Atlantic regions in North America. We located milkweed plants in a total of 69 populations (Fig. 4, Table S1), with *A. incarnata* present at 30% and *A. syriaca* at 97% of all locations visited. Both host plant species occurred at 16 locations but weevil damage occurred exclusively on one of the plant species. We observed weevil damage in 62% of all locations. Locations with *A. syriaca* plants were 1.8 times more likely to have weevil damage than *A. incarnata* plants.

We found that *R. lineaticollis* damage changed from poking and girdling to trenching as latitude increased on *A. syriaca* (Fig. 4b–d, above 41° N). While *R. annectens* poked and girdled the *A. incarnata* plants throughout the range, with minimal levels of trenching on these plants (a total of 10 trenched *A. incarnata* plants found at two sites. Figure 4a–c). From the seven sites with *A. incarnata* plants, we found poking as the major type of damage with five of these sites also showing signs of girdling. Neither host plant nor latitude explained variation in the presence of poking (Fig. 4a,b. GLMM: n = 46, latitude, host, and interaction $P > 0.05$). Nonetheless, trenching presence was explained by an interaction between latitude and the type of host plant (*A. syriaca*) present at the site (Fig. 4a,b. GLMM: $n = 46$, latitude $P = 0.08$, host $P = 0.33$, and interaction $P = 0.02$).

When we considered the frequency of stems with weevil damage and the damage type, we found consistent results with

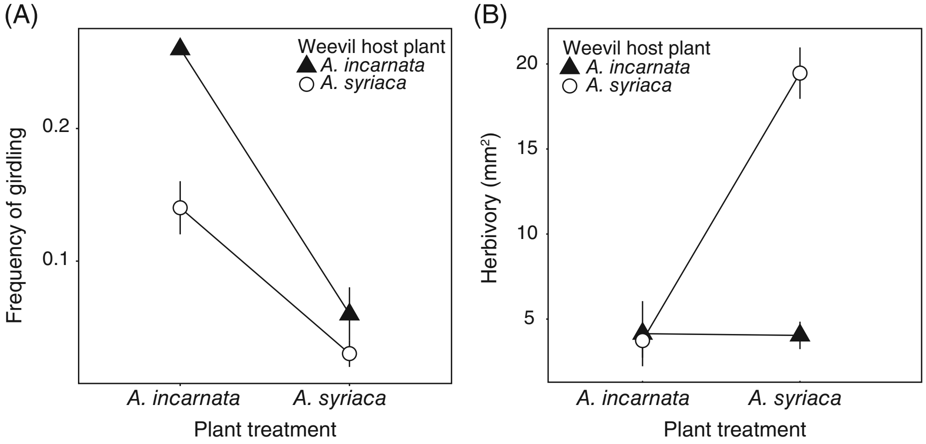


Fig 5. (a) Plot of average frequency ± SE of girdling by *Rhyssomatus* during the acceptance trials. Black triangles show average damage by weevils that were collected on *A. incarnata* and open circles show the average damage inferred by weevils collected on *A. syriaca*. The treatment plant is the plant the weevils fed on for a period of 48 h. (b) Plot of mean leaf area (mm^2) ± SE consumed of *A. syriaca* and *A. incarnata* leaves by *Rhyssomatus* on a 24-hour period. Open circles show consumption by weevils that were collected on *A. syriaca* and triangles show the preference of weevils that were collected on *A. incarnata*.

the presence-absence data on weevil damage. About 70% of the plants in a patch were not damaged by the stem weevil and of those that were damaged, most showed one type of oviposition damage (Fig. 4c,d). Only five sites had both trenching and girdling: two sites where weevils oviposited on *A. incarnata* and three sites on *A. syriaca* (Table S1).

Behaviour experiment 1: Host plant acceptance

We tested host plant acceptance in a greenhouse bioassay with both weevil types and both host plant species, using leaf damage and oviposition scars as signs of acceptance. *Rhyssomatus annectens* weevils were five times more likely to girdle *A. incarnata* than *A. syriaca* (Fig. 5a). Nonetheless, these weevils were as likely to damage *A. syriaca* plants as *R. lineaticollis* weevils. These results showed that *R. annectens* accepts *A. incarnata* more readily but might still use *A. syriaca* plants. *Rhyssomatus lineaticollis* produced 55% less damage on *A. incarnata* plants compared to *R. annectens*, showing stronger acceptance for their host plant, *A. syriaca*.

There was no significant difference in consumption of leaf tissue by weevils collected from different host plants (data not shown; GLMM: observations $n = 202$, and weevil groups $n = 101$; treatment plant, host plant, and interaction $P > 0.05$). However, there was more girdling by both weevil types when using *A. incarnata* compared to when weevils were using *A. syriaca* (Fig. 5a, GLMM: observations $n = 202$, and weevil groups $n = 101$; treatment plant: $P = 0.05$, host plant: $P > 0.05$, interaction: $P = 0.05$).

Behaviour experiment 2: Host plant preference

The host plant preference assay with leaf discs of *A. incarnata* and *A. syriaca* in 2015 showed that *R. lineaticollis* were choosier than *R. annectens* (Fig. 5b). *Rhyssomatus annectens* showed no preference for leaf consumption: these weevils ate about 4 mm^2 of tissue on leaves of both plants (mean ± se *A. incarnata*: 4.2 ± 0.8, *A. syriaca*: 3.8 ± 0.42). The other weevil species, *R.lineaticollis*, showed a nearly five-fold preference for *A. syriaca* leaves (mean ± se; *A. incarnata*: 4.2 ± 0.7, *A. syriaca*: 19.4 ± 1.39, GLMM: observations $n = 237$, and weevil groups $n = 119$; treatment plant $P > 0.05$, host plant $P > 0.05$, treatment plant * host plant $P < 0.005$).

Discussion

Insect host preferences and oviposition behaviours can evolve at the population level in response to host plant variation, generating optimal conditions for insect specialization and speciation (McLeish *et al.*, 2007; Thompson *et al.*, 2017). Our results show that two closely related and co-distributed milkweed stem weevil species varied in their host preference and oviposition patterns on two closely related and co-distributed host plants. These two weevil species show a high level of specialization across the species range, with variability in oviposition behaviours and possibly variation in host plant use when the preferred plant was not available.

Weevil identity

Species identity of the weevils was generally associated with the different host plants and, to some extent, with oviposition phenotypes. Morphological data and at least one molecular gene phylogeny supported that *R. annectens* weevils collected on *A. incarnata* are more closely related to each other than to *R. lineaticollis* weevils collected on *A. syriaca* (Alonso-Zarazaga & Lyal, 1999; Arnett *et al.*, 2002). Weevils that trench before oviposition appear to be a subset of *R. lineaticollis*, and this population level differentiation needs further study.

We sequenced the mitochondrial CoxI gene, which is typically used as a barcode to explore species level differences (Folmer *et al.*, 1994; Park *et al.*, 2010), as well as the more conserved nuclear arginine kinase gene (Marvaldi *et al.*, 2002, 2009). Some level of incongruence is expected when comparing phylogenetic topologies using mitochondrial and nuclear markers because of the different rates of molecular evolution and mode of inheritance (Moore, 1997). Other hypotheses have been proposed to explain differences in gene topologies, such as female dispersal bias, where females might be the main dispersers (Moore, 1997). While this is possible in the *Rhyssomatus* system, there is no evidence of distant dispersal for these weevils (St Pierre & Hendrix, 2003; Grainger *et al.*, 2016). More likely scenarios include differences in rates of molecular evolution or a history of hybridization events. The CoxI topology might be more aligned with contemporary changes in allele frequencies than the nuclear ArgK, which might be more aligned to a deeper evolutionary history of the species in question (Shaw, 2002). This scenario is concordant with each milkweed stem weevil speciating on one of the two host plants, as shown by the distinct 'annectens clade' in the ArgK phylogeny. However, the annectens clade was not completely recovered in the CoxI phylogeny, perhaps due to contemporary changes in population dynamics.

Consequently, hybridization might also be possible in this system given the overlapping ranges observed and the variation on milkweed abundance (specifically *A. incarnata* and *A. syriaca*) after glaciation and deforestation in the northeast (St Pierre & Hendrix, 2003; Boyle *et al.*, 2019). This latter situation is perhaps more intriguing due to observed morphological differences in *R. annectens* populations −some of which may indeed represent a hybrid following more widespread and thorough sampling−. The incongruence in mitochondrial and nuclear molecular markers also alludes to this scenario, whereby *R. lineaticollis* females might oviposit in *A. incarnata* (Fig. 3b), the offspring of which then interbreed with *R. annectens*.

While the combined evidence of morphological, molecular, and ecological data warrants the two species status for *R. annectens* and *R. lineaticollis*, further morphological analyses of the internal morphology and a more comprehensive molecular study should be conducted to understand the evolutionary dynamics of these weevils. For instance, while differences in body and genitalia size can be a result of plant quality, they can also be a result of adaptive changes associated with oviposition in different host plants as has been shown in other internal plant feeders (Joy & Crespi, 2007; Althoff, 2013).

Weevil and host plant survey

There are two main types of oviposition behaviours in milkweed stem weevils: *R. annectens* lay their eggs by poking around the stem or by haphazardly poking the plant. In contrast, *R. lineaticollis* lay eggs either by poking or trenching the stem of the plant. In the case of *R. lineaticollis*, this difference in oviposition behaviour was associated with latitude, with 'trenchers' being dominant in the north (Fig. 4). The type of oviposition behaviour was highly conserved at each location, with very few sites harbouring more than one type of oviposition behaviour. These differences in oviposition behaviour could have drastic effects on larval performance and survival. If oviposition behaviour is a latex avoidance behaviour, larvae could die without the proper avoidance behaviour. Ultimately, changes in oviposition behaviour could lead to morphological divergence and population specialization in these insects as it has been shown in the size and shape variation of the ovipositor in yucca moths (Althoff, 2013). It could also lead to ecological specialization driven by different interactions with the host plant, predators, or other herbivorous insects (Van Zandt & Agrawal, 2004).

It is unclear to us whether the observed differences in behaviour are a response to plant genotype, environmental cues, biotic interactions, or a weevil genotype-by-environment interaction. However, a previous study on oviposition preference of northern *R. lineaticollis* showed that weevils prefer to lay eggs on thicker stems of *A. syriaca*, suggesting that variation in plant traits might be an important factor (Agrawal & Van Zandt, 2003). We also know that there is a genetically based latitudinal cline in milkweed's defenses (Woods *et al.*, 2012; also observed in this study, Fig. S1), including latex exudation, which has been shown to decrease the performance of other herbivorous insects (Agrawal & Konno, 2009). We hypothesize that the differences we observed in oviposition behaviour may be a way of manipulating the plant to avoid latex in the oviposition area as suggested by Fordyce and Malcolm (2000). Similarly, Agrawal and Van Zandt (2003) observed a negative correlation between latex production and weevil oviposition for a population of trenching weevils in southern Ontario.

Host plant acceptance and preference

Both weevil species are able to eat and survive on both species of plants as adults. *Rhyssomatus annectens*, especially, do not seem to have strong host plant fidelity. They will feed on leaves and damage the stems of both host plants at similar rates, as shown in the preference and acceptance trials (Fig. 5b). This is contrary to what we expected since many of the field locations where we collected *R. annectens* had both plant species growing in close proximity, but we observed weevil damage only on *A. incarnata* and weevils were not found foraging on *A. syriaca*. The only significant preference found for *R. annectens* was that they girdled more often on *A. incarnata* than on *A. syriaca* plants (Fig. 5a), suggesting that host specialization for *R. annectens* is mediated by oviposition behaviour and no other types of plant use (e.g., adult foraging, larvae feeding), or by presence of natural enemies (Berlocher & Feder, 2002). However, *R. lineaticollis* consistently preferred leaves from *A. syriaca* and in general used its host plant more often than *A. incarnata* (Fig. 5b). Hence, *R. lineaticollis* is more strict in its use of alternative host plants than *R. annectens*. This again was a surprising result as *R. lineaticollis* is associated with several milkweed species in the literature (Price & Wilson, 1979); thus, we expected this species to be less discerning in plant choice. Weevils that are in northern populations, where trenching is more common, mostly drive this preference for *A. syriaca* plants, suggesting a role for adaptation to local conditions. An alternative hypothesis is that the native host plant, i.e., the plant from which the weevils were collected, greatly influences their behavioural choices (Knolhoff & Heckel, 2014). Although we were not able to remove developmental or behavioural effects induced by the native host plant, we mitigated this possibility in our acceptance trials by randomizing the order of treatment plants. In addition, in the second year of our preference experiment, we provided leaves from both plant species for at least 3 days before the trials started.

Apart from variation in host plant use, there are several unresolved issues in the literature of *Rhyssomatus* spp. that specialize on milkweeds regarding the type of oviposition behaviour and number of generations per year (St Pierre & Hendrix, 2003; Van Zandt & Agrawal, 2004). There are records of *Rhyssomatus* oviposition in seedpods (Price & Wilson, 1979; Fordyce & Malcolm, 2000), a behaviour that we observed more often in southern locations of *A. syriaca* and more generally on *A. incarnata*. In several cases, we found pupae inside seedpods after the larvae consumed the surrounding seeds, suggesting that some populations might have a second brood by ovipositing in seedpods. However, northern populations might largely be limited to one brood due to colder and earlier winters. Clinal variation in the number of broods per season has been observed in other insects (Beck & Apple, 1961; Glover *et al.*, 1992; Thomas *et al.*, 2003). If that is the case for milkweed stem weevils, the number of broods produced, alongside the challenges and nutritional benefits conferred by a seed diet, could play an important role in host specialization (Canfield *et al.*, 2008).

Concluding speculation

The geographic variation in host fidelity, use, and oviposition behaviour supports strict specialization for the milkweed stem weevils, with *R. annectens* specializing on *A. incarnata* and *R. lineaticollis* on *A. syriaca*. However, some of the variations in our data from greenhouse experiments suggest that *R. annectens* could use *A. syriaca* as a host (see behavioural experiments). Since *A. syriaca* and *A. incarnata* can co-occur in the same field, it is easy to imagine a scenario where *R. annectens* weevils do not find a new patch of *A. incarnata* and end up ovipositing on the more common *A. syriaca* plants. This scenario is consistent with specialization hypotheses such as the ’confusion’ or the ’oscillation’ hypotheses. These hypotheses require individuals to lay eggs indiscriminately, allowing for range expansion and a higher chance of finding a new suitable host plant (Larsson & Ekbom, 1995; Janz & Nylin, 2008).

Our survey suggests that *A. incarnata* plants are less common than *A. syriaca* in the northeastern U.S.A. and are less likely to have weevil damage on the stems. Currently, a highly debated topic in the conservation efforts to preserve monarch butterflies is whether milkweed abundance has decreased over time (Zaya *et al.*, 2017; Agrawal & Inamine, 2018; Boyle *et al.*, 2019). Studies on milkweed abundance show that while *A. syriaca* seems to be more common and has reached a stable level of abundance, other species like *A. incarnata* show a more drastic decline over the past 50 years (Boyle *et al.*, 2019). It is possible that milkweed stem weevils might not as frequently encounter their preferred host plant in the region and both weevils species might be competing for the more abundant *A. syriaca*.

In conclusion, our study supports host plant specialization in the evolution of the milkweed stem weevils, with *R. annectens* specializing on *A. incarnata* and *R. lineaticollis* on *A. syriaca*. Clinal variation in ovipositon patterns in these weevils is also consistent with the hypothesis of adaptation to host plant defenses or other local conditions. However, the CoxI molecular data show a more complex pattern with some clades of weevils that are clustered by host plant and others by geographic origin, suggesting that other factors such as isolation by distance or admixture might be at play.

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Data availability statement

The data that support the findings of this study are openly available in figshare at https://doi.org/10.6084/m9.figshare.c.4972220, reference Arcila Hernández *et al.*, 2020 number 9.

Supporting Information

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

Appendix S1. Supporting Information

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