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Exploring host-associated differentiation in the North American native cranberry fruitworm, *Acrobasis vaccinii*, from blueberries and cranberries

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Abstract

The factors explaining host-associated differentiation (HAD) have not yet been fully characterized, especially in agricultural systems. It is thought that certain characteristics within a system may increase the probability for HAD to occur. These characteristics include relatively long-standing evolutionary relationships between insects and their host plants, endophagy, and allochrony in host-plant phenologies. We assessed the status of these characteristics as well as the presence of HAD in the cranberry fruitworm, *Acrobasis vaccinii* Riley (Lepidoptera: Pyralidae), a pest associated with blueberry and cranberry in eastern North America. We reveal the occurrence of two distinct populations of *A. vaccinii* that are allochronically isolated by the phenological stage of their respective host plants (cranberries or blueberries). Laboratory-reared *A. vaccinii* adults collected from blueberries emerge at least 1 week earlier than adults from cranberries and the antennal sensitivity of adults to host-plant volatiles differs between *A. vaccinii* collected from blueberry and cranberry. Despite finding characteristics indicative of HAD, we did not detect a genetic signature of HAD in *A. vaccinii*. These findings suggest that HAD may occur through behavioral and phenological mechanisms before there is sufficient genetic variation to be detected.

Introduction

Speciation in herbivorous insects may result from shifts to novel hosts (Bush, 1969; Winkler & Mitter, 2008) or from co-cladogenesis with the host-plant species they exploit (Silvieus et al., 2008). Both of these processes result in the formation of genetically distinct insect lineages that are associated with different host-plant species, a special case of ecological speciation referred to as host-associated differentiation (HAD) (Bush, 1969; Feder et al., 1988; Abrahamson & Blair, 2008). HAD can be considered as an intermediate step in the process of speciation (Dres & Mallet, 2002; Nason et al., 2002). Although HAD has been studied extensively in two model systems, the *Solidago* system (Abrahamson & Weis, 1997; Abrahamson et al., 2003; Blair et al., 2005; Stireman et al., 2005) and the apple/hawthorn system (Bush, 1969; Feder et al., 1988, 1994, 1997, 2003), the frequency and the factors explaining its occurrence remain unclear. However, recent evidence suggests that HAD is more common than it was previously thought (Stireman et al., 2005; Dickey & Medina, 2010).

One of the mechanisms that may generate and maintain HAD is allochronic isolation of herbivorous insects associated with different host-plant species (Feder et al., 1993; Bush, 1994). For example, a mean difference of ca. 4 weeks in the availability of apples and hawthorns reproductively isolates apple maggot populations associated

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with these two host-plant species (Feder et al., 1993; Berlocher & Feder, 2002). Examples of allochrony in host-plant phenology are usually accompanied by varying host-plant fidelity (i.e., the tendency of an individual herbivore species to remain associated with their host-plant species of origin). Host fidelity is thought to increase the incidence of HAD (Groman & Pellmyr, 2000; Thomas et al., 2003). Differences in host-plant preference among insect populations of the same species associated with different host-plant species may also contribute to prezygotic reproductive isolation, ultimately leading to assortative mating among individuals that show host fidelity (Feder et al., 1994).

Systems involving native plant–insect associations provide an ideal opportunity to study HAD. The probability of finding HAD is thought to be greater in native than in non-native systems because of longer associations with native host-plant species, providing more opportunities for host associations to form (Dickey & Medina, 2010). HAD can also be facilitated by the presence of specific traits, such as endophagy (Medina, 2012). Insects that feed inside plant structures are subject to strong selection pressures specific to a particular host; these include limits in nutrient availability and chronic exposure to plant chemical defenses. In such insect–plant interactions, there is selection against hybridization between individuals with and without adaptive alleles. This type of selection often coincides with selection for host preference, which may drive adaptive alleles to fixation (Thompson & Pellmyr, 1991). To date, for example, all lepidopterans exhibiting HAD [i.e., *Gnorimoschema gallaesolidaginis* Riley, *Spodoptera frugiperda* (JE Smith), and *Ostrinia nubilalis* (Hübner)] are endophagous (Pashley et al., 1987; Nason et al., 2002; Thomas et al., 2003), whereas exophagous Lepidoptera have failed to show HAD (RF Medina & P Barbosa, unpubl.; G Shlichta & P Barbosa, unpubl.).

Currently, it remains unclear how common HAD is in agricultural systems and how it may impact pest control practices (Medina, 2012). To answer these questions, it is necessary to be able to predict which types of species may be more prone to showing it. One way to know how common HAD is, is by providing an array of model systems, such as the one involving *Solidago* species, in which several species from different orders could be assessed for the presence of HAD in the same species pairs. For example, studies done in hickories have shown that HAD may be relatively common among parthenogenetic Sternorrhyncha associated with pecan [*Carya illinoinensis* (Wangenh.) K. Koch] and water hickory [*Carya aquatica* (Michx. f.) Nutt.] (Dickey & Medina, 2010, 2012). Most of the studies on HAD in agricultural systems involve parthenogenetic aphid species (Via et al., 2000; Vialatte et al., 2005; Lozier et al., 2007; Peccoud et al., 2009), or endophagous lepidopterans (Pashley, 1986; Thomas et al., 2003). However, recent studies have found evidence of HAD in native non-parthenogenetic and exophagous hemipterans (Barman et al., 2012; Medina et al., 2012).

The cranberry fruitworm, *Acrobasis vaccinii* Riley (Lepidoptera: Pyralidae), is univoltine and feeds on native *Vaccinium* (Ericaceae) species. It is a significant pest of two North American crops: the highbush blueberry (*Vaccinium corymbosum* L.) and the cranberry (*Vaccinium macrocarpon* Ait.) (Ericaceae) (Beckwith, 1941; Hutchinson, 1954; Tomlinson, 1970; Averill & Sylvia, 1998). Commercial production of these two plant species began about 100 years ago (Hancock et al., 2008), creating novel and large areas of relatively uniform habitats for *A. vaccinii* where populations can complete their entire lifecycle on a single host-plant species for several generations. Both berry species do best in acidic, well-drained, porous, and sandy-loam soils (Roper & Vorsa, 1997) and their production can thus overlap geographically (USDA, Plants Database). Blueberries and cranberries differ significantly in their phenology and in their major areas of production in the eastern USA, with blueberries flowering and producing fruit about a month before cranberries. This is important because *A. vaccinii* eggs are laid on young fruit soon after petal fall and the larvae feed internally on the developing fruit; therefore, the insect's lifecycle is closely intertwined with the timing of flower and fruit development. This temporal asynchrony between blueberry and cranberry phenologies and the consequent adaptations necessary for host-associated populations of fruitworms to track their respective host-plant species have the potential to generate host-associated, reproductively isolated populations. Lastly, other insects in the blueberry–cranberry system bear the genetic signature of HAD. For example, the native cecidomyiid midge, *Dasineura oxycoccana* Johnson (Diptera: Cecidomyiidae) shows complete assortative mating (Cook et al., 2011) that has resulted in HAD and cryptic speciation on blueberries and cranberries (Mathur et al., 2012). This finding is relevant because it suggests that the differential selection pressures presented by blueberry and cranberry are strong enough to produce HAD in at least one insect associated with these plant species.

The present study tested for HAD among sympatric *A. vaccinii* populations associated with blueberries and cranberries in New Jersey, USA. *Acrobasis vaccinii* has several characteristics that make it an ideal candidate for testing HAD: it is a native endophagous insect feeding on geographically overlapping, native host plants with allochronically distinct phenologies. Also, the status of HAD in *A. vaccinii* may be relevant to pest management practices because, even though it attacks both blueberries

and cranberries across its geographic distribution, it is commonly reported as a major pest only in one of the crops in different geographic regions of New Jersey (Marucci, 1966). The present study also assessed the geographic genetic population structure of *A. vaccinii* across three USA states, viz., New Jersey, Michigan, and Massachusetts. Michigan mainly produces blueberries whereas Massachusetts produces mostly cranberries. Both of these North American regions report *A. vaccinii* as an important pest in their respective producing crop (Averill & Sylvia, 1998; Mallampalli & Isaacs, 2002). In New Jersey, *A. vaccinii* occurs in both blueberries and cranberries but it is only a significant pest in blueberries (Marucci, 1966).

Our main objective was to test for host-associated differentiation and for the presence of traits commonly invoked to explain its existence (i.e., allochrony in peak flight activity and odor preferences for host-associated volatiles in blueberry and cranberry *A. vaccinii* populations). In addition, we searched for evidence of local adaptation. The genetic signatures of both HAD and local adaptation were assessed using AFLP.

Materials and methods

Field monitoring

A 2-year (2007–2008) study was conducted to investigate for possible phenological differences in adult flight activities between blueberry and cranberry populations of *A. vaccinii* in New Jersey. Field abundance of *A. vaccinii* was monitored with white plastic delta traps (PHEROCON® VI Trap; Trécé, Adair, OK, USA) baited with *A. vaccinii* pheromone lure (Trécé). Traps were placed on 16 commercial highbush blueberry farms (eight in Burlington County and eight in Atlantic Co.) and eight cranberry farms in Burlington Co. No traps were placed in cranberries in Atlantic Co. because cranberries are almost exclusively grown in Burlington Co. In blueberries (cv. Duke or Bluecrop), traps were placed from the 2nd week in May (peak bloom), when *A. vaccinii* flight activity typically begins, through the 1st week in August (end of harvest). One trap was placed per blueberry farm near a wooded area, attached to a ca. 2 m tall pole and at canopy level. In cranberries (cv. Stevens), traps were also deployed from the 2nd week in May (pre-bloom) through the 1st week in August (fruit maturation). Each trap was placed in an individual cranberry bog (two traps per farm), attached to a ca. 70 cm tall pole, with the bottom of the trap at 25–30 cm above canopy level. All traps were checked weekly and the number of *A. vaccinii* moths was recorded. Degree-day accumulations were calculated using a base temperature of 10 °C and the Hammonton, New Jersey (Atlantic Co.), and Chatsworth, New Jersey (Burlington Co.), RISE (Resource Information Serving Everybody) stations operated by the South Jersey Resource Conservation and Development Council (www.sjrcd.org/rise/); initiation of heat units began on 23 January because complete, uninterrupted data collections were available from that date forward.

Data on the number of *A. vaccinii* moths captured per trap were analyzed using ANOVA with the computer package MINITAB 16 (Minitab, State College, PA, USA). Numbers of moths captured per trap were compared using a factorial analysis with location (blueberry – Atlantic Co., blueberry – Burlington Co., and cranberry), date, and year. Before ANOVA, the variances were checked for homoscedasticity and the data checked for normal distribution. The data met ANOVA assumptions. The means were separated by an honestly significant difference (HSD) Tukey test.

Adult emergence studies

This 4-year study (2006–2009) compared adult emergence periods following post-diapause development for *A. vaccinii* populations from blueberries and cranberries. *Acrobasis vaccinii* adults (n = 850) were obtained by rearing larvae from infested fruit (Mcdonough et al., 1994). These infested fruits were collected from eight sites in New Jersey, one site in Massachusetts, and two sites in Michigan (Table 1) at the end of the growing season in September–October of 2006, 2007, and 2008. *Acrobasis vaccinii* overwinters as a larva in hibernacula, which are formed after larvae complete their development in the fruit and drop to the soil. Berry samples were spread on screens suspended 4 cm over moistened sand (1–2 cm deep) in 5.7-l (34 × 21 × 12 cm) plastic shoeboxes (Sterilite, Townsend, MA, USA) in a protected screen-house for 4–5 weeks to allow larvae to develop and drop from the berries. After hibernacula were formed, the sand below each sample was sieved through a #16 mesh (six strands per cm) and the hibernacula were collected. Hibernacula were then transported to the laboratory, where they were placed into 200-ml Styrofoam cups (Solo, Lake Forest, IL, USA) with moist *Sphagnum* peat moss (Premier horticulture, Quakertown, PA, USA). The cups were then stored in environmental chambers (L14:D10) with a uniform temperature program (Figure 1). The peat moss was watered throughout storage as needed to maintain moisture to prevent the hibernacula from desiccating. When adults emerged in the spring of the following year, they were sexed immediately and transferred into 75% ethanol vials. The location and date of collection, host plant,

Table 1 Cranberry fruitworm, *Acrobasis vaccinii*, collected from different commercial farms in North America in 2006 and 2007; NJ = New Jersey; MA = Massachusetts; MI = Michigan

	State	County	Host plant	Total no. individuals used for AFLP analysis	STRUCTURE and PCA ID	Coordinates	
						Latitude	Longitude
1	NJ	Burlington	Blueberry	11	NJ4	39°47′37.65″N	74°31′40.73″W
2	NJ	Burlington	Cranberry	33	NJ5	39°57′06.01″N	74°30′02.21″W
3	NJ	Atlantic[1]	Blueberry	23	NJ2	39°41′56.99″N	74°44′27.50″W
4	NJ	Atlantic[1]	Blueberry	26	NJ3	39°34′26.03″N	74°46′53.51″W
5	NJ	Burlington	Blueberry	10	NJ1	39°49′44.84″N	74°34′22.80″W
6	MA	Barnstable	Cranberry	56	MA	41°41′30.68″N	70°05′18.47″W
7	MI	Allegan	Blueberry	9	MI	42°31′34.82″N	86°12′58.33″W
8	MI	Ottawa	Blueberry	4	MI	43°01′28.85″N	86°12′19.86″W
9	NJ	Burlington[1]	Cranberry	13	NJ6	39°45′29.98″N	74°32′32.73″W
10	NJ	Burlington[1]	Cranberry	7	NJ7	39°44′29.59″N	74°32′02.69″W
11	NJ	Burlington[1]	Cranberry	5	NJ7	39°39′45.25″N	74°29′59.78″W
	Total				197		

[1]Insects collected from different farms.

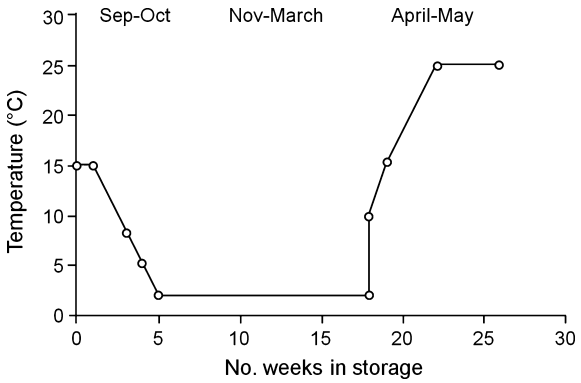

Figure 1 Temperature regime used in environmental chambers for determining differences in time of emergence among cranberry fruitworm, *Acrobasis vaccinii*, populations collected from different locations and host plants.

emergence time, and sex were recorded for each individual moth. Specimens were then sent to Texas A&M University in College Station, TX, and stored at −80 °C until DNA extraction.

Data on *A. vaccinii* adult emergence were analyzed using ANOVA (Minitab 16), with host (blueberry/cranberry), sex (male/female), and year (2007/2008/2009) as main factors blocked by state (NJ/MI/MA). Before ANOVA, the variances were checked for homoscedasticity and the data checked for normal distribution. The data met ANOVA assumptions. The means were separated by Tukey HSD test.

Electroantennogram (EAG) studies

We determined the antennal receptivity of adult *A. vaccinii* from blueberry and cranberry populations to 27 selected synthetic volatile compounds previously found in blueberry and cranberry buds and flowers (Szendrei et al., 2009; Rodriguez-Saona et al., 2011) by EAG. These volatiles are emitted from plants prior to and during bloom, which coincides with *A. vaccinii* emergence and peak flight activity (Averill & Sylvia, 1998). *Acrobasis vaccinii* larvae were collected in 2007 from blueberry and cranberry fields in New Jersey. Berries infested with *A. vaccinii* larvae were collected in early July (blueberries) and in mid-August (cranberries). Hibernacula were stored in environmental chambers as described above. Emerging moths were collected and sexed daily until no more adults emerged. For EAG recordings, the insect head was carefully removed and a reference electrode was inserted into its base with a glass capillary filled with physiological saline solution (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, 0.2 g $NaHCO_3$, in 1 l H_2O) (Malo et al., 2004). The distal end of the antenna was inserted into the tip of the recording glass capillary electrode. Each replicate was made with one moth antenna. The signals generated by the antenna were passed through a high-impedance amplifier (NL 1200; Syntech, Hilversum, The Netherlands) and displayed on a monitor by Syntech software for processing EAG signals. A stimulus flow controller (CS-05; Syntech) was used to generate a stimulus at 1-min intervals. A current of humidified pure air (0.7 l per min) was constantly directed onto the antenna

through a 10-mm-diameter glass tube. *Acrobasis vaccinii* adults from blueberries or cranberries of each sex were used for each compound in these tests (n = 7–10 per host/sex/compound; 3–8 days old).

Dilutions of the synthetic compounds were prepared in HPLC-grade hexane to make 10 μg μl^{-1} solutions. All synthetic products used in these studies were purchased from Fluka Sigma-Aldrich (St. Louis, MO, USA) and Bedoukian Research (Danbury, CT, USA). The chemical products showed a purity of 97% checked by GC, with the exception of linalool oxide and farnesene, which are a mixture of isomers and ocimene and had a purity of 70%. A standard aliquot (1 μl) of each test dilution was pipetted onto a piece of filter paper (0.5 × 3.0 cm, Whatman No. 1) exposed to air for 20 s to allow the solvent to evaporate, then inserted into a glass Pasteur pipette or sample cartridge and left for 40 s before applying. A new cartridge was prepared for each insect. To present a stimulus, the pipette tip containing the test compound was inserted through a side hole located at the midpoint of a glass tube through which humidified pure air flowed at 0.5 l per min. The duration of stimulus was 1 s. The continuous flow of clean air through the airflow tube and over the preparation ensured that odors were removed immediately from the vicinity. The synthetic compounds were presented in random order. Control stimuli (hexane) were presented at the beginning and end of each EAG analysis. The peak EAG amplitudes of the response to each tested chemical were analyzed using ANOVA (Minitab 16). The EAG amplitude responses of *A. vaccinii* from blueberry and cranberry were compared using a factorial analysis with chemical products, host origin and sex with the data previously ln(x) transformed to meet the assumptions of normality and homogeneity of variances. The means were separated by Tukey HSD test.

Genetic population structure

Amplified fragment length polymorphism (AFLP) (Vos et al., 1995) analyses were conducted on a total of 197 insects (Table 1), with 114 individuals originating from cranberry and 83 from blueberry. AFLP markers were used to assess HAD and to determine the genetic population structure of *A. vaccinii* collected from three USA states (i.e., NJ, MI, and MA) from two host plants (cranberry and blueberry). DNA was extracted from whole adult individuals using a Qiagen DNEasy Tissue Kit (Qiagen, Valencia, CA, USA) following the recommended protocol (Qiagen, 2002). The quantity (ng of nucleic acid per μl of solution) and purity (ratio of sample absorbance at 260 and 280 nm) of DNA were measured using a NanoDrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Digestion of genomic DNA by *Eco*RI and *Mse*I and ligation of oligonucleotide adaptors (Applied Biosystems, Foster City, CA, USA) were accomplished in a single reaction. Each reaction contained a minimum of 50 ng μl^{-1} of DNA. Pre-selective polymerase chain reaction (PCR) amplification was performed using the Applied Biosystems AFLP Pre-selective Mix (Applied Biosystems). The PCR program for pre-selective amplification was: 95 °C for 1 min followed by 20 cycles of 95 °C for 10 s, 56 °C for 30 s, and 72 °C for 1.5 min, with a final hold at 75 °C for 5 min. All samples were stored at 4 °C following pre-selective amplification on a thermal cycler and before selective amplification. The amplified product was then diluted 20-fold using 15 nM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA. For the selective amplification of restriction fragments, pre-prepared custom primers for recognition of *Eco*RI and *Mse*I adaptors were used. The three primer pairs selected were M-CAT and E-ACT, M-CTC and E-ACT, and M-CAT and E-AAC. Fragments were visualized by attaching a fluorescent dye to the 5′ end of each *Eco*RI selective amplification primer with no modification made to the *Mse*I primer. The PCR program for the selective amplification consisted of an initial warm-up at 95 °C for 30 s, 12 cycles of 95 °C for 10 s, 65 °C for 40 s with a lowering of 0.7 °C per cycle, 72 °C for 1.5 min, followed by 35 cycles of 95 °C for 11 s, 56 °C for 40 s, 72 °C for 1.5 min, and finally a hold of 75 °C for 5 min before storing the samples at 4 °C.

AFLP markers were separated by capillary electrophoresis by a 3100 Genetic Analyzer from Applied Biosystems and analyzed using GeneMapper® software (Applied Biosystems, 2005). Markers with a dye signal greater than 100 luminescent units were considered to be present. Each AFLP band was considered a locus and was assumed to have two possible alleles (i.e., 0 for no allele present and 1 for an allele present). The statistical reliability of the number of specimens and primer combinations necessary to detect genetic differences in this study was assessed using SESim values (Medina et al., 2006).

To determine the population structure of *A. vaccinii*, we analyzed AFLP markers using STRUCTURE v2.2 (Pritchard et al., 2000; Falush et al., 2007). STRUCTURE is a Bayesian population assignment method that assumes within-population loci are in Hardy–Weinberg and linkage equilibrium and uses a Markov chain Monte Carlo algorithm to group individuals into populations by measuring the probability that a sample genome belongs to a distinct population. The method is designed to assign individuals to a population in such a way as to eliminate violations of Hardy–Weinberg and linkage equilibrium

assumptions. The output of STRUCTURE is the natural log probability of the genome data of a sample (X) given the number of clusters (K) assumed, or [ln Pr(X|K)]. Where parameter estimates indicated K>1, the ad hoc ΔK statistic (Evanno et al., 2005) was used to predict the most likely number of clusters (K) in the data. This involves calculating the second order rate of change in [ln Pr(X|K)]. Evanno et al. (2005) showed that the K corresponding to a spike in this value accurately predicts the number of populations represented by the data. The STRUCTURE burn-in period was fixed at 10 000 with a run length of 10 000 under the admixture model with correlated and uncorrelated allele frequencies. To determine the number of populations present in the data (K), twenty replications were completed for each K value between 1 and 12.

Nei's unbiased genetic distances among sites and within host-plant species were calculated in AFLPsurv 1.0 (Vekemans, 2002) using the methods of Lynch & Milligan (1994) to test geographic and host-dependent population structure, respectively. An analysis of molecular variance, AMOVA (Excoffier et al., 1992), and a Principal Coordinate Analysis (PCA) were performed using GenAlEx v6.4 (Peakall & Smouse, 2006) in order to compare the partition of genetic variability among populations grouped by host plant and by the location from which the samples were obtained. Permutations tests were conducted with 9 999 random permutations of the data.

Results

Moth phenology

Over the 2 years of this study, the number of moths per week caught in different fields was significantly different ($F_{2,544}$ = 25.02, P<0.001). On average 1.28 ± 0.21 (SEM) moths were caught in pheromone traps in blueberry fields from Atlantic Co., 0.42 ± 0.07 in traps placed in blueberry fields from Burlington Co., and 0.14 ± 0.03 in traps placed in cranberry fields. *Acrobasis vaccinii* abundance also differed depending on the time of year ($F_{12,544}$ = 8.23, P<0.001), but not between years ($F_{1,544}$ = 1.00, P = 0.319). There was a significant location*date interaction ($F_{24,544}$ = 5.79, P<0.001), so that moth abundance in the different locations was influenced by time of year. Over 2 years of field monitoring in New Jersey, two peak abundances of *A. vaccinii* adults were observed in blueberry fields (pointed out by different arrows; Figure 2), indicating the presence of two potential populations: an early population that peaks in mid-May and a late population that peaks in the second half of June. These two population peaks were present in blueberry farms from Burlington Co. but not in blueberry farms in Atlantic Co. In cranberry fields, the peak flight of adult moths was in mid-June and coincided with the late blueberry population (Figure 2). All other interactions were non-significant (P>0.05).

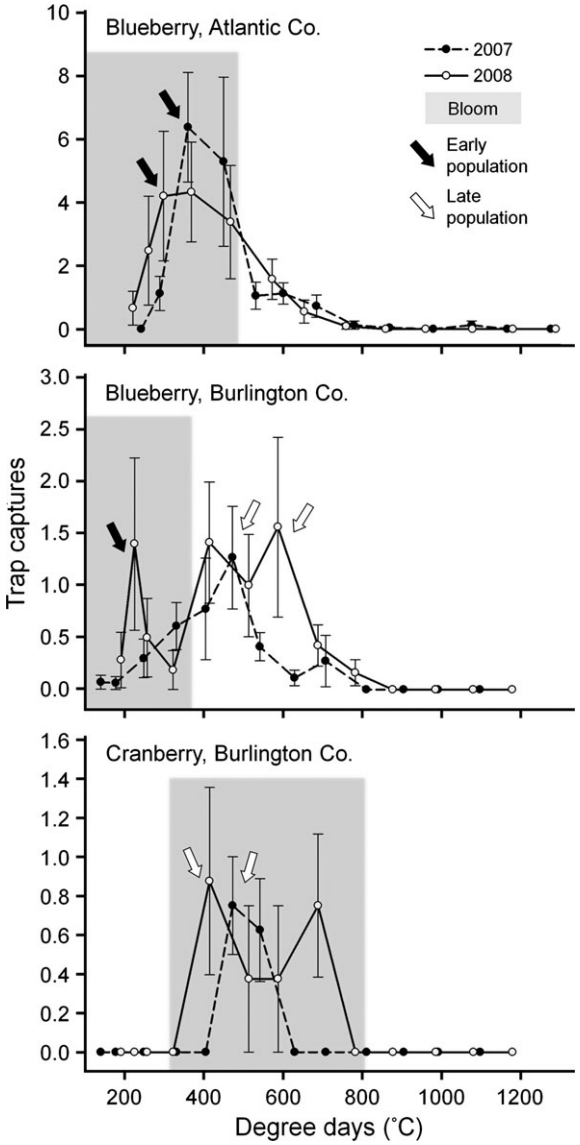

Figure 2 Average (± SEM) number of cranberry fruitworm, *Acrobasis vaccinii*, males caught in pheromone traps over 3 years in New Jersey.

Adult emergence

Time of adult emergence was affected by the host ($F_{1,836}$ = 190.57, P<0.001), year ($F_{2,836}$ = 64.18, P<0.001) and geographic origin, i.e., state ($F_{2,836}$ = 238.51, P<0.001). On average, *A. vaccinii* emerged 10.2 ± 2.1 (SEM) days (range: 7–15 days) earlier from blueberry than from cranberry hosts in our environmental chambers over

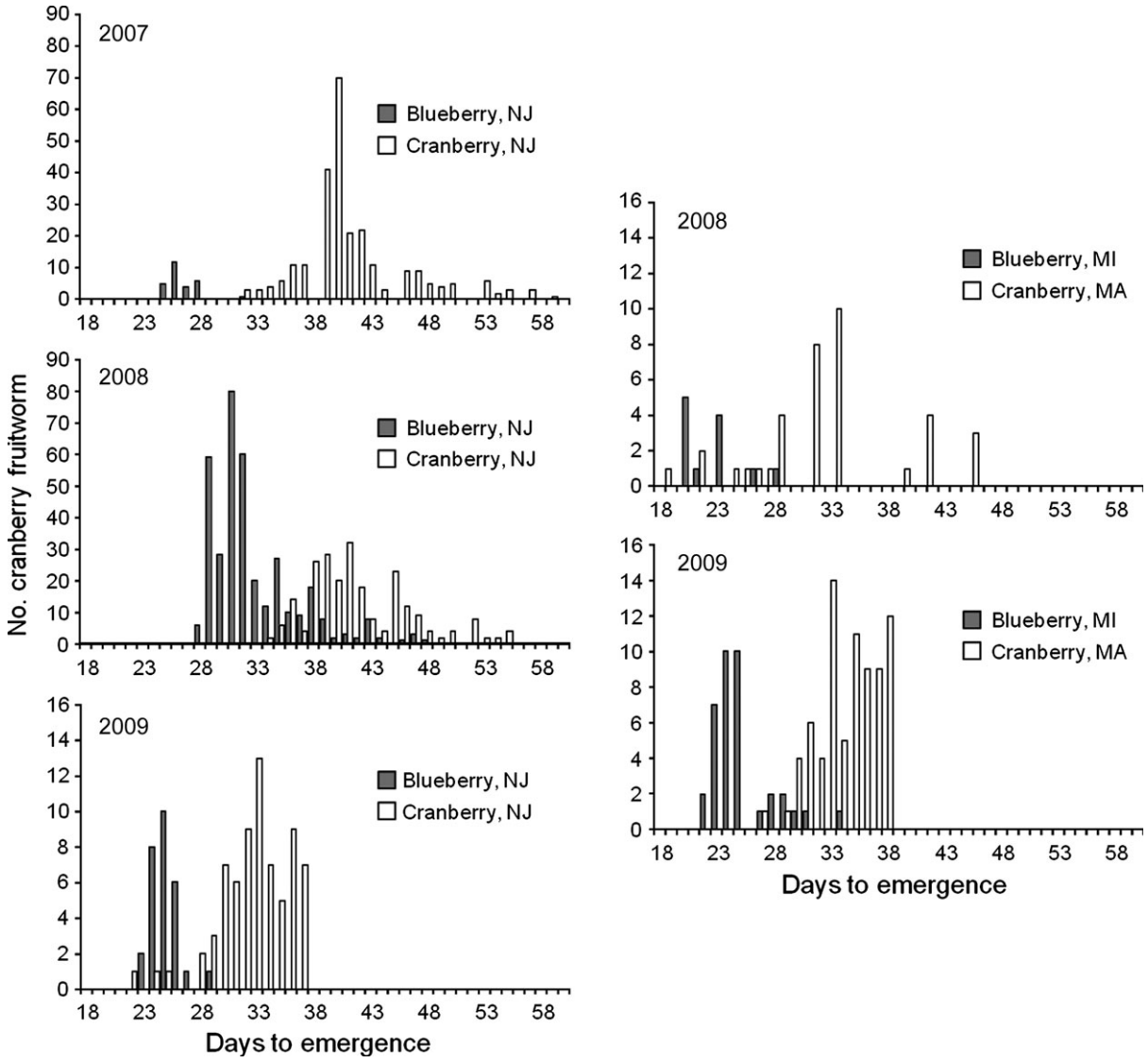

Figure 3 Total number of cranberry fruitworm, *Acrobasis vaccinii*, emerging in environmental chambers over 3 years. Hibernacula were from New Jersey (NJ), Massachusetts (MA), or Michigan (MI) and were either collected in cranberry or blueberry fields.

3 years (2007–2009) (Figure 3). The magnitude of these differences varied by year ($F_{2,836}$ = 10.75, P<0.001); so that in 2007, 2008, and 2009 the difference in adult emergence between cranberry and blueberry *A. vaccinii* populations was 13.9, 8.9, and 9.9 days, respectively. All other 2-way interactions, as well as the host*sex*year interaction, were not significant (P>0.05).

Antennal sensitivity

Volatile compounds from blueberries and cranberries elicited different EAG responses from *A. vaccinii* ($F_{26,891}$ = 6.73, P<0.001; Figure 4). There were also clear differences in the antennal responses to host-plant volatiles between males and females ($F_{1,891}$ = 13.85, P<0.001). Males had stronger EAG responses to non-anal and caryophyllene oxide than females (P≤0.05). Moreover, there were differences in the antennal responses between blueberry and cranberry populations ($F_{1,891}$ = 22.65, P<0.001); however, this effect was influenced by sex ($F_{1,891}$ = 8.03, P = 0.005). EAG responses to only two of the 27 host-plant volatiles differed between cranberry and blueberry populations, i.e., significant host origin effect. Female *A. vaccinii* from cranberry and blueberry responded differently to cinnamyl alcohol (P = 0.046). Both sexes from cranberries responded more strongly to ocimene as compared with those from blueberries

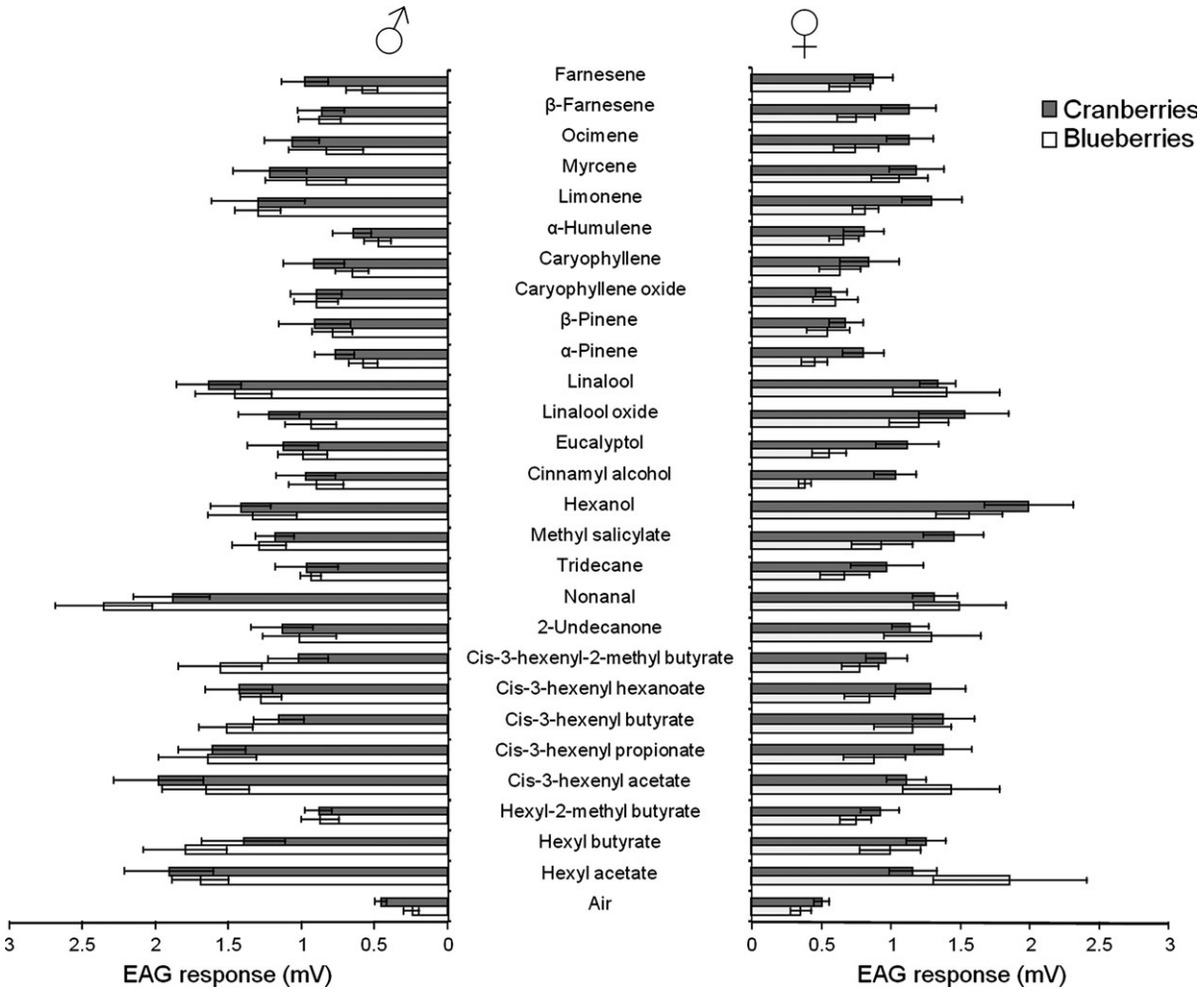

Figure 4 Electroantennogram responses (mean ± SEM) of male (left) and female (right) cranberry fruitworm, *Acrobasis vaccinii*, collected in cranberry or blueberry fields to various host-plant volatiles.

(P = 0.03). There were no significant compound*sex, compound*host origin, or compound*sex*host origin interactions (P>0.05).

Genetic population structure

We found two genetically distinct populations of *A. vaccinii*, with the highest assignment of the second order rate for K = 2 (91%; Figure 5), indicating that two populations best explained the data. These genetic differences, however, were not associated with host plant or with geographic distribution (Figure 5). The relative abundance of the identified genotypes was the same (ca. 70:30) for *A. vaccinii* collected from blueberry in Michigan and New Jersey. This ratio was somewhat different for insects from cranberry hosts from Massachusetts (75:25) compared to New Jersey (84:16) (Figure 5).

The PCA indicated that principal coordinates 1 and 2 explained 73.05% of the molecular variation. Principal coordinate 1 strongly segregates *A. vaccinii* individuals into the same populations delineated by STRUCTURE (Figure 6). AFLPSurv F_{ST} statistics between host plants of origin were not significant, but F_{ST} between collecting sites was 0.354 (P = 0.0085, Table 2). Expected heterozygosity was similar among host populations (Table 3). The results of the nested AMOVA showed that 96% of the molecular variance was explained by collecting site (P = 0.029; Table 4).

Discussion

Acrobasis vaccinii on blueberries and cranberries present several of the characteristics that, in theory, would have

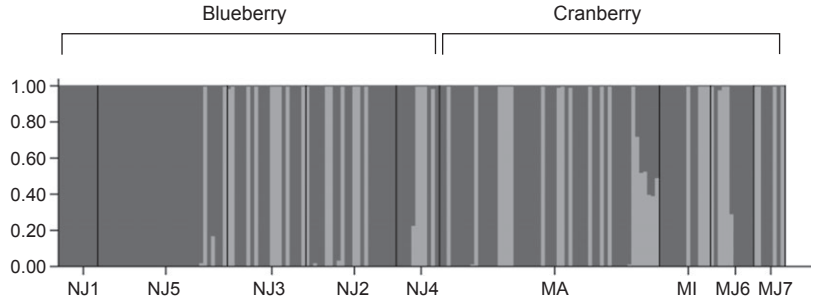

Figure 5 Population structure of cranberry fruitworm, *Acrobasis vaccinii.* Each vertical line represents one individual. The proportion of color in each line corresponds to the probability that an individual is a member of a particular population. Results were obtained using STRUCTURE 2.2 (Pritchard et al., 2000). The most likely number of clusters was two according to the ΔK method of Evanno et al. (2005). The genetically distinct populations found did not correspond to host-plant associations (blueberry and cranberry) or to geographic populations [New Jersey (NJ1-7), Massachusetts (MA) and Michigan (MI)].

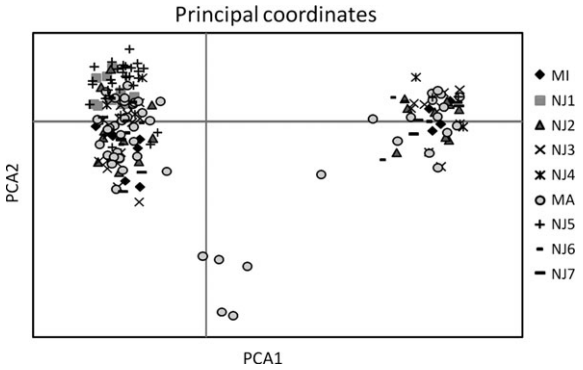

Figure 6 Eigenvectors of principal coordinates 1 (x-axis) and 2 (y-axis) for cranberry fruitworm, *Acrobasis vaccinii*. Symbol shapes denote collecting sites and symbol shading denote host-plant species. Coordinate 1 separates the STRUCTURE delimited populations [New Jersey (NJ1-7), Massachusetts (MA), and Michigan (MI)].

Table 2 Genetic differentiation by host plant and collection site in cranberry fruitworm, *Acrobasis vaccinii*

Source of differentiation	F_{ST}	P
Host plant	0.0005	0.29
Site collected	0.0354	0.0085

The F_{ST} by host plant and overall F_{ST} by site within each host plant were estimated using AFLPsurv.

made it an ideal system in which one would expect HAD to occur. However, although *A. vaccinii* is a native univoltine herbivore that shows allochronic separation based on the distinct fruiting phenologies of two related host-plant species, we did not detect the genetic signature of HAD in this species. If host preferences existed in *A. vaccinii* populations, we would expect to observe a difference in antennal sensitivity to specific compounds found in cranberry and blueberry volatiles. Here, we found differences in the olfactory responses of adult *A. vaccinii* between cranberry and blueberry populations; however, these were found for only 2 of 27 volatiles tested. Further studies are needed to determine the effect of these compounds, and particularly of host fruit compounds, on *A. vaccinii* host preference. If most of the characteristics postulated to explain HAD were present, then why was HAD not found in this system?

The role of endophagy increasing the likelihood of HAD has been attributed to the intimate relationship between endophagous insects and their host plants (Medina, 2012). This may select for specialization of host-associated populations of endophagous insects to specific host-plant tissues at specific levels of maturity in the host-plant species they attack, which we did not evaluate. Furthermore, endophagous insects have adaptations to survive immersed inside plant tissues (Komatsu & Akimoto, 1995; Mopper et al., 2000). Thus, insects of the same species associated with different host-plant species may differ in the adaptations required to survive inside their

Table 3 Diversity statistics for cranberry fruitworm, *Acrobasis vaccinii*, by host plant

Host	n	No. AFLP loci genotyped	No. polymorphic loci	% polymorphic loci	Hj
Blueberry	77	255	87	34.1	0.12165
Cranberry	108	255	94	36.9	0.12152

Hj, expected heterozygosity (Nei's gene diversity).

Table 4 AMOVA for cranberry fruitworm, *Acrobasis vaccinii*, from different collecting sites and host plants

Source of variation	Variance components	% variation	P
Within collecting site	20.153	96	0.029
Among collecting sites	0.795	4	0.038
Among host plants	0.00	0	NA

AMOVA, analysis of molecular variance. Significance testing was done using 9 999 permutations of the binary distance parameter δPT, an analog of F_{ST} in GenAlEx 6.2.

respective host plants. Finally, endophagous insects may get protection against their natural enemies by concealing inside relatively inaccessible host-plant structures (e.g., inside fruits, galls, stems) (Brown et al., 1995; Feder, 1995). Thus, endophagous insects that do not leave their concealment and feed inside plant tissues may have a selective advantage. If plant tissues differ in plant chemistry, nutrition, or the kind of natural enemies they attract, then specialization on different host plants could be expected to result in HAD.

The lack of evidence for HAD in *A. vaccinii* may have to do, in part, with the fact that the association of this insect with cranberries and blueberries is not as 'intimate' as in the endophagous insects in which HAD has been found so far (Medina, 2012). Unlike other Lepidoptera in which HAD has been found such as in the European cornborer (*O. nubilalis*) or the goldenrod elliptical-gall moth (*G. gallaesolidaginis*), *A. vaccinii* often infests more than one fruit during its larval development (Marucci, 1966; Averill & Sylvia, 1998). That is, its association with its host plant may be less intimate as in the case of those insect species that stay within the same plant structure during their entire larval development. Completion of larval development within the same individual host has been postulated as a factor that may increase the likelihood of HAD (Kerdelhue et al., 2002). For example, in the same host-plant system, *D. oxycoccana*, a cecidomyiid fly, shows strong HAD (Mathur et al., 2012). This is likely because this endophagous maggot feeds and completes its entire larval development inside a single developing bud of cranberry or blueberry.

Furthermore, in fruit feeders showing HAD such as in *Rhagoletis pomonella* (Walsh) in apple and hawthorne, an eclosion difference of 10 days is enough to temporally isolate populations associated with different host-plant species. The differences in eclosion times in *A. vaccinii*, although comparable in absolute terms, may not constitute a reproductive barrier strong enough to reduce gene flow between cranberry-associated moths and blueberry-associated moths.

The likelihood of HAD also depends on the ecological distance among the host-plant species involved (Heard et al., 2006). In a HAD context, ecological distance could be defined as the difference in the intensity of key selective traits among two or more host species. Within this framework, differential selection acting on cranberry-associated and blueberry-associated populations of *A. vaccinii* may not be strong enough for HAD to occur. Also, selection pressures may be acting on traits unrelated to the formation of reproductive isolating barriers. In addition, if there is no cost of producing 'hybrids' or if instead there is hybrid vigor, HAD will also be unlikely to occur. To test this possibility, mating experiments among insects associated with different hosts and comparisons of *A. vaccinii* fitness on cranberry and blueberry with their hybrids' fitness should be conducted.

Finally, although no host-associated lineages were detected, we distinguished two genetically distinct populations of *A. vaccinii* at our study sites. These two genotypes could not be associated with geographic origin or with host-plant use. In the blueberry maggot, *Rhagoletis mendax* Curran (Diptera: Tephritidae) there seems to be at least two genetically distinct populations that differ in flight periods (Teixeira & Polavarapu, 2003). Thus, it is possible that the genetic differences found in the cranberry fruitworm could reflect phenological differences among the individuals caught for this study. This seems unlikely though, because insects collected from cranberries and blueberries were all collected during the same period at the same developmental stage. Alternatively, the cryptic genotypic differentiation found in the cranberry fruitworm, may reflect genetic differentiation due to *A. vaccinii* association with host-plant species not considered in this study (e.g., wild blueberries and huckleberries), or could be explained by factors that we did not measure, such as differential bacterial associations on distinct *A. vaccinii* genotypes (Medina et al., 2011).

In conclusion, despite presenting the characteristics that are commonly associated with HAD, we found no strong evidence for HAD's genetic signature in *A. vaccinii* associated with blueberries and cranberries, which suggests that the factors thought to explain HAD are more complex than previously suspected. From a pest management perspective, it is important to know if HAD is present because if pests on different host plants are genetically distinct they may differ in traits relevant to their control (e.g., insecticide resistance, susceptibility to natural enemies). As we currently lack reliable predictors, generalist pest species should be tested for HAD whenever its presence may interfere with control measurements (e.g., when managing vegetation adjacent to crops, when designing refuges for transgenic crops).

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