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Spatial and genetic structure of host-associated differentiation in the parasitoid *Copidosoma gelechiae*

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Abstract

Host-associated differentiation (HAD) appears to be an important driver of diversification in the hyperdiverse phytophagous and parasitoid insects. The gallmaking moth *Gnorimoschema gallaesolidaginis* has undergone HAD on two sympatric goldenrods (*Solidago*), and HAD has also been documented in its parasitoid *Copidosoma gelechiae*, with the intriguing suggestion that differentiation has proceeded independently in multiple populations. We tested this suggestion with analysis of Amplified Fragment Length Polymorphism (AFLP) markers for *C. gelechiae* collections from the midwestern and northeastern United States and eastern Canada. AFLP data were consistent with the existence of HAD, with between-host F_{ST} significant before Bonferroni correction in two of seven sympatric populations. AMOVA analysis strongly rejected a model of HAD with a single historical origin, and thus supported the repeated-HAD hypothesis. *Copidosoma gelechiae* shows significant host-associated divergence at a number of allozyme loci (Stireman *et al.*, 2006), but only weak evidence via AFLPs for genome-wide differentiation, suggesting that this species is at a very early stage of HAD.

Introduction

Phytophagous insects and their associated parasitoids constitute a large fraction of the Earth's total biodiversity, and explaining the astounding diversity of phytophagous and parasitic clades is an important challenge for evolutionary ecologists. A large and rapidly growing body of literature has focused on the evolution of new insect diversity via host shifting and subsequent host-associated differentiation (HAD) by both phytophagous insects (e.g. Stireman *et al.*, 2005; Feder & Forbes, 2008; Funk & Nosil, 2008) and their natural enemies (e.g. Jager & Menken, 1994; Kankare *et al.*, 2005; Smith *et al.*, 2006; Stireman *et al.*, 2006; Marussich & Machado, 2007; Abrahamson & Blair, 2008; Forbes *et al.*, 2009). Mounting evidence suggests that HAD can proceed rapidly, perhaps even in sympatry, and has occured or is occuring in numerous and ecologically diverse insect lineages (Berlocher & Feder, 2002; Dres & Mallet, 2002). However, it remains unclear what ecological factors determine whether an insect lineage associated with multiple hosts undergoes HAD rather than remaining a host generalist, and in particular, how often the occurence of HAD in a phytophagous insect species results in 'cascading' HAD among parasitoids of that species (Stireman *et al.*, 2006).

A powerful model system for the study of HAD among phytophagous insects and parasitoids is provided by the insect community associated with the goldenrods *Soldago altissima* and *Solidago gigantea*. Among the 100+ insect herbivores feeding on one or both goldenrods (Root & Cappuccino, 1992; Fontes *et al.*, 1994) there are at least four cases of HAD leading to the occurence of host-races or host-specialist cryptic species on each goldenrod (Stireman *et al.*, 2005). Furthermore, in at least two cases, there is evidence for cascading HAD, or genetic differentation of a parasitoid attacking a host-race or cryptic species pair on the two goldenrods (Stireman *et al.*, 2006). One of these two cases is particularly interesting: the encyrtid wasp *Copidosoma gelechiae*, which

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is the most common parasitoid of the gallmaking moth *Gnorimoschema gallaesolidaginis*. *Gnorimoschema gallaesolidaginis* has strongly differentiated host races, or possibly cryptic species, on its two hosts (Nason *et al.*, 2002; Stireman *et al.*, 2005). Stireman *et al.* (2006) found that collections of *C. gelechiae* from *G. gallaesolidaginis* larvae on the two goldenrod hosts showed subtle but significant allozyme differentiation. Furthermore, Stireman *et al.* (2006) suggested that *C. gelechiae* had undergone HAD repeatedly, with independent differentiation at three widely separated sites (in Minnesota, Ontario and New Brunswick). This suggests that HAD might occur remarkably easily, and so has major implications for our understanding of insect diversification.

Stireman *et al.*'s (2006) conclusions were limited to three *C. gelechiae* populations and nine allozyme markers, and therefore our picture of host specialization and HAD in *C. gelechiae* is incomplete. Here, we greatly expanded sampling both of the species' geographic range and of its genome, collecting material from 21 sites across a rough east–west transect over 2 000 km long. We generated 940 AFLP (amplified fragment length polymorphism; Vos *et al.*, 1995) markers for our collections. Together, the geographic and genetic scale of our assays allowed us to test for host-associated genetic differentiation across much of the *C. gelechiae*'s range and across broad regions of its genome. We asked two general questions about geographic and host-associated genetic structure in *C. gelechiae*. First, we asked whether AFLP markers indicated genome-wide differentiation between collections from *S. altissima* and *S. gigantea*, either at locations where Stireman *et al.* (2006) documented allozyme differentiation or at newly sampled sites. Second, we asked whether any genetic structure present was more consistent with a single historical origin of HAD (origin of a novel host form, followed by its dispersal across the sampled range) or with repeated local origins of HAD. To aid comparison of the results of our AFLP analyses to those obtained by Stireman *et al.* (2006) using allozymes, we also subjected their allozyme data to several additional analyses. Based on the joint AFLP and allozyme results we suggest that *C. gelechiae* is in the earliest stages of HAD, and offers an exciting opportunity to study the genetic and geographic structure of nascent biodiversity in an insect parasitoid.

Materials and methods

Study organisms

Copidosoma gelechiae Howard (Hymenoptera: Encyrtidae) is a primary parasitoid of *Gnorimoschema gallaesolidaginis* Riley (Lepidoptera: Gelechiidae), which in turn is a gallmaking herbivore of the two goldenrod species *Solidago altissima* L. (late goldenrod) and *S. gigantea* Ait. (tall goldenrod). The goldenrod hosts are closely related members of the *S. canadensis* complex (Asteraceae: Astereae). They are widespread perennials and are common, and often sympatric, across much of North America (Semple & Cook, 2006). These plants are often abundant in prairies, at forest edges, along roadsides, in abandoned fields and in empty commercial lots.

Gnorimoschema gallaesolidaginis is a common univoltine gallmaker on *S. altissima* and *S. gigantea* through most of their range. Larvae bore into host-plant stems and induce spindle-shaped galls; pupation begins in mid-summer with emergence occurring around the beginning of September. *Gnorimoschema gallaesolidaginis* populations on *S. altissima* and *S. gigantea* are genetically distinct and represent either well-established host races or young cryptic species (Nason *et al.*, 2002; Stireman *et al.*, 2005). Moths on *S. gigantea* are sometimes treated as *G. jocelynae* Miller (Miller, 2000).

Copidosoma gelechiae is the most common primary parasitoid of *G. gallaesolidaginis* (on both goldenrod hosts) across most of its range (S.B. Heard, unpublished). The wasp oviposits in *G. solidaginis* eggs, but grows most actively late in the larval development of its host. *Copidosoma gelechiae* is polyembryonic, with up to about 200 larvae arising from a single egg (Patterson, 1915). Although larvae from each *C. gelechiae* egg are clones, occasionally superparasitism results in a mixed brood (all male, all female, or mixed sex, depending on whether one or both eggs were fertilized). Parasitized *G. gallaesolidaginis* larvae do not pupate, instead continuing to feed and growing significantly larger than unparasitized larvae. *Copidosoma gelechiae* larvae pupate in late summer and adult wasps, which are 0.5–2 mm long, emerge in the last week of August.

Copidosoma spp. can also be found in *Gnorimoschema* hosts on other host plants, including *Gnorimoschema salinaris* on *Solidago sempervirens*. The taxonomic identity of *Copidosoma* on *G. salinaris* has not yet been documented. Other *Copidosoma* also parasitize nongallmaking Lepidoptera; among these is the well-studied *Copidosoma floridanum*, which parasitizes *Trichoplusia ni*. The complete host ranges of *C. gelechiae* and of the genus *Copidosoma* are unknown.

Collections

In August of 2005, we collected *C. gelechiae* from sites in the mid-western and northeastern US and two Canadian provinces (Fig. 1), roughly along an east–west transect from Minnesota to New Brunswick. This does not span the entire range of *C. gelechiae*; although that range is poorly known, we suspect it covers most or all of the range of the host insect *G. gallaesolidaginis* (Miller, 2000). Exact locations for all collection sites are reported in Appendix I. Sites from Stireman *et al.* (2005) were re-sampled. We preferred sites where we could collect *C. gelechiae* from *Gnorimoschema* galls on both goldenrod hosts (henceforth referred to as 'sympatric sites'), but in order to achieve appropriate geographical sampling, we

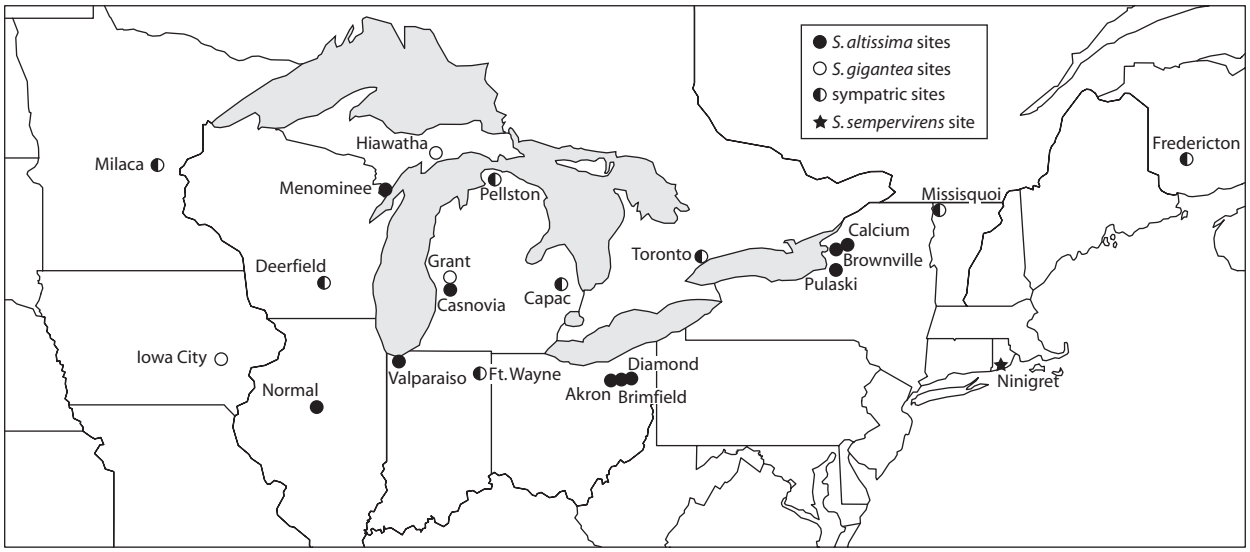

Fig. 1 *Copidosoma gelechiae* collection sites. Sites marked with half-filled circles had collections from both goldenrod hosts ('sympatric sites'); filled circles indicate sites with collections only from *Soldago altissima*, and open circles indicate sites with collections only from *Soldago gigantea*. Star marks collection site for *Copidosoma* sp. from *Solidago sempervirens*.

also included sites with only one host plant present (or with both plants present, but *Gnorimoschema* galls on only one). We refer to sites of the latter kind as 'allopatric' sites, but we acknowledge that there may be individuals of the alternate host within the dispersal range of emerging adults.

At each collection site, we collected and opened *G. gallaesolidaginis* galls, removing the gallmaker larvae and inspecting them for the presence of parasitoid (*C. gelechiae*) pupae. Parasitized larvae were immediately preserved in 95% ethanol. Some gallmaker larvae showed signs of infestation with *C. gelechiae* (much larger body size, reduced mobility and flexibility), but did not yet show visual evidence of *C. gelechiae* pupae. These larvae were held alive until *C. gelechiae* pupae developed and were then preserved in 95% ethanol. Preserved samples were stored at ambient temperatures (20–30 °C) until DNA extraction. Representatives of two additional *Copidosoma* species were also obtained to serve as outgroups in phylogeographic analyses. We collected *Copidosoma* sp. from *Gnorimoschema salinaris* larvae galling *Solidago sempervirens* (from Ninagret Conservation Area, RI, USA). Preserved *C. floridanum* were obtained from Dr Michael R. Strand (University of Georgia).

DNA extraction, AFLP reactions, and electrophoresis

We extracted DNA using Qiagen® (Valencia, CA, USA) DNeasy blood and tissue DNA extraction kits. A single *C. gelechiae* pupa was dissected from each host larva, in order to eliminate the possibility of combining genetic material from nonclones in the case of a mixed brood. Samples were air dried for 15 min and then ground in liquid nitrogen. DNA extraction followed the kit protocol, except that samples were incubated at 55 °C overnight, eluted just once with 25 μL of elution buffer AE, and the elution buffer was allowed to sit on the membrane for 5 min before the final centrifugation. An aliquot from each sample was run on a 1.5% agarose electrophoresis gel and stained with ethidium bromide to determine DNA quality. Samples showing fragmented or degraded DNA were re-extracted.

Procedures for AFLP analysis were based on those of Vos *et al.* (1995) following the modifications of Segraves & Pellmyr (2004). Genomic DNA was cut with the *Eco*RI and *Mse*I restriction endonucleases and then adapter sequences were ligated onto the fragments with T4 DNA ligase (Promega, Madison, WI, USA). Preselective amplification was conducted using the primers Mse+C and Eco+A, and a second selective amplification step was performed using six primer combinations in two multiplex reactions (Mse+CAC or Mse+CTT with each of Eco+AAG, Eco+AAC and Eco+ACA; see Segraves & Pellmyr, 2004). Eco+3 primers were labelled with one of three 'lightsaber' dyes (black/blue/green; Synthegen, Houston, TX, USA). Fragment lengths for the resulting labelled PCR products were determined via electrophoresis on a Beckman Coulter (Fullerton, CA, USA) CEQ6000 sequencer.

AFLP data analysis

Raw fragment length data were filtered with software developed by Abdo *et al.* (2006). This software identifies fluorescence peaks associated with AFLP fragments automatically, enabling quick and consistent scoring of

large data sets. The filtering algorithm first standardizes the dataset by calculating relative peak areas. Next, the standard deviation of peak areas is calculated. Any peaks more than three standard deviations above the mean fluorescence are scored as representing true AFLP fragments and are excluded from further calculations. This process is iterated until no more peaks can be scored, and any remaining fluoresence peaks are considered as background noise (Abdo *et al.*, 2006).

Amplified Fragment Length Polymorphism fragment data were used in three complementary analyses. First, we conducted analyses of molecular variance (AMOVAS) using Arlequin® V 2.0 (Schneider *et al.* 2000). AMOVA analyses apportion genetic variation to levels of hierarchichal organization – in this case, host affiliation and site. We compared AMOVA analyses specifying sites nested within host affiliations and specifying host affiliations nested within sites. The former hierarchical structure is appropriate if a single HAD event was followed by dispersal of the novel host race, whereas the latter structure is appropriate either if HAD proceeded independently in each population or if there is no host-associated differentiation, but some geographical differentation. We compared the explanatory power of the alternative models using the corrected Akaike Information Criterion, following calculations outlined in Halverson *et al.* (2008). The AMOVA analysis used data for seven sympatric sites. Collections from Ft. Wayne, IN, included only two broods from *S. altissima* and were excluded from the AMOVA analyses.

Second, we calculated F_{ST} values between *C. gelechiae* collections from *S. altissima* and *S. gigantea* at the same seven sympatric sites used in the AMOVAS, using AFLP-Surv (Vekemans *et al.*, 2002). Estimates of allelic frequencies were computed using the 'Bayesian method with nonuniform prior distribution of allelic frequencies' (Zhivotovsky, 1999), and Hardy–Weinberg genotypic proportions were assumed. F_{ST} values significantly different from zero would identify populations with barriers to gene flow between host-associated types. In order to reduce inflation of type I error when performing these tests on multiple locations, we checked *P*-values for significance following sequential Bonferroni correction. This procedure has been criticized as being too conservative (Moran, 2003; Nakagawa, 2004) so cautious interpretation of these results would be appropriate. As an alternative way to test for significant local differentiation, we also used AMOVA to calculate the between-hosts genetic variance component V_a, using permutation (1000 replicates) to test for V_a values significantly greater than zero. The results of this procedure differed only slightly from the tests using F_{ST}, and so are not reported.

Third, as a way to visualize phylogeographic structure in our data we calculated Nei-Li genetic distances among pairs of populations using AFLP-Surv (Vekemans *et al.*, 2002). We then used the NEIGHBOR program of Phylip 3.67 (Felsenstein, 2005) to create a neighbour-joining tree based on the Nei-Li distances. This analysis included data from all our collections, both sympatric and allopatric, although one using only sympatric collections (not shown) leads to similar interpretation. Analyses using the two alternative outgroups generally resulted in identical ingroup topologies, so we report only results using *Copidosoma* from *S. salinaris*. Under the single-HAD-event hypothesis, we would expect populations from one of the two hosts to cluster together (e.g. Stireman *et al.*, 2005, tree for *G. gallaesolidaginis*); in contrast, under the multiple-HAD-events hypothesis, we would expect populations from the two hosts at each site to cluster. We calculated bootstrap support for each node as a measure of confidence in tree structure.

Allozyme data analysis

Sampling and allozyme genotyping of *C. gelechiae* populations are described in Stireman *et al.* (2006) for sympatric collections from *S. altissima* and *S. gigantea* at Fredericton (nine polymorphic loci), Milaca (eight polymorphic loci) and Toronto (seven polymorphic loci). For each sympatric site, Stireman *et al.* (2006) tested for significant differentiation at individual loci using exact genic tests (Raymond and Rousset 1995) and over all loci using an exact genotypic test (Goudet *et al.*, 1996), finding significant genic tests for a subset of loci (different subsets at each site) and a significant genotypic test at each site. For more direct comparison to our AFLP analyses, we used Stireman *et al.*'s (2006) allozyme data to estimate F_{ST} (and 95% bootstrap confidence limits) between sympatric populations using Weir & Cockerham's (1984) θ as calculated by the program GDA (Lewis & Zaykin, 1999). Stireman *et al.* (2006) reported site-specific estimates of F_{ST} but not their statistical significance.

Stireman *et al.*'s (2006) finding that genic tests were significant for different subsets of loci at different sites indicates HAD at multiple genomic regions and suggests that overall significance was not the result of selection affecting genetic variation at a few specific loci or closely linked genomic regions. To more directly evaluate the potential for selection to explain differentiation at individual allozyme loci, we subjected F_{ST} estimates for each locus and at each site to the Lewontin & Krakauer (1973) test of neutrality, as advocated in several recent papers (Vitalis *et al.*, 2003; Whitlock, 2008). Because the L–K test uses the expected neutral distribution of single-locus F_{ST} values in the range 0–1, we constrained any negative variance components obtained from GDA to 0 to obtain, for each site, single- and multi-locus estimates of F_{ST} in the range 0–1. Using the constrained multi-locus F_{ST} as the mean F_{ST} value in the L–K test (with 1 degree of freedom), for each site we rejected neutrality for an individual locus if its estimated F_{ST} was greater than or equal to the upper 95% critical value of the expected neutral distribution. Because we employed the L–K test

many times, we checked *P*-values for significance following sequential Bonferroni correction.

Results

AFLP markers

We identified and scored 940 AFLP markers, of which typically 400–600 were polymorphic among individuals within a site by host species combination.

AMOVAS

In each AMOVA model, less than 4% of the total genetic variation is assigned to the host plant and site levels, with the remainder assigned to the lowest level in the hierarchical structure (within host plants, or within sites; Table 1). The analysis nesting sites within host plants provided a negative variance component for host plants (Table 1A), and the alternative model nesting host plants within sites was strongly favoured ($\Delta AIC_c = 9.5$). This analysis suggests that while there is relatively little structure in the genetic data, the structure present is best accomodated by a model grouping collections from the two host plants together within sites.

Table 1 AMOVA analyses partitioning genetic variance in *Copidosoma* among sites and host plants. Model (b) is strongly favoured, with AIC analysis indicating essentially no support for model (a) ($\Delta AIC_c = 9.5$).

Source of variation	d.f.	Sum of squares	Variance	Percent of total variance	*P*
(a) Sites nested within host plants					
Host plants	1	92.4	0*	0*	0.81
Sites within host plants	12	1380	3.11	3.7	< 0.0001
Within sites	143	11 700	81.5	96.3	
Total	156	13 200	84.6	100	
(b) Host plants nested within sites					
Sites	6	864	2.61	3.1	< 0.0001
Host plants within sites	7	610	0.511	0.6	0.10
Within host plants	143	11 700	81.5	96.3	
Total	156	13 200	84.6	100	

*A small but negative variance component is set to zero; this negative estimate was not significantly different from zero.

Local F_{ST}s

Amplified Fragment Length Polymorphism genetic differentiation between sympatric *C. gelechiae* collections from *S. altissima* and *S. gigantea* was generally subtle (Table 2), with an average F_{ST} of 0.012. Before Bonferroni correction, F_{ST} values were significantly greater than zero at two sites (Missisquoi, $F_{ST} = 0.043$; and Pellston, $F_{ST} = 0.028$). Following Bonferroni correction, none of the seven F_{ST} values was significant, although the highly conservative nature of the correction makes interpretation of this result difficult. For the sites re-sampled from Stireman *et al.* (2006); Fredericton, Milaca, and Toronto), our nonsignificant AFLP estimates of F_{ST} do not confirm the significant differentiation observed for allozyme markers using genic and genotypic exact tests. However, allozyme estimates of F_{ST} for these sites were also less conclusive than the genic and genotypic tests, with F_{ST} significantly greater than zero at only one site (Toronto, $F_{ST} = 0.0510$ with 95% limits 0.0060–0.0677).

Phylogeography

The neighbour-joining tree of all *C. gelechiae* populations (Fig. 2) shows no strong grouping based on either sites or host affiliations. We were not able to infer with confidence the relationships between populations (bootstrap support for all nodes < 70%; data not shown). This lack of recognizable genetic structure was surprising and led to some concern that the AFLP data were too noisy or

Table 2 Population specific F_{ST} values for seven sympatric sites. For AFLP data, bold font indicates *P*-values of F_{ST} estimates significant before Bonferroni correction (none are significant after correction). For allozyme data, bold font indicates confidence limits about F_{ST} estimates that exclude zero.

	AFLP data				Allozyme data			
Population	No. broods (*altissima*/*gigantea*)	Polymorphic loci (*altissima*/*gigantea*)	F_{ST}	*P*	No. broods (*altissima*/*gigantea*)	Polymorphic loci (*altissima*/*gigantea*)	F_{ST}	95% CI†
Capac	20/21	521/504	< 0.001	0.95	–	–	–	–
Deerfield	10/10	523/510	0.0056	0.49	–	–	–	–
Fredericton	15/22	455/438	0.0060	0.16	44/58	9/7	0.0029	–0.0095–0.029
Milaca	4/8	446/559	< 0.001	0.97	71/66	7/8	0.0143	–0.0028–0.036
Missisquoi	9/5	471/457	0.043	**0.030**	–	–	**–**	–
Pellston	8/5	477/452	0.028	**0.05**–0.06*	–	**–**	**–**	–
Toronto	10/11	559/511	< 0.001	0.71	44/41	5/7	0.0510	**0.0061–0.068**

**P*-value range arises because significance is assessed via bootstrapping. For all other populations, the *P*-value range does not span 0.05, and only the central value is reported.

†95% bootstrap confidence limits; GDA does not provide *P*-values.

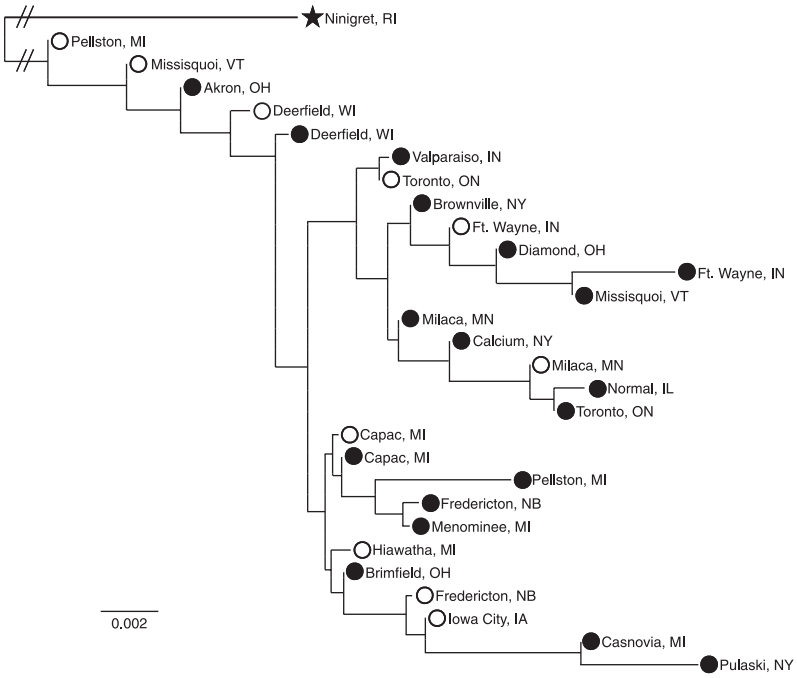

Fig. 2 Estimated phylogeographic relationships among sampled *Copidosoma gelechiae* populations. Tree was constructed via neighbour-joining based on Nei-Li distances (indicated as scale bar). Bootstrap support for all nodes < 70%. The outgroup (at which the root is forced) is *Copidosoma* sp. collected from *Solidago salinaris*. Filled circles indicate collections from *S. altissima* and open circles indicate collections from *S. gigantea*.

otherwise unreliable. However, when analyzed as individuals (UPGMA tree not shown) the outgroup individuals clustered together, whereas individuals from other populations were intermixed. This suggests that our markers can reveal population structure, but that AFLP differentiation between sympatric collections from *S. altissima* and *S. gigantea* is absent or extremely subtle at this (genetic) scale.

L–K tests of allozyme neutrality

Lewontin–Krakauer tests of single-locus F_{ST} estimates (Table 3) were significant for one of nine polymorphic allozyme loci at Fredericton (PGI $F_{ST} = 0.0570$, $P = 0.016$) and one of eight polymorphic loci at Milaca (ACOH $F_{ST} = 0.0727$, $P = 0.040$). No tests were significant after sequential Bonferroni correction for multiple tests within sympatric sites.

Table 3 Lewontin–Krakauer tests of allozyme locus neutrality at three sympatric sites studied by Stireman *et al.* (2006). *P*-values in bold were significant at the 0.05 level. None were significant following sequential Bonferroni correction for multiple tests within sites. Negative F_{ST} values (none less than −0.015) were constrained to zero.

	Fredericton		Milaca		Toronto	
Locus	F_{ST}	*P*	F_{ST}	*P*	F_{ST}	*P*
ACOH	0.0237	0.120	0.0727	**0.040**	–	–
G3PDH	0.0000	1.000	0.0000	1.000	0.0085	0.068
HADH3	0.0000	1.000	0.0254	0.223	0.0132	0.611
HK	0.0082	0.362	–	–	–	–
IDH	0.0157	0.207	0.0588	0.064	0.0136	0.607
LDH	0.0000	1.000	0.0000	1.000	0.0679	0.249
MDH	0.0000	1.000	0.0343	0.157	0.0707	0.239
PGI	0.0570	**0.016**	0.0000	1.000	0.0054	0.745
PGM	0.0161	0.200	0.0054	0.574	0.0008	0.900

Discussion

Our AFLP results provide at most weak evidence of host-associated divergence (HAD) at the whole-genome scale in *C. gelechiae*, but in combination with evidence of HAD from allozymes (Stireman *et al.*, 2006) they do shed useful light on the geographic structure of differentiation. Our AMOVA analysis strongly favours a model of genetic structure with host affiliation nested within sites (Table 1; as did Stireman *et al.*'s (2006) similar analysis). This is clearly inconsistent with a model of HAD involving a single host shift followed by dispersal of a novel host form – that is, the model that was supported for *C. gelechiae*'s host, *G. gallaesolidaginis* (Nason *et al.*, 2002; Stireman *et al.*, 2005). Instead, it is consistent either with the absence of HAD but the presence of some geographic differentiation, or with the occurence of HAD independently at multiple sites (as argued by Stireman *et al.*, 2006). These two possibilities are best separated by site-by-site analysis of reproductive isolation (Table 2). The results of such analyses, calculating Wright's F_{ST} from our AFLP data, are suggestive of HAD at Missiquoi and Pellston (F_{ST} significant before, but not after, Bonferroni correction) but provide no evidence for genome-wide differentiation at the other sites.

These AFLP results constrast with Stireman *et al.*'s (2006) analysis of differentiation at three sympatric sites based on allozyme markers (7–9 polymorphic loci/site). Stireman *et al.* (2006) found strong evidence for HAD in *C. gelechiae* at all sites using both genic and genotypic exact tests. This, along with AMOVA analyses favouring grouping of populations by site and then by host affiliation, suggested that *C. gelechiae* had undergone HAD, and that differentation had occurred independently at least three times. We expected that our F_{ST}-based analyses of AFLP and allozyme markers would confirm the occurrence of local differentiation, but they do so only weakly: when assessed using F_{ST}, differentiation based on AFLP markers was nonsignificant at all three of Stireman *et al.*'s (2006) sites whereas allozyme differentiation was significant only at one of them (Table 2). The apparent conflict between the strong allozyme results reported by Stireman *et al.* (2006) and the weak AFLP and allozyme results of this study has at least three potential explanations: the differences might be attributable to (i) the nature of expected differentiation during early stages of HAD, (ii) issues of statistical power arising from the inheritance and sampling of the two classes of genetic markers, or (iii) issues of power arising from the different analyses of differentiation used.

First, discrepancies between allozyme and AFLP-based measures of differentiation might not be unexpected at early stages of HAD, when divergence may often be driven by strong selective pressures and consequent changes at a relatively small number of loci (Coyne & Orr, 2004; Gavrilets, 2006) – loci that encode phenotypically important information, or are closely linked to other loci that do. These few divergent regions of the genome will be surrounded by large areas of neutral or nearly neutral DNA (coding and noncoding) that have not undergone sufficient divergence to be easily distinguished. Over time, and once a sufficient level of reproductive isolation has evolved, divergence is expected to spread across the rest of the genome (Coyne & Orr, 2004), including loci under weak or no selection and including noncoding regions (which are sampled by AFLP but not by allozyme markers). Because allozymes reflect the genetic variation only of coding regions, it is possible that the relatively strong differentiation revealed by the exact genic and genotypic tests of Stireman *et al.* (2006) reflect greater average effects of selection on allozyme vs. AFLP loci. However, for two reasons this seems unlikely to be the case for *C. gelechiae*. First, Stireman *et al.*'s genic tests were significant for different subsets of loci at different sites, suggesting that if selection is operating on these loci it is doing so inconsistently across geographic locations. Second, Lewontin and Krakauer tests conducted here on Stireman *et al.*'s (2006) allozyme data revealed only two potential cases of selection (Table 3), neither of which was significant after correction for multiple tests.

Second, the difference between studies may be attributable to differences in the markers employed and the sampling of genetic information. Simulations by Goudet *et al.* (1996) indicate that when genetic differentiation between populations is low, as is the case for host-associated populations of *C. gelechiae*, the power to detect differentiation using F_{ST} benefits strongly from sampling many individuals per populations. They suggest that getting better estimates of local allele frequencies increases the power of the test to detect small differences in allele frequencies. While we assayed a large number of AFLP loci, the number of individuals sampled per host-associated population was modest (4–22; in accord with many AFLP studies). In contrast, Stireman *et al.* (2006) assayed far fewer allozyme loci but sampled 44–71 individuals per population. Furthermore, estimates of local allele frequencies can be affected by the mode of inheritance of the marker system. While allele frequencies at codominant loci (such as allozymes) can be estimated without bias, allele frequency estimates at dominant loci (such as AFLPs), can be biased in small populations (Lynch & Milligan, 1994). Still, these observations are unlikely to entirely explain the observed differences between AFLP and allozyme loci for HAD in *C. gelechiae*. One reason is that AFLP F_{ST} estimates were highest and significant (before sequential Bonferroni correction) not at the sites with the largest sample sizes (Capac and Fredericton) but at two sites (Missisquoi and Pellston) with barely half as many sample individuals (Table 2). Another reason is that AFLP and allozyme estimates of F_{ST} were in fact quite similar (Table 2). Therefore, inherent differences between AFLP and allozyme markers are unlikely to explain the apparent conflict between our results and those of Stireman *et al.* (2006).

The third, and most likely, explanation for the contrast between our results and those of Stireman *et al.* (2006) is a difference in analyses of differentiation. In this study, we focussed on F_{ST} analyses amenable to both AFLPs and allozymes, but Stireman *et al.* (2006) used exact genic and genotypic tests of differentiation using allozymes (such tests cannot be applied to dominant markers such as AFLPs). Goudet *et al.* (1996) tested the power of exact allelic and genotypic tests and F_{ST}-estimator tests of population differentiation, and found them to have similar power for balanced data sets. Therefore, we expected to be able to expand on the well-supported allozyme evidence of geographically independent HAD reported by Stireman *et al.* (2006) with our F_{ST}-estimator tests of differentiation using AFLPs and allozymes. Instead, whether employing AFLP or allozyme data, our F_{ST}-based tests appear to have less power to detect differentation than Stireman *et al.*'s (2006) exact allelic and genotypic tests. The reason for this is unclear but may be related to F_{ST}-estimator tests being less sensitive than the exact tests to variation in the frequency of low frequency alleles.

The wide geographic scope of our study (Fig. 1) was designed to test Stireman *et al.*'s (2006) hypothesis that HAD in *C. gelechiae* had proceeded independently at the three sites they sampled. Although AFLP markers provided only weak evidence for HAD itself, our AMOVA analysis strongly rejects a hypothesis of HAD with a single historical origin. As the existence of HAD is strongly supported by Stireman *et al.*'s (2006) allozyme data, we interpret our AFLP results as supporting the independent-origins hypothesis. Intriguingly, our results even suggest that HAD may have begun independently at more sites than the three studied by Stireman *et al.* (2006). The two sympatric sites at which we found weak evidence of HAD lie midway between pairs of sites from Stireman *et al.*, 2006 (Pellston, MI between Milaca and Toronto, and Missisquoi, VT between Toronto and Fredericton; Fig. 1), and in neither case do we see any evidence that host-associated collections at our new sites group with collections from the same hosts at the sites of Stireman *et al.* (2006) (Fig. 2). A high priority for future research will be to re-sample the Pellston and Missisquoi sites with much larger sample sizes, in order to examine host-associated divergence at the allozyme loci that were informative at other sites for Stireman *et al.* (2006).

Our emerging picture of host evolution in *C. gelechiae* is one of subtle differentation in a species at the very earliest stages of HAD. This offers an exciting opportunity, because the same pair of host plants supports both herbivores (Stireman *et al.*, 2005) and parasitoids (Stireman *et al.*, 2006) at stages of differentiation ranging from host races to cryptic host-specialist species. Differentiation in *C. gelechiae* thus offers a view of the earliest stages of a process that can be studied in its later stages via other insect species in the same ecological setting. Taken together, studies to date of the goldenrod-insect system (e.g. Abrahamson & Weis, 1997, Stireman *et al.*, 2005, 2006; Abrahamson & Blair, 2008) paint a picture of repeated origin of new insect diversity through HAD via processes that are complex and include interesting variation in extent, timing and mechanism.

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Appendix I. Collection locations

Site name	State/ Province	North	West	No. broods from *Soldago altissima*	No. broods from *Soldago gigantea*
Akron	OH	41°02′	81°24′	5	–
Brimfield	OH	41°06′	81°21′	5	–
Brownville	NY	44°00′	76°00′	6	–
Calcium	NY	44°03′	75°50′	9	–
Capac	MI	42°59′	82°56′	20	21
Casnovia	MI	43°15′	85°48′	10	–
Deerfield	WI	43°01′	89°04′	10	10
Diamond	OH	41°06′	81°01′	10	–
Fort Wayne	IN	41°05′	85°02′	2	10
Fredericton	NB	45°57′	66°39′	15	22
Grant	MI	43°20′	85°49′	–	10
Hiawatha	MI	45°53′	86°50′	–	9
Iowa City	IA	41°41′	91°30′	–	7
Menominee	MI	45°16′	87°39′	9	–
Milaca	MN	45°45′	93°39′	4	8
Missisquoi	VT	44°58′	73°12′	9	5
Ninigret	RI	41°22′	71°32′	–	–*
Normal	IL	40°34′	88°57′	10	–
Pellston	MI	45°33′	84°42′	8	5
Pulaski	NY	43°33′	76°07′	5	–
Toronto	ON	43°38′	79°22′	10	11
Valparaiso	IN	41°32′	86°55′	10	–

*Collections (10 broods) from Ninigret were of *Copidosoma* sp. from *Gnorimoschema salinaris* galling *Solidago sempervirens*.

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