Evolutionary radiation of *Asteromyia carbonifera* (Diptera: Cecidomyiidae) gall morphotypes on the goldenrod *Solidago altissima* (Asteraceae)

JOHN O. STIREMAN III1*, ERIC M. JANSON2, TIMOTHY G. CARR3, HILARY DEVLIN1 and PATRICK ABBOT2

1Department of Biological Sciences, Wright State University, 3640 Colonel Glenn Highway, Dayton, OH 45435, USA
2Department of Biological Sciences, Vanderbilt University, Box 351634 Station B, Nashville, TN 37235, USA
3Department of Ecology and Evolutionary Biology, Cornell University, E145 Corson Hall, Ithaca, NY 14853, USA

Received 21 April 2008; accepted for publication 3 June 2008

Population divergence of phytophagous insects is often coupled to host-plant shifts and is frequently attributed to the divergent selective environments associated with alternative host-plants. In some cases, however, divergence is associated with the use of alternative host-plant organs of a single host species. The basis of within-host radiations such as these remains poorly understood. In the present study, we analysed the radiation of *Asteromyia* gall midges occurring both within one host plant species and within a single organ on that host. In this system, four morphologically distinct *Asteromyia* gall forms (morphs) coexist on the leaves of goldenrod *Solidago altissima*. Our analyses of amplified fragment length polymorphism and DNA sequence data confirm the genetic differentiation among midges from three gall morphs and reveal evidence of a genetically distinct fourth gall morph. The absence of clear gall morph related clades in the mitochondrial DNA derived phylogenies is indicative of incomplete lineage sorting or recent gene flow, suggesting that population divergence among gall forms is recent. We assess the likely history of this radiation and use the results of phylogenetic analyses along with ecological data on phenology and parasitism rates to evaluate potential hypotheses for the mode of differentiation. These preliminary analyses suggest that diversification of the *Asteromyia* gall morphs is likely shaped by interactions between the midge, a symbiotic fungus, and parasitoid enemies. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, 95, 840–858.


INTRODUCTION

Insect diversity is strongly associated with phytophagy (Mitter, Farrell & Wiegmann, 1988; Farrell, 1998). At the root of this association are the specialized relationships that most insects have with their host plants. Although some are extreme polyphages, most phytophagous insects are restricted to either one or a small set of related host plant species (Strong, Lawton & Southwood, 1984; Dyer et al., 2007). When successful, shifts between host plants can result in divergent selection, assortative mating, and/or spatial isolation between populations on different hosts (Futuyma & Moreno, 1988; Jaenike, 1990; Berlocher & Feder, 2002). Differential adaptation to ecological niches and reproductive barriers associated with the use of alternative host plants is thought to encourage ecological specialization and eventually lead to the emergence of reproductively isolated host races or species (Feder et al., 1997; Rundle & Nosil, 2005;
Stireman, Nason & Heard, 2005; Funk, Nosil & Etges, 2006).

In this light, the existence of clades that have radiated on a single host plant can be puzzling. Some members of tephrild flies (Condon & Steck, 1997), cone flies (Sachet, Roques & Despres, 2006), pinyon aphids (Favret & Voegtlin, 2004), gall-forming aphids (Inbar & Wool, 1995; Abbot & Withgott, 2004), cynipid wasps (Cook et al., 2002), mordellid beetles (Blair et al., 2005), and gall midges (Jones, Gagné & Barr, 1983; Hawkins, Goeden & Gagné, 1986; Gagné, 1989; Dorchin, Freidberg & Mokady, 2004; Joy & Crespi, 2007) exhibit evidence of within-host radiations, typically involving utilization of different host plant structures or organs (Stone & Schonrogge, 2003). But what new niches and ecological opportunities do these structures provide, and how might they lead to reproductive isolation and speciation? Are within-host radiations scaled-down versions of their between-host counterparts, or do within-host radiations involve qualitatively different mechanisms?

Some evidence from gall midges favours the latter, and suggests that ecological interactions are comparably more persistent over the course of population divergence, and therefore important in shaping within-host rather than between-host radiations (Joy & Crespi, 2007). For between-host radiations, the ecological disadvantages of generalist strategies are principally mediated by selection for efficient host location (Bernays, 2001), physiological tradeoffs associated with host plant use (Jaenike, 1990), mate finding and selection (Diehl & Bush, 1984), and other ecological factors acting during the initial stages of divergence. But, for within-host radiations, direct or apparent competition, shared predators, and other indirect interactions almost certainly persist well past the formation of post-zygotic reproductive isolation (Price, 2005). Given such interactions, the ecological coexistence of such recently diverged lineages is problematic, suggesting the existence of ecological stabilization mechanisms (e.g. temporal niche differentiation, frequency dependent parasitism; Chesson, 2000). At present, generalizations cannot be made with much confidence because only a few within-host radiations have been well-characterized.

In the present study, we use genetic data to investigate the diversification of an apparent within-host adaptive radiation in the North American goldenrod (Solidago)-galling cecidomyiid, Asteromyia carbonifera (Osten Sacken). Unlike most other known within-host radiations, for A. carbonifera, the available evidence to date reveals no obvious partitioning of alternative host structures. Populations of this species, morphologically-distinguishable by their gall morphologies, occur on the same plant structure (leaves) and may even be found on the same individual leaf, within centimeters of one another. In addition, predators and mutualists may be playing crucial roles in the origin and coexistence of these genetically distinct populations. As in a number of other cecidomyiid taxa, A. carbonifera is obligately associated with a symbiotic fungus on its host plant, and may not directly feed on the host plant at all (Gagné, 1968; Bissett & Borkent, 1988). Similar to many other gall-forming taxa, A. carbonifera also experiences high levels of mortality due to parasitoids (Weis, 1982a). Thus, the key selection pressures driving population divergence in this system may not be those between insect and plant, but between insect and symbiotic fungus, and possibly a consortium of specialist parasitoids as well.

In a previous survey of A. carbonifera gall morphology and allozyme frequencies, Crego et al. (1990) identified three morphologically and genetically distinct gall forms (hereafter ‘morphs’) of A. carbonifera that appeared to coexist on the host plant Solidago altissima L. Although that study provided initial evidence for a within-host radiation, nothing is known of the phylogenetic history and pattern of divergence of these gall morphs and how they may have arisen. The Cecidomyiidae is among the most diverse families in the Order Diptera, comprising over 5000 described species (Gagné, 2004). Many are economically important pests (e.g. the Hessian fly). However, studies of the mechanisms underlying their evolutionary diversification are rare (Joy & Crespi, 2007). In the present study, we use molecular markers and ecological data to more fully define and understand the diversity of radiating lineages contained within A. carbonifera on the host plant S. altissima and related Solidago species. Given the lack of obvious morphological differentiation of the midges (Gagné, 1968) and the broadly sympatric distribution of gall morphs, this system provides an ideal opportunity to examine the processes involved in the early stages of ecological speciation and adaptive radiation. The study aimed to answer the following primary questions:

1. Is the gall morph-associated reproductive isolation suggested by Crego et al. (1990) supported by mitochondrial and nuclear DNA data? Inference of distinct midge lineages in this system was based upon an allozyme study of a single population employing three loci in which Hardy–Weinberg equilibrium was rejected (Crego et al., 1990). DNA sequence and marker data will allow us to test whether these findings are robust and hold across populations.

2. Is there evidence of additional genetically divergent lineages of Asteromyia on S. altissima or on closely related co-occurring Solidago species?
3. What are the phylogenetic relationships among the gall morph associated populations? No attempt was made by Crego et al. (1990) to quantify or characterize the extent of divergence or the phylogenetic history of these lineages. With additional DNA data, we can assess whether gall morph-associated populations represent monophyletic clades indicative of an adaptive radiation.

4. What ecological conditions may have facilitated the divergence of these populations? Has phenological isolation facilitated population divergence? Have natural enemies played a role in gall-morph diversification?

**MATERIAL AND METHODS**

**Biology of A. carbonifera**

The goldenrod-galling cecidomyiid *A. carbonifera* forms blister galls on the leaves of *Solidago* spp. throughout North America (Gagné, 1968, 1989). *Asteromyia carbonifera* undergoes multiple generations each season from June to August/September and overwinters in the gall as mature larvae (Weis, 1982a). Adults are short lived, and are unlikely to survive for longer than a few days in nature (Weis, Price & Lynch, 1983; Yukawa & Rohfritsch, 2005; J. O. Stireman, unpubl. data). Eggs are glued to the underside of *Solidago* leaves, and newly-hatched larvae burrow into the leaf epidermis, initiating gall development. As known for several genera of Cecidomyiidae, *A. carbonifera* maintains an intimate association with a fungus; specifically, a lineage of the generalist filamentous ascomycete plant pathogen/endophyte, *Botryosphaeria* sp. (Borkent & Bissett, 1985; Bissett & Borkent, 1988; E. M. Janson, unpubl. data). Asexual fungal spores (conidia) appear to be carried by adult females in specialized abdominal invaginations associated with the ovipositor (mycangia), and it is suspected that these are deposited on *Solidago* leaves during oviposition (Borkent & Bissett, 1985). Fungal hyphae proliferate through the leaf tissue and form much of the final gall structure. Preliminary evidence from sterol analyses (E. M. Janson, unpubl. data) and in-vitro culturing of *Asteromyia* larvae (J. J. Heath, unpubl. data) indicate that the fungus is fed upon by the midge larvae. Larvae ultimately come to lie on the leaf mesophyll in a central gall chamber, surrounded by a layer of fungal mycelium.

Crego et al. (1990) identified three morphologically distinct gall forms caused by *A. carbonifera* on the common old-field goldenrod *S. altissima* (as *S. canadensis*) in Illinois. They termed these ‘flat’, ‘cushion’, and ‘irregular’ morphs (Gagné, 1968; Fig. 1). In addition to differing in external shape, these galls consistently differ in the mean number of chambers per gall, position of larvae within the gall, the position of the gall on the leaf, and several other physical characteristics (Gagné, 1968; Crego et al., 1990). Each gall morph can be found sympatrically and syn-
topically, co-occurring relatively frequently on the same ramet or individual leaf (J. O. Stireman & E. M. Janson, unpubl. data). Crego et al. (1990) also demonstrated that individuals of these gall morphs collectively had allozyme allele frequencies that deviated significantly from Hardy–Weinberg equilibrium, a pattern not observed for within-gall morph analyses. Moreover, they found no evidence of hybridization among gall morph-associated populations (Crego et al., 1990). This was interpreted as evidence for complete reproductive isolation among gall morph-associated populations, but random mating within gall morph-associated populations. Crego et al. (1990) identified a fourth gall morph, ‘crescents’, but no analyses of this form were conducted.

_Asteromyia carbonifera_ is attacked by at least five different species of hymenopteran parasitoids: _Torymus capite_ (Hüber), _Platygaster_ sp., _Baryscapus_ (Tetristichus) _fumipennis_ (Girault), _Aprostocetus_ (Tetristichus) _tesserus_ (Burkes), and an unidentified eulophid (Weis, 1982a). As in many gall-inducing Diptera and other insects (Hawkins, 1994), absolute parasitism rates are high, ranging from 40–60%, with population specific parasitism rates sometimes reaching over 80% (J. O. Stireman, unpubl. data). As part of normal gall development, fungal hyphae differentiate into a hard, black tissue layer (stroma) (Batra, 1964), which appears to prevent successful attack by _T. capite_ (Weis, 1982b), and possibly other parasitoid species.

**COLLECTION OF STUDY ORGANISMS**

_Asteromyia carbonifera_ galls were collected between September and October 2005 from _S. altissima_ ramets from five sites in southwestern Ohio: Aullwood Audubon Center (AA), Cedar Bog (CB), Pearl Fen (PF), Siebenthaler Fen (SF), and Zimmerman Prairie (ZP) (see Supporting Information, Appendix S1). Additional _Asteromyia_ collections were made from the plant species _Solidago rugosa_ Mill., a common associate of _S. altissima_ and _Euthamia graminifolia_ (L.) Nuttall, a distant goldenrod relative, in Ohio (see Supporting Information, Appendix S1). For these collections, leaves bearing mature galls were collected haphazardly along trails or field edges. Only a single galled leaf was collected per individual ramet in an attempt to broaden population sampling. Galls were transported in a cooler to the laboratory where they were photographed and dissected. For each gall, parasitism status, number of larvae, and developmental stage were recorded and the morphotype (flat, cushion, crescent or irregular) was designated based on traits previously described in Crego et al. (1990). Larvae and pupae dissected from a subset of these galls were stored in a (−20 °C) freezer, without preservative or in 95% ethanol until DNA was extracted. Additional collections of _A. carbonifera_ from _S. altissima_ and _S. rugosa_ were made opportunistically across much of the northeastern and north-central USA (NE/NC) by T.G.C. from 1997 to 2002 (see Supporting Information, Appendix S1; hereafter referred to as NE/NC samples). _A. carbonifera_ larvae and pupae were dissected from these galls and fresh frozen in a −80 °C freezer until extraction.

**GALL MORPHOLOGY**

The following traits were measured from photographs of all galls from the Ohio collections (\( N = 106 \)): length, width, basal area, luminosity (brightness, measured as luma), deviation from circularity, number of galls per leaf, distance from leaf edge to gall center, and position of gall on the leaf (base, middle, terminus). Deviation from circularity was measured as 1 – \( [a_i/a_a] \) where \( a_i \) is the area (\( \text{mm}^2 \)) of gall \( i \) and \( a_a \) is the area of the smallest circumscribing circle around gall \( a \). All measurements were conducted in Adobe Photoshop CS2 and standardized with respect to 0.5 × 0.5 cm grids of engineering paper on which the leaves were photographed. Gall morph data recorded from photographs were analysed using a multivariate general linear model in OpenStat (Miller, 2007). Tukey–Kramer post-hoc tests were used for pairwise comparisons of trait values for each _A. carbonifera_ gall morph. Gall height and width were excluded from analyses due to their strong correlations with gall area (0.778 and 0.752, respectively). Principle components analysis was also conducted to visualize differences in gall morphology. This data rotation did not result in easily delineated unambiguous clusters corresponding to gall morphs and results are not shown. A discriminant function analysis indicated relatively poor separation of gall morphs (see Results).

**COLLECTION OF DNA DATA**

DNA was extracted from _A. carbonifera_ using Puregene DNA Purification System, Cell and Tissue Kit (Gentra Systems Inc.), according to the manufacturer’s protocol. The entire pupa or larva was used for DNA extraction. Standard polymerase chain reactions (PCR) were conducted following conditions outlined in Stireman et al. (2005). Primers LCO1490 and HCO2198 (Folmer et al., 1994) were used, with an annealing temperature of 57 °C, to amplify 700 bp of the cytochrome oxidase subunit I fragment. The PCR reaction was carried out using a Mastercycler Gradient (Eppendorf Scientific). For the NE/NC samples (as well as some later samples collected by J.O.S.) the primers Barb1 and S1718 (Simon et al., 1994) were used to amplify an approximately 1800 bp fragment.
spanning much of cytochrome oxidase subunits I and II, as well as the intervening leucine tRNA gene. For the larger 1800 bp sequences, conditions were identical and the annealing temperature was also 57 °C.

PCR reactions from the Ohio collection were purified using the Wizard SV Gel and PCR Clean-Up System by Promega. The protocol for DNA purification by centrifugation was followed. The PCR reactions from the NE/NC collection were purified using an EXO/SAP enzymatic cleanup (Dugan et al., 2002). Sequencing reactions were carried out using ABI Prism Big Dye 3.1 using standard procedures and run on a 3730 DNA Analyzer from Applied Biosystems, Inc. Sequences were inspected for miscalls and edited using Chromas Pro and Codon Code Aligner, for the Ohio and NE/NC collections, respectively. Further manual alignment was conducted using MacClade, version 4.08 (Maddison & Maddison, 2005). The Ohio collection included 82 total samples with 620 bp used in analyses and the NE/NC collection consisted of 58 samples with 1837 bp used in analyses.

The amplified fragment length polymorphism (AFLP) protocol from Vos et al. (1995) was modified accordingly to quantify genetic variability of A. carolifera. For each individual in the Ohio collection, genomic DNA was digested using two different restriction enzymes, EcoRI (rare cutter) and MseI (frequent cutter) (Vos et al., 1995). The samples were diluted five-fold and used as a template for pre-selective amplification. A pre-selective amplification was performed using two primers complementary to the adapters and the restriction site sequences (ECO + A primer and MSE + C primer). Amplification conditions were: 75 °C for 2 min, 94 °C for 30 s, 56 °C for 1 min, 72 °C for 2 min, for a total of 20 cycles, and finishing with 60 °C for 30 min and held at 4 °C. Three additional bases were added to the primers for selective amplification, FAM ECO-RI +AAC primer, HEX ECO-RI +ACA primer and Mse +CAA primer. The EcoRI primer was fluorescently labelled to visualize the DNA during migration (Vos et al., 1995). Samples were again diluted five-fold. Selective amplification conditions were: 94 °C for 2 min, 94 °C for 30 s, 65 °C for 30 s (reduced by 1 °C per cycle), 72 °C for 2 min, repeat ten times excluding 94 °C for 2 min; 94 °C for 30 s 56 °C for 30 s, 72 °C for 2 min, repeat last cycle 35 times, and follow with 60 °C for 30 min and holding at 4 °C. AFLP samples were run on a 3730 DNA Analyzer (Applied Biosystems).

Genetic data analysis

Analysis of AFLP data

AFLP bands were scored and analysed with GENEMAPPER, version 3.7 (Applied Biosystems). Scoring was performed without reference to population or gall morph. All loci with bands of strength greater than 50 fluorescent units and size greater than 50 bp were scored and used in analyses. These relatively permissive conditions were used to maximize the amount of AFLP data at the same time as minimizing the use of arbitrary or biased standards for band inclusion. Although the data set may thus include some random or erroneous bands, this noise is unlikely to influence genetic or phylogenetic interpretation. Banding was weak and sporadic for eight samples that were excluded from analyses.

Phylogenetic analysis of the AFLP data set was conducted in PAUP 4b10 (Swofford, 2002) by coding each band as a dichotomous character (0/1) and searching for trees using maximum parsimony. The parsimony analysis consisted of 100 replicate searches of trees generated by stepwise addition using TBR branch swapping. Robustness of branches was evaluated with 1000 bootstrap replicates.

The parsimony analysis resulted in 20 trees differing in the basal relationships of the gall morphs. To visualize this uncertainty and determine whether distance-based methods resulted in similar phylogenetic relationships, we conducted another analysis using the SPLITSTREE 4.8 software, which may provide a more appropriate representation of relationships at the intraspecific level. A splits diagram represents all inferred splits in a network diagram and is composed of parallel edges, rather than a pruned bifurcating tree representing only a consensus of the optimal tree or trees (Huson & Bryant, 2006). We derived an uncorrected p-distance matrix from the binary AFLP data. A Neighbour-joining (NJ) algorithm (‘NeighborNet,’ Bryant & Moulton, 2004) was then used to construct an unrooted dendrogram, which is a visualization of the equal-angle split transformation we performed on the AFLP distance matrix.

The number of polymorphic loci (at the 5% level), proportion of polymorphic loci, and expected heterozygosity (H; under Hardy–Weinberg genotypic proportions) of each gall morphotype was calculated using AFLPSurv (Vekemans, 2002) employing the method of Lynch & Milligan (1994). The mean-expected genetic diversity (Hw) across all populations and the components of variance in Hw due to sampling of loci, individuals and populations were also estimated. FST was calculated over all gall morphotypes and between all gall-morph population pairs. The significance of the FST values (i.e. significantly greater than zero) and 95% confidence intervals intervals were calculated via permutation tests employing 1000 permutations. Bonferroni corrections were applied due to multiple statistical tests resulting in an adjusted α of 0.0083. AFLPSurv was also used to calculate Ni’s genetic distances (sensu Lynch & Milligan, 1994) with 1000 bootstraps, which was used to estimate a popu-
loration tree via the Neighbour module in PHYLLIP, version 3.6 (Felsenstein, 2004). The relative isolation of populations associated with geography versus gall morph was assessed using analysis of molecular variance (AMOVA), as implemented in GenAlEx, version 6.0 (Peakall & Smouse, 2006). The AMOVA was performed on a Dice index similarity matrix constructed in DistAFLP (Mougel et al., 2002). Grouping levels were geographic populations and gall morph, and significance was established by 1000 permutations of the data.

**Analysis of mitochondrial (mt)DNA data**

For both mtDNA datasets (0.6 and 1.8 kb), models of nucleotide substitution were selected according to the AIC using the program MODELTEST (Posada & Crandall, 1998), with likelihoods of successively more complex models estimated relative to an initial NJ tree (Bio NJ, HKY distances, rate variation with gamma = 0.5). Maximum likelihood (ML) searches were conducted using PHYML (Guindon & Gascuel, 2003). ML tree searches with the 620 bp data set employed an HKY model of substitution (Hasegawa, Kishino & Yano, 1985) with a t/ti ratio of 2.401, a gamma shape parameter of 0.545 (Ncat = 4), and proportion of invariant sites (Pinvar) of 0.225 (nucleotide frequencies were estimated via ML). A general time-reversible model of substitution was employed in phylogenetic analysis of the 1.8 kb data set (Table 1) with gamma shape parameter of 0.013 (Ncat = 4). For both data sets, branch support was evaluated using 1000 bootstraps (with nucleotide models as above). The tree was rooted with sequences from Asteromyia euthamiae Gagné.

We used DNAsp 4.10.9 (Rozas et al., 2003) to characterize populations and assess genetic differentiation of gall morphs using both sets of mtDNA sequence data. We estimated haplotype diversity, \(H_D\) (Nei, 1987), and nucleotide diversity with Jukes–Cantor Correction, \(\pi\) JC (Lynch & Crease, 1990), for each population, and estimated overall values of \(K_s\) and \(K_{ST}^*\) (where \(K_s\) is a weighted average sequence diversity within populations and \(K_{ST}^*\) is a measure of within population sequence variation relative to sequence variation in the total population; Hudson, Boos & Kaplan (1992a) and \(F_{ST}\) and \(N_m\) (Hudson, Slatkin & Maddison, 1992b). \(F_{ST}\) and \(K_{ST}^*\) were estimated for each pairwise combination of gall morphotypes and significance of \(K_{ST}\) was estimated with 1000 permutations (Hudson et al., 1992a). AMOVA was used in ARLEQUIN, version 3.1 (Excoffier, Laval & Schneider, 2005) to test whether significant geographic isolation was evident among populations at the local (Ohio) scale, and whether genetic variation associated with gall morphology is independent of geography. Individuals were grouped by sampling site, and significance of variance components (e.g. \(F_{ST}\), \(F_{SC}\), \(F_{CT}\)) was assessed with 1000 randomized permutations of the data among groups and populations. The crescent morphs were excluded from the AMOVA due to their clear divergence from other morphs in the phylogenetic reconstructions and their overly strong influence on the results of the AMOVA. Small regional sample sizes and lack of representation of all morphs within all regions prohibited rigorous evaluation of the roles of isolation and geography for the more widespread NE/NC samples. Flat gall morphs collected from *S. gigantea* host plants were lumped with flat gall morphs from *S. altissima* in all analyses based upon initial observations of no apparent host-plant related genetic structure (Stireman et al., 2005).

**Ecological data collection and analysis**

In the summer of 2006, we marked 110 mature galls representing all four morphotypes along a 50 m transect at one of our gall collecting sites (Huffman MetroPark). Assessment of maturity was based on gall size and coloration. We selected the galls along the transect haphazardly, but we attempted to disperse sampling along the transect to maximize genetic independence of galls and ramets. In some cases, multiple galls from the same ramet were sampled and in such cases this association was recorded. The galls were examined daily between approximately 10.00 h and 14.00 h for 30 days for signs of adult emergence (i.e. holes and pupal exuviae). After 30 days, all galls were collected and dissected to confirm emergence and identify any uneclosed individuals. Although pupal exuviae are often evident when *A. carbonifera* eclose, they are

---

Table 1. Instantaneous rate matrix used in maximum likelihood analyses of the 1.8 kb northeastern and north-central mitochondrial DNA data set

<table>
<thead>
<tr>
<th>From (\downarrow) to (\rightarrow)</th>
<th>A</th>
<th>C</th>
<th>G</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-0.75113</td>
<td>0.08089</td>
<td>0.398861</td>
<td>0.28163</td>
</tr>
<tr>
<td>C</td>
<td>0.27280</td>
<td>-2.05410</td>
<td>0.01934</td>
<td>1.76196</td>
</tr>
<tr>
<td>G</td>
<td>1.51349</td>
<td>0.02233</td>
<td>-1.62146</td>
<td>0.08563</td>
</tr>
<tr>
<td>T</td>
<td>0.26836</td>
<td>0.49789</td>
<td>0.02095</td>
<td>-0.78723</td>
</tr>
</tbody>
</table>
sometimes dislodged from the gall. Therefore, it was frequently difficult to discern midge eclosion from parasitoid eclosion.

Eclosion phenology of galls in the field was analysed by calculating the number of days to eclosion since the initiation of the survey and comparing these values for different gall morphs using analysis of variance (ANOVA). We used the first instance of eclosion from galls in which multiple eclosures were recorded to ensure independence and minimize inclusion of data from parasitoids, which tend to exhibit extended development times relative to their host midges (J. O. Stireman, unpubl. data). Histograms of eclosion times were constructed for visual comparison of gall morph eclosion patterns.

Frequencies of parasitism were estimated relative to gall morph for samples from six populations in Southwestern Ohio (Huffman MetroPark (HMP), Koogler prairie (KWP), and Beaver Creek Wetland (BCW), as well as the sites AA, PF, and ZP from which samples for DNA were collected; see above). Each gall was dissected as described above and examined for signs of parasitism. Galls with very young larvae were excluded from analyses due to the limited amount of time they had been exposed to parasitoids and the difficulty in assessing parasitism. Parasitism frequencies were estimated on a per individual basis, calculated as the number of Asteromyia chambers occupied by parasitoid larvae or pupae divided by the number of chambers dissected. These represent underestimates, as at least some of the parasitoids attack mature galls (Weis, 1982a) and many endoparasitoids were likely undetected. The significance of differences in parasitism among gall morphs was assessed using a series of chi-square tests employing a Bonferroni corrected α of 0.0083, to correct for multiple tests.

**RESULTS**

**AFLP ANALYSIS**

We obtained an AFLP data set of 562 polymorphic loci (at the 5% level) out of 917 total loci for 92 individuals. Gall morphs exhibited similar levels of polymorphism and expected heterozygosity (Table 2).

Twenty most parsimonious trees of length 1751 (belonging to three ‘islands’) were recovered in the parsimony analysis of the AFLP data set. These 20 MPTs indicated: (1) a monophyletic clade of *A. carbonifera* with respect to *A. euthamiae* (99% bootstrap support); (2) the existence of a crescent clade (89% support); (3) a cushion clade (99% support); and (4) a flat morph clade in most (80% of MPTs) and one or two clades of irregular morphs (the cryptic diversity in irregular morphs is highlighted by the bold red shading of branches shown in Fig. 2A). The cushion and crescent morphs were recovered as monophyletic, but irregular and flat clades were not perfectly matched to gall morphology because two apparent flat morphs were nested well within the irregular morph clade (Fig. 2A). Three individuals from *S. rugosa* consistently formed a distinct monophyletic group (100% support); however, its position on the tree was labile with respect to the gall morphs on *S. altissima*. The NeighborNet tree inferred using SPLITSTREE is highly consistent with the results from the parsimony analysis and provides a visual summary of both the unambiguous patterns and uncertainties (Fig. 2A). Each gall morph as well as the *Asteromyia* samples from other host species (*S. rugosa, E. graminifolia*) form distinct clades, with somewhat ambiguous relationships among them. A clear split is observed between cushions + flats morphs and crescent + irregular morphs (Fig. 2A, bold black shading), a pattern also recovered in the NJ tree estimated using Nei’s genetic distances (Fig. 2B). The pattern of genetic variation in the AFLP samples did not appear to correspond to geography at the local scale measured in this analysis (Fig. 2A).

The mean ± SE within-gall morph expected heterozygosity was 0.169 ± 0.0047 (Table 2). Much of the variance in this statistic was due to the between population component (74.2%), with smaller contributions of variance due to sampling of loci (22.8%) and individuals (3%). The overall estimated *F*$_{ST}$ among populations was 0.1430 (Table 3), providing clear evidence of gall-morph associated genetic structure. Pairwise estimates of population differentiation (*F*$_{ST}$) based on the AFLP data were in the range 0.088–0.196 (Table 3). All of these estimates were significantly greater than expected based on permutation tests (*P* < 0.001 for all pairwise tests). In the AMOVA of AFLP data based on similarity indices, gall morph was responsible for approximately 35% of the total genetic variation, whereas population (sampling site) was responsible for 3% (Table 4). Both of these vari-

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>NPL*</th>
<th>PLP†</th>
<th><em>H</em>_ ± SE‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>92</td>
<td>562</td>
<td>57.9</td>
<td>0.169 ± 0.0047</td>
</tr>
<tr>
<td>Crescent</td>
<td>19</td>
<td>477</td>
<td>42.0</td>
<td>0.161 ± 0.0054</td>
</tr>
<tr>
<td>Cushion</td>
<td>23</td>
<td>399</td>
<td>43.5</td>
<td>0.160 ± 0.0057</td>
</tr>
<tr>
<td>Flat</td>
<td>16</td>
<td>505</td>
<td>45.8</td>
<td>0.177 ± 0.0050</td>
</tr>
<tr>
<td>Irregular</td>
<td>24</td>
<td>423</td>
<td>46</td>
<td>0.161 ± 0.0054</td>
</tr>
</tbody>
</table>

* *NPL* Number of polymorphic loci at 5% level.
† Proportion of polymorphic loci at 5% level (PLP).
‡ Expected heterozygosity (*H*).
ance components were significantly greater than expected, but most of the genetic variation was found within gall morphs and within populations (62%).

**mtDNA ANALYSIS**

Substantial levels of mtDNA haplotype diversity were observed within gall morphotypes at both the local and regional levels (Table 5; regional estimates are higher due to the larger sequences). However, levels of genetic variation of cushion morphs were lower and crescent morph nucleotide diversity was elevated relative to the other gall morphotypes at the regional scale.

One ML tree was recovered for the Ohio data set including 82 sampled individuals (–LnL: 2053.45; Fig. 3). A clearly-resolved and well-supported clade

---

**Figure 2.** A, NeighborNet tree from 917 AFLP markers showing phyletic relationships between alternative gall morphs on *Solidago altissima*, collected across five sites in central Ohio. Included are galls collected from two other host plants: *Solidago rugosa* (pink shading) and *Euthamia graminifolia* (green shading). The black and red edges highlight splits between cushion + flat and irregular + crescent clades (black), and an internal split within the irregular clade (red). For further details on how the network was constructed, and the significance of the highlighted splits, see text. B, Neighbour-joining tree of gall morphotypes using Nei’s genetic distances calculated from AFLP data. Numbers above lines are bootstrap percentages (1000 replicates).

**Table 3.** Pairwise estimates of population differentiation (*F*~ST~ ± SE) between gall morph populations in Ohio for amplified fragment length polymorphism data

<table>
<thead>
<tr>
<th>Population</th>
<th>Crescent</th>
<th>Cushion</th>
<th>Flat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crescent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cushion</td>
<td>0.196 ± 0.030</td>
<td>0.097 ± 0.043</td>
<td>0.0884 ± 0.041</td>
</tr>
<tr>
<td>Flat</td>
<td>0.110 ± 0.009</td>
<td>0.194 ± 0.018</td>
<td></td>
</tr>
<tr>
<td>Irregular</td>
<td>0.155 ± 0.029</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All estimates departed significantly from zero with a probability, *P* < 0.0001 (Bonferroni corrected α = 0.0083).
% the percentage of variance explained by each sampling level. Significance of $\Phi$ statistics (molecular analogs of Fisher's $F$ statistics) are based on 1000 permutations of samples.

Table 4. Summary of analysis of molecular variance analysis of amplified fragment length polymorphism data using dice genetic distances

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>SS</th>
<th>MS</th>
<th>Estimated variance</th>
<th>%</th>
<th>Statistic</th>
<th>Value</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among morphs</td>
<td>3</td>
<td>0.556</td>
<td>0.185</td>
<td>0.009</td>
<td>35%</td>
<td>$\Phi_{RT}$</td>
<td>0.355</td>
<td>0.010</td>
</tr>
<tr>
<td>Among populations/morphs</td>
<td>8</td>
<td>0.149</td>
<td>0.019</td>
<td>0.001</td>
<td>3%</td>
<td>$\Phi_{PR}$</td>
<td>0.044</td>
<td>0.030</td>
</tr>
<tr>
<td>Within populations</td>
<td>66</td>
<td>0.986</td>
<td>0.015</td>
<td>0.015</td>
<td>62%</td>
<td>$\Phi_{VT}$</td>
<td>0.383</td>
<td>0.010</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>1.691</td>
<td>0.219</td>
<td>0.024</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Population data for each gall morphotype based on mitochondrial DNA data for the Ohio (northeastern and north-central) data sets

<table>
<thead>
<tr>
<th>Population</th>
<th>$N$</th>
<th>$H_s$</th>
<th>$\pi$JC</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>73/40</td>
<td>0.755/0.979</td>
<td>0.0122/0.0178</td>
</tr>
<tr>
<td>Crescent</td>
<td>15/16</td>
<td>0.476/0.966</td>
<td>0.0053/0.0141</td>
</tr>
<tr>
<td>Cushion</td>
<td>22/6</td>
<td>0.476/0.533</td>
<td>0.0048/0.0008</td>
</tr>
<tr>
<td>Flat</td>
<td>13/13</td>
<td>0.525/0.987</td>
<td>0.0060/0.0042</td>
</tr>
<tr>
<td>Irregular</td>
<td>23/5</td>
<td>0.656/0.900</td>
<td>0.0065/0.0069</td>
</tr>
</tbody>
</table>

$H_s$ haplotype diversity (Nei, 1987); $\pi$JC, nucleotide diversity with Jukes–Cantor Correction (Lynch & Crease, 1990).

(98% bootstrap support) of crescent-form A. carbonifera galls was present, but other gall morphs failed to differentiate into distinct clades. Two clades consisting primarily of irregular morphs exist, but these clades are not strongly supported (72% and 42%, respectively), and the former contains at least one individual of another morph (flat). The three A. carbonifera samples from S. rugosa form a distinct clade (85% bootstrap support). As in a previous study (Stireman et al., 2006), S. gigantea samples do not form a distinct clade, and exhibit sequences identical to those present in the S. altissima samples. The AMOVA excluding crescent morphs revealed that: (1) most genetic variation was found within gall morphs and local populations (92.9%); (2) little differentiation is present among sites (2.1%, $F_{SC} = 0.022$, $P = 0.515$); and (3) significant, but slight differentiation associated with gall morph exists (5.06%; $F_{CT} = 0.51$, $P = 0.023$).

ML analysis of the geographically diverse NE/NC sequences obtained a tree (–LnL: 4651.92) similar in structure to that reconstructed for the Ohio sequences (Fig. 4). Again, a crescent-morph clade sister to the remaining A. carbonifera samples is well resolved (90% bootstrap support). The remaining gall morphs are not resolved into single well-supported clades, but some gall-morph related structure is hinted at by subclades of geographically separated cushions, irregulars, and flats. Again, a clade of S. rugosa associated A. carbonifera is resolved (100% bootstrap support), but other S. rugosa samples are scattered throughout the tree, and the three crescent-morph samples from S. rugosa are clearly allied with the S. altissima crescent morph. Although sampling was too limited for a robust AMOVA, apparent geographic population structure is suggested in the phylogenetic reconstruction by clustering of some samples relative to geographic region (e.g. clades of Northeastern flats, Northeastern crescents, Ohio irregular morphs; Fig. 4).

Tests of genetic differentiation based on mtDNA sequence data implemented in DNAsp 4.10.9 (Rozas et al., 2003) revealed significant evidence of genetic isolation among morphs for both Ohio ($K_{ST}^* = 0.463$, $P < 0.001$; $F_{ST} = 0.667$, $N_m = 0.13$) and NE/NC ($K_{ST}^* = 0.287$, $P < 0.001$; $F_{ST} = 0.618$, $N_m = 0.15$) data sets. As expected given the reconstructed phylogenies, crescents are distinctly differentiated from all other gall morphs. In addition, despite the absence of monophyletic clades of other gall morphs in the phylogenetic reconstructions, significant genetic differentiation was also observed between irregulars and cushions and irregulars and flats in both Ohio and NE/NC data sets (Table 6).

GALL SHAPE

Multivariate analysis of gall morph characteristics (area, deviation from circularity, number of chambers, number of galls per leaf, distance to leaf edge, position along leaf axis) from photos confirms morphological differentiation observed by Crego et al. (1990) (multivariate analysis of variance: Wilks $\lambda = 0.348$, $F_{21,135} = 6.692$, $P < 0.0001$). Gall morphs differed significantly in all traits except position along leaf axis (Table 7). However, most of the traits primarily served to distinguish crescent morphs from all others (see Supporting Information, Appendix S2). By contrast to the findings of Crego et al. (1990), the flat, cushion, and irregular gall morphs are not resolved...
Figure 3. Maximum likelihood tree of *Asteromyia carbonifera* populations in southwest Ohio based on 600 bp of mitochondrial DNA gene for cytochrome oxidase subunit I, with taxon names coloured according to gall morph (blue, cushion; red, flat; green, irregular; orange, crescent; black, *Solidago rugosa* and *Solidago gigantea*). Numbers along branches indicate bootstrap support for clades (1000 replicates). Outgroups (*Asteromyia euthamiae*) are omitted for greater clarity of branching patterns; note that the ingroup is supported by 100% of bootstrap replicates.)
Figure 4. Maximum likelihood tree of Asteromyia carbonifera populations across the north central and north-eastern USA (NC/NE) based on 1800 bp of mtDNA genes for cytochrome oxidase subunits I and II, with taxon names coloured according to gall morph (blue, cushion; red, flat; green, irregular; orange, crescent; black, Solidago rugosa and Solidago gigantea). Numbers along branches indicate bootstrap support for clades (1000 replicates). As in Fig. 3, outgroups are omitted for greater clarity of branching patterns.
into isolated clusters when plotted on major principal components analysis axes (not shown), and thus galls could not be objectively separated into distinct morphs based on the traits measured from photographs. Discriminant function analysis resulted in 'correct' placement of gall morphs based on morphological traits with the following frequencies: 85% of crescent morphs, 64% of cushion morphs, 21% of flat morphs, and 78% of irregular morphs.

ECOLOGICAL DATA

Analyses of eclosion dates revealed no evidence of temporal isolation among gall morphotypes when all eclosion events were recorded (ANOVA: $F_{3,126} = 1.602$, $P = 0.192$) and when only the first eclosion event per gall was analysed (ANOVA: $F_{3,92} = 1.364$, $P = 0.259$). The average number of days to eclosion was slightly longer for crescents (mean: 11.8) than other morphs (mean = 8.5–9.0) but, generally, gall morphs exhibited similar, rather broad, distributions of eclosion times (Fig. 5). Interestingly, eclosion times for galls on the same leaf (presumably siblings) were no more similar on average than for random pairs of galls (5.3 versus 5.6 days respectively) and eclosion times of multiple midges from the same gall also varied widely (mean difference = 2.5 days, range = 0–10 days).

Table 6. Pairwise estimates of population differentiation ($F_{ST}$ and $K_{ST}^{*}$) between gall morphotype populations for mitochondrial DNA sequence data

<table>
<thead>
<tr>
<th>Population</th>
<th>$F_{ST}$</th>
<th>$K_{ST}^{*}$</th>
<th>$F_{ST}$</th>
<th>$K_{ST}^{*}$</th>
<th>$F_{ST}$</th>
<th>$K_{ST}^{*}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ohio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crescent</td>
<td>0.807</td>
<td>0.677***</td>
<td>0.000</td>
<td>0.002 ns</td>
<td>0.036</td>
<td>0.072**</td>
</tr>
<tr>
<td>Cushion</td>
<td>0.501</td>
<td>0.576***</td>
<td>0.098</td>
<td>0.088***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flat</td>
<td>0.484</td>
<td>0.548***</td>
<td>0.082</td>
<td></td>
<td>0.000</td>
<td>0.002 ns</td>
</tr>
<tr>
<td>Irregular</td>
<td>0.737</td>
<td>0.256***</td>
<td>0.122</td>
<td>0.044 ns†</td>
<td>0.048</td>
<td>0.045*‡</td>
</tr>
<tr>
<td>Northeastern and north-central</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crescent</td>
<td>0.681</td>
<td>0.250***</td>
<td>0.122</td>
<td>0.044 ns†</td>
<td>0.048</td>
<td>0.045*‡</td>
</tr>
<tr>
<td>Cushion</td>
<td>0.659</td>
<td>0.160**</td>
<td>0.207</td>
<td>0.192**</td>
<td>0.048</td>
<td>0.045*‡</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.005; ***P < 0.001.
†P = 0.0520.
‡Not significant with Bonferroni corrected α = 0.0083.

Table 7. Mean ± SD for gall traits according to gall morph and results of multivariate analysis of variance (MANOVA) of morphological differentiation of gall forms

<table>
<thead>
<tr>
<th>Gall morph</th>
<th>N</th>
<th>Area</th>
<th>Deviat</th>
<th>Luma</th>
<th>Pos.</th>
<th>Edge</th>
<th>N cham</th>
<th>N galls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crescent</td>
<td>33</td>
<td>9.9 ± 4.0</td>
<td>0.46 ± 0.11</td>
<td>0.51 ± 0.18</td>
<td>2.1 ± 0.7</td>
<td>1.07 ± 0.38</td>
<td>1.0 ± 0.0</td>
<td>6.2 ± 4.9</td>
</tr>
<tr>
<td>Cushion</td>
<td>23</td>
<td>23.7 ± 10.7</td>
<td>0.26 ± 0.20</td>
<td>0.71 ± 0.21</td>
<td>2.0 ± 0.6</td>
<td>1.96 ± 1.03</td>
<td>1.6 ± 0.6</td>
<td>2.7 ± 1.7</td>
</tr>
<tr>
<td>Flat</td>
<td>13</td>
<td>26.8 ± 15.7</td>
<td>0.34 ± 0.15</td>
<td>1.35 ± 2.68</td>
<td>1.8 ± 0.8</td>
<td>1.82 ± 1.23</td>
<td>2.6 ± 1.6</td>
<td>1.8 ± 1.6</td>
</tr>
<tr>
<td>Irregular</td>
<td>37</td>
<td>13.6 ± 6.7</td>
<td>0.32 ± 0.16</td>
<td>0.59 ± 0.15</td>
<td>1.9 ± 0.6</td>
<td>1.59 ± 0.87</td>
<td>1.9 ± 1.2</td>
<td>3.8 ± 2.5</td>
</tr>
</tbody>
</table>

MANOVA

<table>
<thead>
<tr>
<th>Trait</th>
<th>F</th>
<th>d.f.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20.11</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>8.70</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>2.54</td>
<td>3</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>0.74</td>
<td>3</td>
<td>0.531</td>
</tr>
<tr>
<td></td>
<td>6.19</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10.20</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>8.565</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Traits: area of gall (mm); deviation from circularity (Deviat.); luminosity (Luma; brightness); position on leaf (Pos.; base = 1, middle = 2, terminus = 3); distance from gall center to leaf edge (Edge); the number of chambers per gall (N cham); and the number of galls of a given morph per leaf (N galls).
Parasitism frequencies across all galls varied widely across the six sites surveyed, in the range 4–32% with an overall rate of 16.5%. This variation was likely due in part to the differing developmental stages of galls at different sites. For three of the six sites, parasitism varied significantly among gall morphs, with flat morphs tending to experience the highest rates of parasitism (30.3% overall) and irregular morphs the lowest (12.6% overall; Fig. 6).

Figure 5. A histogram indicating the number of days from initial marking to eclosion of midges for each Asteromyia carbonifera gall morph in the field.

Figure 6. A histogram showing parasitism rates of each gall morph across six sampling sites in Ohio (HMP, Huffman MetroPark; PF, Pearl Fen; AA, Aullwood Audubon Center; KWP, Koogler prairie; ZP, Zimmerman Prairie; BCW, Beaver Creek Wetland). Significant variation in parasitism among gall morphs at each site is indicated: **P < 0.01; ***P < 0.001; or n.s. when P > 0.05. Numbers below the columns indicate sample sizes for each gall morph.

Parasitism frequencies across all galls varied widely across the six sites surveyed, in the range 4–32% with an overall rate of 16.5%. This variation was likely due in part to the differing developmental stages of galls at different sites. For three of the six sites, parasitism varied significantly among gall morphs, with flat morphs tending to experience the highest rates of parasitism (30.3% overall) and irregular morphs the lowest (12.6% overall; Fig. 6).

DISCUSSION

GENETIC DIFFERENTIATION OF ASTEROMYIA GALL MORPHS

Our analyses characterized cryptic diversity in sympatric populations of the fungus-associated gall midge, A. carbonifera within the host plant S. altissima. Phylogenetic analyses of mtDNA and AFLP data generally confirm the findings of previous work
(Crego et al., 1990) indicating that genetically differentiated populations form distinct galls on the same host-plant organ. However, both the mtDNA and AFLP data suggest the existence of gene flow or mismatching between midge and gall morph. Gene flow estimates based on mtDNA data were substantial for some morphs, and parsimony analyses of the AFLP data did not always result in perfectly monophyletic clades of midges characterized by distinctive gall morphology. We cannot discriminate whether these incongruities represent gene flow, some form of fungal mutualist switching among midge populations (see below), or incorrect assessment of gall morphs. The lack of clearly monophyletic gall morph clades in the mtDNA based ML trees suggests either incomplete lineage sorting or mitochondrial gene flow. This latter hypothesis is consistent with previous studies that examined genetic structure of organellar and nuclear DNA in a given taxon, which found considerably greater levels of gene flow in the organellar DNA (Smith, 1992; Howard et al., 1997; Shaw, 2002; Schef- fer & Hawthorne, 2007).

Interestingly, the one gall morph in our samples that was not examined by Crego et al. (1990) is the most morphologically and genetically distinct. Crescent morph gall midges form a clearly differentiated, well-supported clade in analyses of both AFLP and mtDNA data. This clade is highly divergent in mtDNA analyses (1.6–1.8% net sequence divergence from other gall morphs depending upon the data set) and is placed as a sister group to all other gall morphs, including those collected from other Solidago species in the ML analyses of mtDNA.

Crescent morphs appear to display the greatest level of genetic diversity (π nucleotide diversity), an observation that may be related to the occurrence of these morphs on S. rugosa and S. gigantea, and possibly on other Solidago species as well (J. Heath & T. G. Carr, unpubl. data). Flat morphs appear to occur on S. gigantea (a species broadly sympatric with S. altissima) with no apparent genetic differentiation among host plants (Stireman et al., 2006; Figs 3, 4). Solidago rugosa hosts a ‘flat-like’ morph of somewhat unique morphology that exhibits equivocal evidence of genetic differentiation (Figs 2, 3, 4). The fine-scale mtDNA and AFLP analyses of Ohio populations suggest the existence of a unique lineage on S. rugosa, yet the regional scale mtDNA analysis does not support a monophyletic S. rugosa clade. The occurrence of multiple geographically congruent lineages on this host interspersed among S. altissima haplotypes in this phylogenetic reconstruction suggests repeated colonization of S. rugosa from S. altissima. However, given the failure of the mtDNA sequence data to resolve gall morphs that were resolved with AFLP data, caution is warranted in interpreting phylogenetic patterns indicated by mtDNA, which may be indicative of incomplete lineage sorting.

**Asteromyia gall morphology**

Unlike other gall midges (Rohfritsch, 1992), it is unclear whether Asteromyia larvae themselves cause significant deformation of plant tissue (Camp, 1981). Thus variation in Asteromyia gall form may be more a function of distinctive fungal morphologies than the specific biochemical interactions between midge and plant, as in most other insect galls. We photographed all galls before dissection precisely so that gall morph could be confirmed, but our measurements based on these photographs do not allow objective separation of gall morphs into discrete phenotypic clusters (aside from crescent morphs). Additional traits such as gall thickness and position of larvae within the gall appear to be necessary for objective discrimination of galls (these traits had among the highest loadings on the axes in PCA analyses; Crego et al. (1990)), suggesting that they may be experiencing the strongest divergent selection pressures. The number of chambers per gall also varies consistently with gall morph but this pattern is partly obscured by fusion with neighboring galls. Our observations of young galls indicate irregular morphs are probably always solitary (one larva per gall), but the galls typically occur in closely spaced groups that frequently fuse as they grow larger (J. O. Stireman & J. Heath, unpubl. data).

Because gall morphology is not a perfectly reliable guide to A. carbonifera diversity, genetic data are particularly valuable in recovering undiagnosed lineages. Geographically unexplained genetic structure in the mtDNA data sets and multiple lineages of irregular morphs reconstructed in parsimony analyses of AFLP data suggest that additional genetically distinct populations may exist, undetected by morphological descriptions. It also is likely that the similarity between gall morphs is not so much the issue as the failure to find an adequate composite descriptor. For example, the close relationship between crescent and irregular gall morphs indicated by NJ analyses of AFLP population data is supported by their similarity in size and coloration, and the usual occurrence of a single larva (though irregular galls frequently fuse with one another; see above). Flat morphs resemble cushion morphs, with which they are clustered in AFLP analysis, in the general large size of the galls, the frequency of galls with multiple larvae, and in the thicker layer of fungal hyphae surrounding the larvae (Crego et al., 1990). Thus, a number of biological attributes of the galls support the general trends in the genetic data.
It may be that each of the A. carbonifera populations is associated with a distinct fungal genotype that is responsible for the observed morphological variation in galls. This could provide a solution to the apparent conundrum of genetic differentiation and coexistence of gall morphs in the absence of apparent ecological isolating mechanisms, if coevolved associations with distinct fungal genotypes serves to isolate gall morphs either pre- or post-zygotically. However, this hypothesis is not borne out by preliminary analyses of fungal DNA sequence data from several genes (ITS, beta-tubulin, EF1α, and 28S rDNA), which shows no evidence of differentiation of fungal genotypes across gall morphs or host-plants (E. M. Janson & T. G. Carr, unpubl. data).

**ORIGIN AND MAINTENANCE OF REPRODUCTIVE ISOLATION IN A. CARBONIFERA**

Within-host radiations of herbivorous insects provide unique opportunities to explore the relationship between specialization, ecology, and evolutionary divergence. In the extreme, with in situ origins of divergent lineages, they have the potential to provide positive evidence of sympatric speciation. However, no example of within-host radiation has been characterized in sufficient detail to exclude allopatric origins (Coyne & Orr, 2004), and allopatry is clearly important in many cases (Condon & Steck, 1997; Sachet et al., 2006). One compelling pattern, however, is that, whether or not the initial divergence originated in situ, pre-zygotic isolating mechanisms (phenology and organ shifts within hosts) are in place that likely explain the maintenance of isolation.

Because of their extreme spatial proximity, an ecological basis to the observed genetic divergence in A. carbonifera is likely. However, all gall morphs are found on the same plant part, on the same host plant species. Thus, A. carbonifera does not fit the pattern established by other within-host radiations, or even other gall midges (Joy & Crespi, 2007). The obvious suspicion is that S. altissima has been colonized only recently by previously fragmented ancestral populations of A. carbonifera (Coyne & Orr, 2004), a possibility supported by the fact that A. carbonifera can be found on related Solidago species. We can begin to address this hypothesis (and are in the process of a more thorough evaluation). First, our sampling indicates that each of the four gall morphs that coexist on S. altissima has a wide geographic distribution spanning at least the north-eastern quarter of the USA. Anecdotal observations and evidence from herbarium sheets (T. G. Carr, unpubl. data) suggest that they coexist across a large portion of the range of S. altissima. However, apparent boundaries appear to exist for irregular and cushion morphs, as they have not been found in the extreme north-eastern USA (T. G. Carr, unpubl. data).

An allopatric scenario of divergence would be supported by significant geographic isolation of populations which would suggest limited dispersal of A. carbonifera. Cecidomyiid gall midges are generally considered to have limited capacities of dispersal associated with directed flight (Briggs & Latto, 2000), but passive dispersal with wind currents may be an important means of dispersal (Pendleton & Teetes, 1994) and long distance (e.g. > 1 km) dispersal of cecidomyiids is an established fact (Yukawa & Rohfritsch, 2005). The AMOVA analysis of Ohio populations suggests a large degree of isolation relative to gall morph (35%) but very little (yet statistically significant) differentiation between sites (3%). Evidence of geographic structure was also suggested by some relationships inferred in the broader geographic sampling (e.g. Ohio haplotypes pairing; Fig. 4), although other relationships countered these patterns (e.g. pairing of haplotypes from Illinois and Pennsylvania) and the limited sampling makes it difficult to interpret geographic structure. There is some suggestion of a north-eastern origin and subsequent westward expansion of crescent morphs based on the basal position and diversity of mtDNA haplotypes from Maine in the north-eastern USA (ME) and derived haplotypes in more western states of Ohio, Kentucky, and Missouri (Fig. 4), but this hypothesis needs to be tested more rigorously.

Even if allopatric isolation has played an important historical role in fostering divergence, what maintains the contemporaneous isolation of the distinct lineages on S. altissima? Post-zygotic mechanisms may be at work, but this would be unusual for such young taxa. Seasonal temporal isolation has been found to underlie premating isolation in a number of plant-associated insects (Wood, 1993; Marshall & Cooley, 2000; Simon et al., 2000; Abbot & Withgott, 2004; Joy & Crespi, 2007). Our analyses of eclosion time in the field provided no evidence of phenological differences among gall morphs. We found little evidence for tight synchrony in emergence dates of galls of a particular morph and even among individuals within a particular gall. Protandry or protogyny could be responsible in part for the variation in eclosion dates (Yukawa & Rohfritsch, 2005), but the magnitude of the variation is surprising, given the relatively short adult lifespans characteristic of Cecidomyiidae (Weis et al., 1983; Yukawa & Rohfritsch, 2005). However, we cannot rule out small-scale ‘micro-temporal’ isolation, as has been described in a few cases (Pashley, Hammond & Hardy, 1992; Sample, 1992; Clifton & Clifton, 1999; Miyatake et al., 2002).

Solidago altissima is known to consist of at least three ploidy races (diploid, tetraploid and hexaploid;
Semple & Cook, 2004; Halverson et al., 2008a). Thus, it could be hypothesized that gall morph differentiation is ploidy-specific, with each gall morph using a particular ploidy race of hosts. Indeed, *S. altissima* ploidy races have been shown to vary significantly in the abundance of *Asteromyia* galls as a whole (Halverson et al., 2008b). However, all four gall morphs were common in Ohio, where only hexaploids are thought to exist (Halverson et al., 2008a), and multiple gall morphs (occasionally all four) have frequently been observed on a single host ramet (J. O. Stireman & B. L. Wells, unpubl. data).

Another plausible ecological cause of the observed phenotypic divergence and reproductive isolation involves pressure from natural enemies. In many plant-galling insects lineages, gall structure, shape, and size have evolved in response to natural enemy pressure (Ronquist & Liljeblad, 2001; Stone & Schonrogge, 2003; Craig, Itami & Horner, 2007). Gall morphologies in this system could evolve in response to enemies in at least two ways: via gall structures that may directly limit access to parasitoids and via selection on clutch size and the dispersion of offspring among leaves (Tabuchi & Amano, 2004). The parasitoid species in this system possess distinct attack strategies for parasitizing *A. carbonifera* galls (Weis, 1982a). *Torymus capite* attacks galls from the periphery, whereas *Tetrastichus* spp. drill their ovipositors through the surface of the galls. Thus, irregular (where larvae tend to be located around the periphery of the gall) and cushion morphs (thick fungal stroma) may be less susceptible to *Tetrastichus* spp., and flats (which tend to produce galls of large area) may be less susceptible to *T. capite*. Indeed, Weis (1982b) previously demonstrated that the fungal stroma can provide some protection from *T. capite* at later stages of gall development (although Weis was apparently unaware of the existence of gall morphs at this time). That the traits most consistent in delimiting gall morphs (see above) may influence susceptibility to parasitoids is suggestive of an important role of these interactions in the evolution of gall morphology. Although we were unable to differentiate parasitoid species in our parasitism surveys, we found that overall parasitism frequency varied significantly among gall morphs within at least some sites, supporting the hypothesis that parasitoid pressure may selectively shape gall morphology. Furthermore, initial results from more extensive and detailed ongoing surveys of parasitism frequencies of *A. carbonifera* indicate that gall morphs vary in susceptibility to particular parasitoid guilds (J. O. Stireman & B. L. Wells, unpubl. data).

The interaction between clutch size (i.e. number of larvae per gall) and gall morphology in the context of natural enemy attack may also bear on the observed phenotypic divergence and evolution of reproductive isolation. Particular gall morphs consistently vary in the number of larvae or larval chambers per gall and the number of galls per leaf (Table 7). Weis et al. (1983) demonstrated that, although individuals that develop from and subsequently contribute to smaller clutches have higher lifetime fitness (i.e. greater fecundity), they are also subject to substantially higher probabilities of parasitism. Thus, theoretically the overall fitness of an individual should be determined by the interaction between the number of siblings sharing its gall (fecundity) and adult mortality between oviposition events (ability to realize fecundity). Multiple optima for clutch size and dispersion of offspring may exist that are dependent on frequencies of parasitism by particular parasitoids, the growth pattern of the fungal mutualist (e.g. thick or thin hyphal layer), and adult mortality risk. Fitness ‘valleys’ between these optima may restrict gene flow between gall morphs.

In summary, *A. carbonifera* gall midges consist of a diverse assemblage of cryptic lineages on *S. altissima*. Unlike many other examples of within-host radiations, phenological isolation does not appear to explain the differentiation of these gall morphs on their goldenrod host. Ultimately, natural enemies may prove far more important than any other factor. However, the diversification and coexistence of *A. carbonifera* lineages remains perplexing. Most known examples of within-host radiations are gall-forming taxa, suggesting that the intimacy of the association of plants and insects must be important in explaining why within-host radiations occur at all. In our study system, however, the degree of intimacy in the relationship between the midge and goldenrod is unclear, as the midge appears to subsist mostly or entirely on the Botryosphaeria fungus (E. M. Janson & J. J. Heath, unpubl. data), which itself belongs to a group of extreme generalist phytopathogens/endophytes (Barr, 1972, 1987; Farr et al., 1989). Instead of manipulating the host plant to form nutritive tissues and protective structures as in most other gall-forming insects (Shorthouse & Rohfritsch, 1992), *A. carbonifera* may manipulate the growth of their mutualistic fungus, which in turn is the primary manipulator of the host plant. Alternatively, *A. carbonifera* may manipulate the plant in more subtle ways, allowing proliferation of the fungus. For *Asteromyia*, understanding the role of the fungal symbiont thus may be the key to resolving mechanisms underlying its radiation (Janson, Stireman, Abbot & Singer, 2008). In essence, the problem may not be to solely explain the within-host radiation of *Asteromyia* on goldenrod, but within-host radiation of *Asteromyia* on its mutualistic fungus. Key future directions for understanding this apparent within-host adaptive radiation include: (1) more
detailed analyses of the specificity and genetic structure of the Botryosphaeria fungal mutualist; (2) determining whether gall morphs differ in their susceptibility to particular parasitoid species and the magnitude and direction of selection that they exert on gall traits; (3) more detailed study of the physiological/ecological relationships between host-plant, fungus, and gall midge and their roles in gall development; and (4) expanded sampling of additional Solidago species to assess the distribution of gall morphs among host plants, evaluate the occurrence of additional genetically distinct populations of A. carbonifera, and measure patterns of parasitoid attack. We are currently pursuing each of these avenues in order to understand the causes and consequences of diversification in this remarkable system.

ACKNOWLEDGEMENTS

We would like to thank Anne M. Royer for aiding in molecular work and gall dissection and Brenda L. Wells for collecting phenological and parasitism data. J. J. Heath shared insights and unpublished data on Asteromyia biology. This work was supported by NSF DEB 0614433 to P.A. and J.O.S. and by a Wright state University research challenge award to J.O.S.

REFERENCES


Barr ME. 1987. Prodomus to class Loculoascomycetes. Published by the author; Amberst, MA.


Rohfritsch O. 1992. Patterns of gall development. In: Short-
house JD, Rohfritsch O, eds. Biology of insect-induced galls. 
Ronquist F, Liljeblad J. 2001. Evolution of the gall wasp-
Rozas J, Sanchez-Del Barrio JC, Messeguer X, Rozas R. 
2003. DnaSP, DNA polymorphism analyses by the coales-
Sachet JM, Roques A, Despres L. 2006. Linking patterns 
and processes of species diversification in the cone flies 
Strobilomyia (Diptera: Anthomyliidae). Molecular Phylo-
genetics and Evolution 41: 606–621.
Sample BE. 1992. Temporal isolation of flight time of two 
sympatric Malacosoma species. Environmental Entomol-
Scheffer SJ, Hawthorne DJ. 2007. Molecular evidence of 
host-associated genetic divergence in the holly leafminer 
Phytomyza glabrichola (Diptera: Agromyzidae): apparent dis-
cordance among marker systems. Molecular Ecology 16: 
2627–2637.
Semple JC, Cook RE. 2004. Chromosome number determina-
ions in fam. Compositae, tribe Asteraeae. VII. Mostly 
eastern North American and some Eurasian taxa. Rhodora 
Shaw KL. 2002. Conflict between nuclear and mitochondrial 
DNA phylogenies of a recent species radiation: What 
mtDNA reveals and conceals about modes of speciation in 
Hawaiian crickets. Proceedings of the National Academy 
Shorthouse JD, Rohfritsch O. 1992. Biology of insect-
induced galls. New York: Oxford University Press.
Simon C, Frati F, Beckenbach A, Crespi BJ, Liu H, 
Flook P. 1994. Evolution, weighting, and phylogenetic 
utility of mitochondrial gene sequences and a compilation of 
conserved polymerase chain reaction primers. Annals of the 
Entomological Society of America 87: 651–701.
Simon C, Tang JM, Dalwadi S, Staley G, Deniega J, 
Unnasch TR. 2000. Genetic evidence for assortative 
mating between 13-year cicadas and sympatric ‘17-year 
cicadas with 13-year life cycles’ provides support for allo-
Smith GR. 1992. Introgression in fishes – significance for 
paleontology, cladistics, and evolutionary rates. Systematic 
Biology 41: 41–57.
Stireman JO III, Nason JD, Heard SB. 2005. Host-
associated genetic differentiation in phytophagous insects: 
General phenomenon or isolated exceptions? Evidence from a 
Stireman JO III, Nason JD, Heard SB, Seehawer JM. 
2006. Cascading host-associated genetic differentiation in 
parasitoids of phytophagous insects. Proceedings of the Royal 
Stone GN, Schonrogge K. 2003. The adaptive significance of 
insect gall morphology. Trends in Ecology and Evolution 18: 
512–522.
Strong DR, Lawton JH, Southwood R. 1984. Insects on 
plants: community patterns and mechanisms. Cambridge, 
MA: Harvard University Press.
Swofford DL. 2002. PAUP* phylogenetic analysis using pars-
imony (*and other methods), Version 4.0b10. Sunderland, 
attack on the number of chambers in multilocular galls of 
two closely related gall midges (Diptera: Cecidomyiidae). 
Vekemans X. 2002. AFLP-SURV, Version 1.0. Belgium: Labo-
ratoire de Genetique et Ecologie Vegetale, Universite Libre 
de Bruxelles.
Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, 
Hornes M, Fritiers A, Pot J, Peleman J, Kuiper M, 
Zabeau M. 1995. AFLP: a new technique for DNA finger-
Weis AE. 1982a. Use of a symbiotic fungus by the gall maker 
Asteromyia carbonifera to inhibit attack by the parasitoid 
Weis AE. 1982b. Resource utilization patterns in a commu-
nity of gall-attacking parasitoids. Environmental Entomol-
ogy 11: 809–815.
Weis AE, Price PW, Lynch M. 1983. Selective pressures on 
clutch size in the gallmaker Asteromyia carbonifera. Ecology 
64: 688–695.
Yukawa J, Rohfritsch O. 2005. Biology and ecology of 
gall-inducing Cecidomyiidae (Diptera). In: Raman A, 
Schaefer CW, Withers TM, eds. Biology, ecology, and evolu-
tion of gall-inducing arthropods. Enfield, NH: Science 

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:
Appendix S1. Samples used mitochondrial DNA analyses with host plants, collection site, and genbank accession number.
Appendix S2. Pairwise post-hoc tests of significant differences in gall morphology for each gall trait using the Tukey-Kramer method in OpenStat (Miller, 2007).

Please note: Blackwell Publishing are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

© 2008 The Linnean Society of London, Biological Journal of the Linnean Society, 2008, 95, 840–858