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ADAPTIVE RADIATION OF GALL-INDUCING INSECTS WITHIN A SINGLE HOST-PLANT SPECIES

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Speciation of plant-feeding insects is typically associated with host-plant shifts, with subsequent divergent selection and adaptation to the ecological conditions associated with the new plant. However, a few insect groups have apparently undergone speciation while remaining on the same host-plant species, and such radiations may provide novel insights into the causes of adaptive radiation. We used mitochondrial and nuclear DNA to infer a phylogeny for 14 species of gall-inducing *Asphondylia* flies (Diptera: Cecidomyiidae) found on *Larrea tridentata* (creosote bush), which have been considered to be monophyletic based on morphological evidence. Our phylogenetic analyses provide strong support for extensive within-host plant speciation in this group, and it demonstrates that diversification has involved numerous shifts between different plant organs (leaves, buds, flowers, and stems) of the same host-plant species. Within-plant speciation of *Asphondylia* is thus apparently facilitated by the opportunity to partition the plant ecologically. One clade exhibits temporal isolation among species, which may have facilitated divergence via allochronic shifts. Using a novel method based on Bayesian reconstruction, we show that the rate of change in an ecomorphological trait, ovipositor length, was significantly higher along branches with inferred shifts between host-plant organs than along branches without such shifts. This finding suggests that *Larrea* gall midges exhibit close morphological adaptation to specific host-plant parts, which may mediate ecological transitions via disruptive selection.

KEY WORDS: **Adaptive radiation, *Asphondylia*, ecological shifts, galling, insect–plant interactions, plant-part specific specialization, speciation.**

Plant-feeding insects have several characteristics that make them useful models for the study of speciation. First, the high diversity of phytophagous insects and the continuum of populations exhibiting various stages of reproductive isolation facilitate comparative analyses of speciation mechanisms (Drès and Mallet 2002). Second, most phytophagous insects are ecologically specialized on particular host-plant resources, and such specialization may facilitate the evolution of reproductive isolation (Jaenike 1989; Caillaud and Via 2000). Third, the developmental timing of phytophagous insect populations can be determined by host-plant resources with different phenologies, such that adults from populations specialized on different host-plant resources may mature and mate at different times, leading to temporal isolation (Feder and Filchak 1999; Groman and Pellmyr 2000).

Shifts to new host-plant species have played a crucial role in the diversification of phytophagous insects (Ehrlich and Raven 1964; Jermy 1984; Farrell and Mitter 1994; Thompson 1994; Mardulyn et al. 1997; Becerra and Venable 1999; Funk et al. 2002). Speciation via host shifting often proceeds via the development of prezygotic isolation, associated with fidelity of mating on the

host plant (Berlocher 2000; Feder et al. 2003). Such prezygotic isolation can lead to the formation of host-plant races exhibiting moderate levels of reproductive isolation, and in time these host races may differentiate into species (Drès and Mallet 2002). Such host-plant shifts and the evolution of host races have been proposed as a common scenario for nonallopatric speciation (Craig et al. 1993; Feder et al. 1994; Futuyma et al. 1995; Berlocher 2000; Groman and Pellmyr 2000; Abrahamson et al. 2001; Craig et al. 2001; Emelianov et al. 2001; Drès and Mallet 2002), although strong support for these mechanisms has remained elusive.

Recent phylogenetic and ecological studies of several clades of phytophagous insects have demonstrated that speciation can also occur in the absence of host-plant shifts (Condon and Steck 1997; Cook et al. 2002; Després et al. 2002). In these cases, speciation is often associated with shifting to different parts of the same host-plant species, such as from leaf to stem, and the evolution of reproductive isolation may often involve phenological separation (Condon and Steck 1997; Després et al. 2002; Ferdy et al. 2002). These patterns of within-host speciation are also not limited to phytophagous insects: for example, Simkova et al. (2004) showed that in a group of monogean parasites of fishes, diversification is explained in part by within-host speciation. Cases of within-host speciation may provide useful insights into speciation, because in these cases the effects of ecology on divergence are likely easier to partition from alternative processes, and divergence may be more likely to involve nonallopatric processes in the evolution of reproductive isolation.

Gall midges (Diptera: Cecidomyiidae) are unusual among phytophagous insects in that taxonomic classifications show that many genera exhibit large groups of putatively closely related species found on a single host-plant species (Jones et al. 1983; Hawkins et al. 1986; Gagné 1989; Gagné and Waring 1990). Gall midges comprise the largest radiation of galling insects (Ronquist and Liljeblad 2001). They form galls on virtually all plant parts (leaves, stems, twigs, buds, flowers, and roots). Cecidomyiids are widely distributed among host plants, occurring on gymnosperms, angiosperms, monocotyledons, and dicotyledons (Gagné 1989). Most cecidomyiids, like other gall-inducing insects (Crespi et al. 1997), are highly host-plant specific, most often feeding only on one part of a single host-plant species (Jones et al. 1983; Hawkins et al. 1986; Gagné 1989). For example, within the large genus *Asphondylia* (247 described species world wide), members of morphologically based species groups, defined by similarities in larval, pupal, and adult characters, are often associated with the same host-plant species (Hawkins et al. 1986; Gagné and Waring 1990).

Current understanding of phylogenetic relationships among the Cecidomyiidae is highly incomplete, such that patterns of host-associated radiations in this group remain largely unexplored (Dorchin et al. 2004). Based on larval, pupal, and adult morphological characters, gall-inducing flies of the *Asphondylia auripila* group are believed to form a monophyletic group in which all of the species feed upon creosote bush (*Larrea tridentata*) (Waring and Price 1989). Members of this group differ in several ecologically important characteristics such as gall morphology, gall position, and ovipositor characteristics. The life histories of these midges are linked to winter rains followed by increasing temperature and rains in the spring and to late summer monsoonal rains. Thus, adults of different species are active (for their very short adult lives of 1–2 days) in spring, summer, or both (Waring and Price 1989). The different species in this group are sympatric over a broad area and widely distributed across the Mojave, Sonoran, and Chihuahuan deserts of North America, and up to 10 species having been collected from a single creosote bush (Waring and Price 1989).

In this study, we investigated the phylogenetic relationships of the "*Asphondylia auripila* group" (Gagné and Waring 1990) of cecidomyiid flies to evaluate hypotheses regarding the role of host-plant use in their diversification. First, we used DNA sequence data from one mitochondrial and three nuclear genes to address the hypothesis that the *auripila* group has evolved wholly or in part via in situ radiation on *L. tridentata.* Second, we analyzed the potential roles of ecology (gall position) and phenology (adult emergence time) in the diversification of this group. Thus, if new species arise in association with changes in gall position, then we expect sister species to exhibit contrasting gall positions. By contrast, if new species arise through phenological separation, then sympatric sister taxa are predicted to be temporally isolated. Alternatively, if neither temporal isolation nor tissue shifts are observed, then new species are more likely to have arisen through divergence resulting from geographic isolation. Finally, we employed independent contrast analysis to test whether evolutionary shifts in gall position (the host-plant part that is galled) are associated with increased rates of change in two ecologically important traits, ovipositor length, and wing length.

Methods

COLLECTION SITES AND METHODS

We collected *Asphondylia* species associated with *L. tridentata* (creosote bush) from sites across southern California, Nevada, Arizona, New Mexico, and Texas between March and September 2001–2005. We also collected six *Asphondylia* species associated with the sympatric host plants *A. atriplicis*, *A. caudicis*, and *A. neomexicana* from saltbush (*Atriplex* spp.), *A. bigeloviabrassicoides* from rabbitbrush (*Chrysothamnus* spp.), *A. websteri* from alfalfa (*Medicago* spp.), and *Asphondylia* spp. from snake weed (*Gutierrezia* spp.) as putative outgroups. Outgroups were chosen

based on previous taxonomic work which identified the saltbush inhabiting *Asphondylia* species as a potential sister group complex to those found on creosote bush, based upon shared morphological character states between these two groups (Gagné and Waring 1990). One additional outgroup, *A. conglomerata* from a species of saltbush (*Atriplex hamalis*), was obtained from Genbank.

Field-collected galls were transported to the laboratory in an ice-filled cooler where they were kept room temperature until adults emerged. Following emergence, adults were preserved whole in 20% dimethyl sulphoxide in a saturated solution of NaCl. Voucher specimens were deposited with the Smithsonian Institution National Museum of Natural History in Washington, DC.

COLLECTION OF DNA DATA

Genomic DNA was isolated using standard phenol chloroform methods (Hillis et al. 1996) from single adult midges of either sex. DNA was extracted from as many individuals for each species as possible (Table 1). We used polymerase chain reactions (PCR) to amplify three nuclear and one mitochondrial gene. A 452 base pair fragment of cytochrome oxidase I (COI) was amplified using primers C1-J-1718 and C1-N-2191 (Simon et al. 1994). A 419 base pair fragment of the internal transcribed spacer region 2 (ITS-2) of nuclear ribosomal DNA (Harris and Crandall 2000) was amplified using primers 5.8sFC and 28s BLD (Simon et al. 1994). A 574 base pair fragment of the Wingless gene (Wg) was amplified using primers 5′wg1 and 3′wg2 (Ober 2003). A 568 base pair fragment of the elongation factor 1 alpha (EF-1∝) gene was amplified using primers EF1aF (AAAATGCCATGGTTCAAAGG) and EF1aR (CGAAATTTGACCTGGATGGT) developed based on an EF-1∝ sequence from *Mayetiola destructor* obtained from Genbank (accession number AF085227). Resulting PCR products were purified using shrimp alkaline phosphatase (SAP) and exonuclease (EXO), and purified PCR products were used in sequencing reactions with an ABI Prism Dye Terminator Cycle (Applied Biosystems, Foster City, CA, USA) sequencing kit.

Table 1. Number of sequences obtained per species per gene (see also Appendix).

Species	Host plant	COI	ITS-2	Wg	EF-1
Asphondylia apicata	*Larrea tridentata*	2	1	0	0
A. rosetta	*L. tridentata*	2	2	2	2
A. florea	*L. tridentata*	2	2	2	2
A. auripila	*L. tridentata*	2	2	2	2
A. foliosa	*L. tridentata*	2	2	2	2
A. resinosa	*L. tridentata*	2	2	2	2
A. barbata	*L. tridentata*	2	2	2	2
A. clavata	*L. tridentata*	2	2	2	2
A. fabalis	*L. tridentata*	2	1	1	1
A. pilosa	*L. tridentata*	2	2	2	2
A. silicula	*L. tridentata*	2	1	0	1
A. villosa	*L. tridentata*	2	2	2	1
A. digitata	*L. tridentata*	1	0	0	0
A. bullata	*L. tridentata*	2	0	0	1
A. caudicis	*Atriplex spp.*	1	1	1	0
A. atriplicis	*Atriplex spp.*	1	1	1	1
A. neomexicana	*Atriplex spp.*	1	0	0	0
A. bigelovia-brassicoides	*Chysothamnus spp.*	1	0	1	1
A. spp.	*Gutierrezia spp.*	1	0	0	1
A. websteri	*Medicago spp.*	1	0	0	0
A. conglomerata	*Atriplex spp.*	1	0	0	0

PHYLOGENETIC ANALYSES

Sequences were aligned using Clustal (Thompson et al. 1994) and adjusted by eye using Se-Al (Rambaut 1996). Protein coding genes were also checked to ensure that they coded and for stop codons in Se-Al (Rambaut 1996). The best-fitting model of sequence evolution was determined for each gene using ModelTest (Posada and Crandall 1998). We also employed MrModeltest 2.2 (Nylander 2004) to identify best models of sequence evolution for each partition for use in Bayesian phylogeny estimation. We first used maximum likelihood (ML) and maximum parsimony (MP) analyses to infer phylogenies for *Asphondylia* species for each gene separately. We employed the heuristic (ML) and branch and bound (MP) searching features of PAUP 4.0b10 (Swofford 2002). ML trees were also reconstructed using Mr Bayes 3.1.2 (Ronquist and Huelsenbeck 2003). To assess support for recovered nodes, we employed bootstrap replicates (500 for ML, 1000 for MP). We employed the incongruence length test (ILD test), as implemented in PAUP* (TBR, 1000 replicates) (Huelsenbeck and Bull 1996; Swofford 2002), to help evaluate the congruence of the trees inferred from the four different genes. To analyze the combined data, we employed a four-partition analysis applying the best-fit model of sequence evolution for each partition using Mr. Bayes 3.1.2 (Ronquist and Huelsenbeck 2003).

Evaluation of the monophyly of *Asphondylia* taxa found on *L. tridentata* is complicated by the large number of ingroup taxa (14) relative to putative outgroup taxa (7) in our dataset, and size of the genus as a whole (67 Nearctic species, 247 world wide). We used several lines of evidence to test the hypothesis of monophyly. First, we considered MP, ML bootstrap values, and Bayesian posterior probability values from the combined tree, for the nodes that corresponded to monophyly of the *Larrea* taxa (Hillis and Bull 1993). Second, we used Shimodaira–Hasegawa (SH) tests and Templeton tests, as implemented in PAUP* (Swofford 2002), to compare the best trees with constraint trees that forced the invasion of the ingroup by one or more outgroup taxa. For example,

the best tree was compared to the best constraint tree that did not contain the grouping (ingroup1, ingroup2, ingroup3, ingroup4) because one or more outgroup species had invaded the combined ingroup.

COMPARATIVE ANALYSES

We predicted that changes in gall position should be associated with accelerated change in an ecomorphological trait (ovipositor length) related to gall induction, but not in change in wing length, a trait closely indicative of body size (Sokoloff 1966; Norry and Vilardi 1996). To best infer changes in gall position, we used Bayesian methods to reconstruct ancestral states for the categorical four-state character gall position (leaf, stem, flower, bud) for each node, using Bayes MultiState (Pagel et al. 2004). This program uses a Markov Chain Monte Carlo approach to sampling phylogenies, and for investigating the parameters of trait evolution, and it calculates a fifth state for the probability that the node does not exist. To calculate the strength of evidence for a shift in gall position at each node, we first calculated the probability of no shift across an internode by summing the product of the probability of each state in each node (e.g., p(leaf) node A* p(leaf) node B + p(flower)node A* p(flower) node B) + . . ., where A and B are the ends of an internode). One minus this probability is a continuous measure of the probability of change for each node that accounts for phylogenetic uncertainty. To quantify the evolution of our ecomorphological trait (ovipositor length), we optimized this trait, and wing length (a measure of body size), on the combined data Bayesian consensus tree (data from Gagnè and Waring 1990) using McPeek's (1995) contrast method. We then used McPeek's (1995) independent contrast test to determine whether higher rates of change in ovipositor length and wing length occurred along branches associated with ecological shifts (changes in gall position) relative to branches lacking ecological shifts. We tested this hypothesis by regressing a measure of the probability of change at each node with independent contrast values. For this analysis, we used the "speciational" model of character evolution, because we assumed that changes in ovipositor morphology take place in association with speciation events rather than continuously over time.

Results

DATASET

The complete dataset of COI, internal transcribed spacer region 2 (ITS-2), wingless (Wg), and elongation factor 1 alpha (EF-1$\propto$) nucleotide sequences for 21 *Asphondylia* species, consisted of 2013 positions (452 COI, 574 Wg, 419 ITS-2, 568 EF-1$\propto$, Table 2). All gene sequences have been deposited in GenBank (Appendix.). Of the 2013 sites, 243 were parsimony informative (118 COI, 47 Wg, 30 ITS-2, 88 EF-1$\propto$). Interspecific pairwise differences within the ingroup ranged from 0.2% to 15.0% for COI, 0.4% to 5.5% for Wg, 0.0% to 2.5% for ITS-2, and 0.5% to 7.8% for EF-1$\propto$. Differences between the ingroup and outgroups were 9.3% COI, 10.5% Wg, 4.8% ITS-2, and 6.6% EF-1$\propto$. Incongruence length difference (ILD) tests showed that all-possible combinations of the different gene regions were compatible ($P = 0.51$).

PHYLOGENIES

Figure 1 shows the ML trees for each of the four gene regions. For each of the four, MP and ML and Bayesian analyses yielded trees of very similar topology. The grouping of the ingroup taxa into five main clades relative to the outgroup taxa was consistent across all genes except EF-1$\propto$ in which one clade (*A. auripila/A. foliosa/A. resinosa*) is moved to the base of the tree with the outgroup taxa (Fig. 1). The topologies of the best trees for COI and Wg exhibited only minor differences. ITS-2 differed in the placement of one leaf galling taxon (*A. villosa*) and in the placement of *A. florea* and *A. rosetta* at the base of the tree. EF1-1$\propto$ differed in the invasion of the ingroup by the putative outgroup taxon *A. atriplicis* and in the placement of the stem galling clade (*A. auripila, A. foliosa,* and *A. resinosa*) at the base of the tree with the outgroup taxon. MP, ML, and Bayesian analyses of the combined dataset yielded similar topologies (Fig. 2).

EVOLUTION OF HOST-PLANT USE

All *Asphondylia* species that induce galls on *L. tridentata* formed a monophyletic group for both the combined dataset and three of the four datasets separately. Support for the node indicating

Table 2. Summary of support for monophyly of clades within the *Asphondylia auripila* group. L, leaf; S, stem; B, bud; and F, flower. Support for each of the five clades is provided: ML, maximum likelihood bootstrap support; MP, maximum parsimony bootstrap support; MCMCMC, Bayesian posterior probability; SH, significance for the SH test; Templeton test, significance level for Templeton test.

A. auripila supported clade	Plant part	MP	ML	MCMCMC	SH test	Templeton test
A. clavata, A. pilosa	L,L	100	100	99	$P < 0.05$	$P < 0.001$
A. silicula, A. fabalis	L,L	100	100	100	$P < 0.05$	$P < 0.001$
A. barbata, A. villosa	L,L	100	100	99	$P < 0.05$	$P = 0.29$
A. rosetta, A. florea, A. apicata	S,F,B	100	100	96	$P < 0.05$	$P < 0.05$
A. resinosa, A. auripila, A. foliosa, A. digitata	S,S,S,L	88	88	88	$P < 0.05$	$P < 0.05$

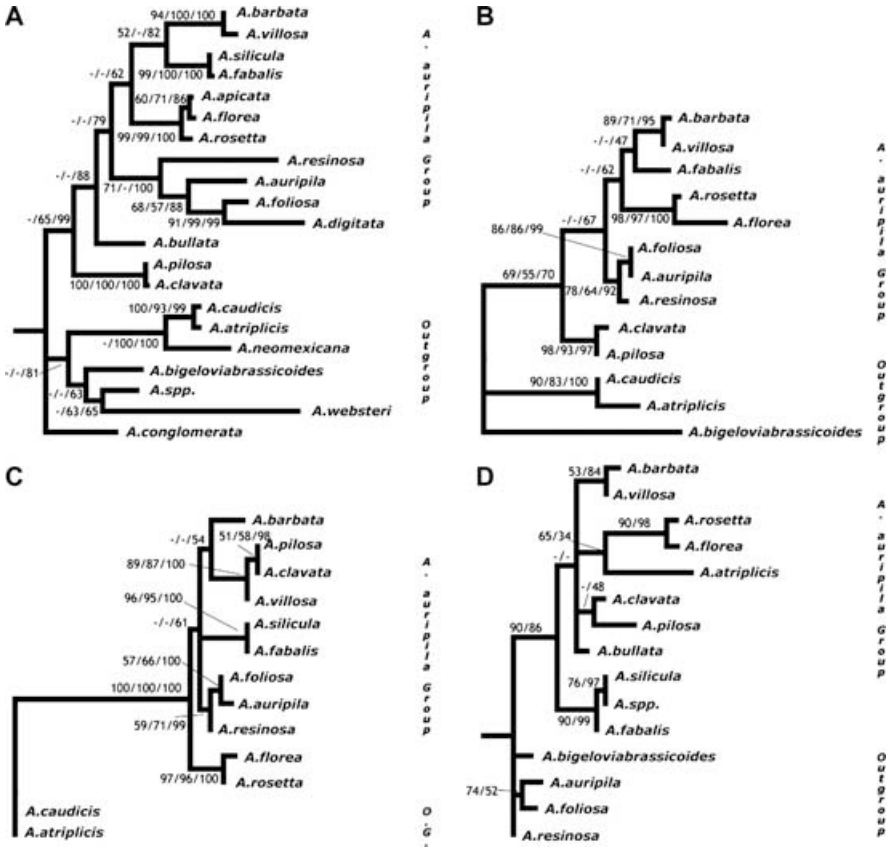

Figure 1. Maximum likelihood (ML) phylogenies for (A) COI, (B) wingless (C) ITS-2, and (D) EF-1. Maximum parsimony, ML, and Bayesian support values are shown for each node. Branch lengths are proportional to the inferred number of substitutions per site.

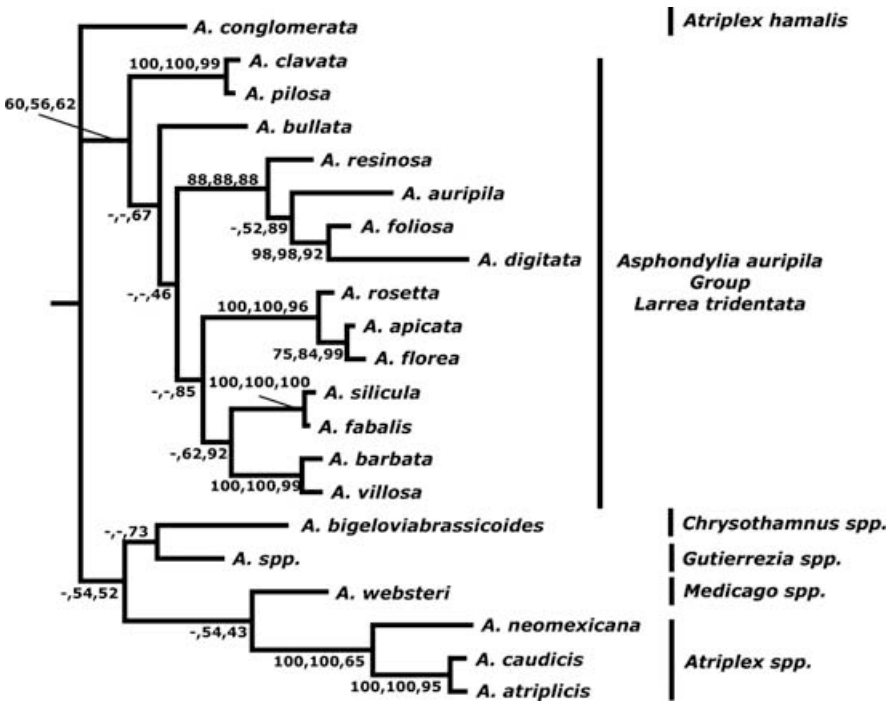

Figure 2. Phylogeny of *Asphondylia auripila* group and outgroups according to a four-partition Bayesian phylogenetic analysis using a separate substitution model for each gene. Numbers above branches are MP bootstrap, ML bootstrap, and Bayesian posterior probabilities. Host genera are delineated at the tips.

monophyly of this entire group varied among genes, being strongest in ITS-2, the most highly conserved gene (100, 100, 100; MP bootstrap, ML bootstrap, and Bayesian a posteriori probabilities, respectively), moderate in Wg (61, 73, 95), weakest in COI (−, 65, 99), nonexistent in EF-1∝, and intermediate but weak in the combined analyses (60, 56, 62). Monophyly of the *Asphondylia* species on *L. tridentata* was strongly statistically supported for the ITS2 data under MP using Templeton test (difference in length = 15, $P < 0.001$) and under ML using SH test (difference in $-\ln L = 48.67$, $P < 0.001$). The ML and MP scores for best trees were better than negative constraint trees, but not significantly so as judged by Templeton and SH tests for the rest of the datasets and for the combined dataset.

Within the *A. auripila* group, five clades consistently formed strongly supported groups as judged by ML and MP bootstrap support, Bayesian posterior probabilities, SH test, and Templeton tests. These five clades consisted of three pairs of leaf-galling sister taxa, the clade containing three species that form galls on different plant parts (*Asphondylia rosetta*, *A. florea*, and *A. apicata)*, and a fifth clade containing four species, three of which form galls on the same plant part but display widely divergent emergence timing (Table 2). These results demonstrate that although support for monophyly of the entire *A. auripila* group of gall midges on *L. tridentata* is not definitive, there is strong evidence for within-host plant speciation within particular clades.

HOST-PLANT COLONIZATION SEQUENCE

Bayesian ancestral state reconstruction of colonization of different host-plant parts yielded several notable inferences (Fig. 3). Leaf galling has apparently evolved twice, *A. digitata* derived within the clade of stem gallers (*A. resinosa, A. auripila,* and *A. foliosa*), and *A. barbata, A. villosa, A. silicula,* and *A. fabalis* from stem galling ancestors (Fig. 3). Flower (*A. florea)* and bud galling (*A. apicata*) each evolved once, but the order under which these transitions occurred is not clear (Fig. 3). In the well-supported clades within this radiation on a single host plant, speciation has apparently occurred in association with shifts to new plant parts three times, and in association with retention of the same host-plant part five times (Fig. 3).

ECOLOGICAL ADAPTATION TO SPECIFIC PLANT PARTS

Evolution of phenology

Most *Asphondylia* species (10 of 14 sampled) found on *L. tridentata* are bivoltine, with adults found in both spring and summer. The remaining four species are univoltine, being found as adults in only the spring, winter, or summer as follows: March–May (*A. foliosa*), August–September (*A. rosetta*, *A. auripila*), and December–February (*A. resinosa*) (Fig. 3). If new species arise through phenological separation, then sympatric sister taxa are expected to be temporally isolated. Among well-supported clades, sister-taxa comparisons for phenology (Fig. 3) show two main

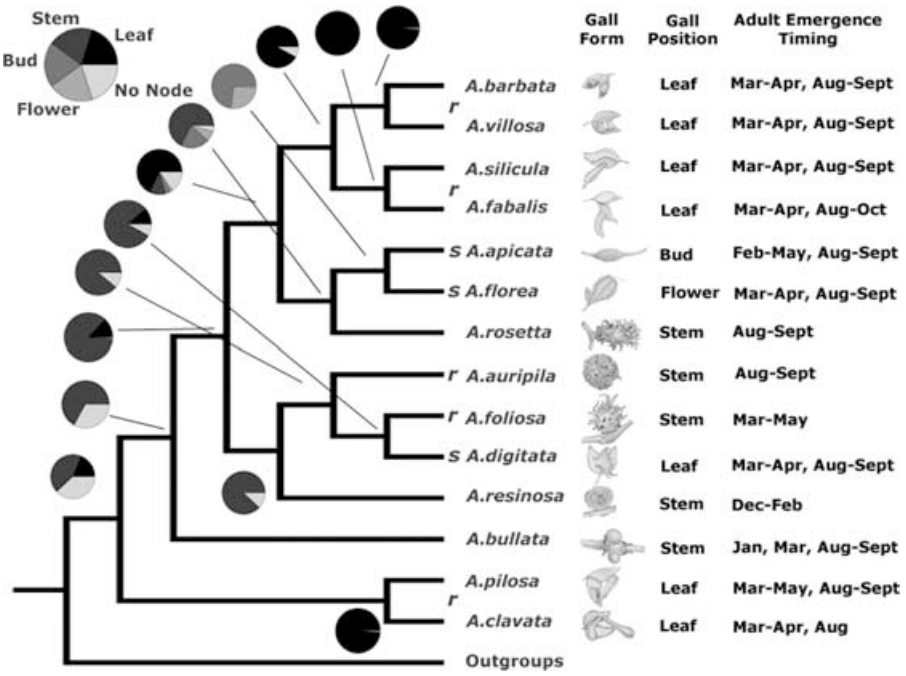

Figure 3. **Phylogeny of *Asphondylia auripila* group based on combined dataset with ancestral gall position reconstruction by Bayesian methods. Drawings of galls for each species, gall position, and the phenology of adult emergence are provided for each species. Speciation events associated with shifts to new plant parts are denoted with an "s" and speciation events associated with retention of the same plant parts are denoted with an "r."**

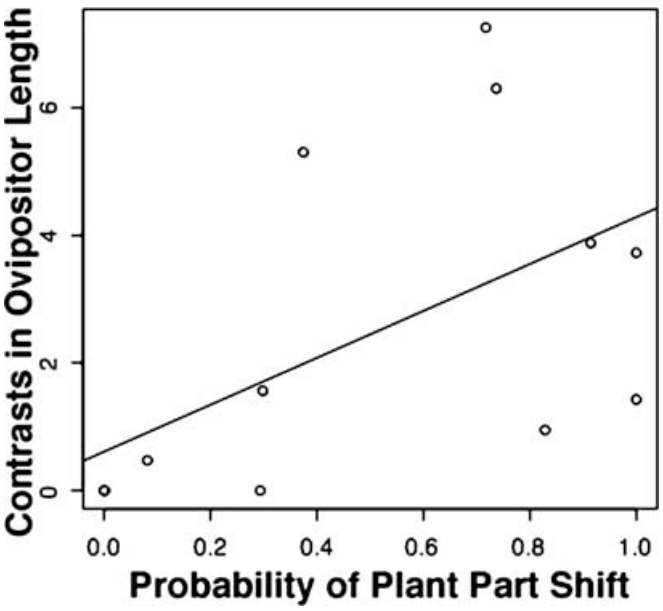

Figure 4. Phylogenetically independent contrast values for *Asphondylia* ovipositor lengths calculated for each node plotted versus an index of the probability of change in host-plant part usage from one node to the next.

patterns: (1) bivoltine sister taxa emerge at the same times (*A. barbata* and *A. villosa; A. silicula* and *A. fabalis*; *A. clavata* and *A. pilosa*; *A. apicata* and *A. florea*) and (2) three of the four univoltine taxa that are phenologically isolated are members of the same clade (*A. resinosa*, *A. auripila*, and *A. foliosa*) and within this clade there is a reversal to bivoltinism (*A. digitata*).

Evolution of ecomorphology

Our Bayesian extension of McPeek's (1995) contrast analysis indicates that ovipositor length underwent higher rates of change along branches where shifts to new plant parts were inferred to be more probable than where shifts were not inferred ($R^2 = 0.26$, $F = 5.054$, df $= 11$, $P < 0.05$). By contrast, there was no difference in rates of change in wing length in relation to shifts in plant part versus retention of the same plant part ($R^2 = 0.13$, $F = 2.802$, df $= 11$, $P = 0.13$, Fig. 4).

Discussion

Four of five phylogenies (each gene and combined), and SH and Templeton tests for ITS2, support the hypothesis derived from morphological data (Gagné and Waring 1990) that the *A. auripila* group has radiated in situ on *L. tridentata*. The tree from the EF-1$\propto$ data did not support the hypothesis of monophyly for the *A. auripila* group as a whole. However, SH and Templeton tests show this tree is not significantly better than a tree constraining the ingroup (*A. auripila* group) to be monophyletic. The contrasting results from different genes, and the nonsignificant SH and Templeton tests, suggest that based on the currently available evidence, support for monophyly of the *A. auripila* group as a whole remains equivocal.

Despite this uncertainty regarding monophyly of the *A. auripila* group as a whole, two lines of evidence strongly support the monophyly of multiple clades within this group. First, Bayesian posterior probabilities, ML, and MP bootstap values indicate strong support for five clades, and some of the sister species in these clades are very closely related (e.g., *A. villosa* and *A. barbata* differ by only 1.3% at COI). Second, SH and Templeton tests significantly support the hypotheses of monophyly of these clades (Fig. 3). Thus, even if the entire *A. auripila* group is not monophyletic, it comprises multiple lineages that show strong evidence for monophyly, which indicates that this group is characterized by a notable degree of within host-plant speciation. Hypotheses regarding monophyly of this clade, and the lineages within it, could be tested further via sequencing of additional *Asphondylia* species from the North American deserts.

POTENTIAL MECHANISMS OF WITHIN HOST-PLANT SPECIATION

Shifts to a new host plant are usually accompanied by adaptations to markedly different plant characteristics, such as plant morphology, chemistry, and phenology (Jaenike 1989; Jaenike 1990; Becerra and Venable 1999; Cook et al. 2002). By contrast, shifts within a host plant may not require such substantial evolutionary change. Other barriers, such as high rates of gene flow, likely inhibit speciation via ecological shifts within a host plant (Ferdy et al. 2002). In *Asphondylia* midges, there are several possible geographic modes and mechanisms of speciation within a single host plant, each of which could result in the partitioning of the plant into a number of finely divided niches.

Divergence under sympatry

Changes in diapause timing could result in sympatric populations shifting in time to exploit the same or a new part of a host plant at a different point in time, effectively generating reproductive isolation. Thus, three species of stem galling *Asphondylia* midges on *L. tridentata* (*A. auripila, A. foliosa, A. resinosa*) in a well-supported clade are phenologically separated from one another (Fig. 3). The emergence timing of these species corresponds to the timing of plant growth associated with rains in winter (*A. resinosa*), spring (*A. foliosa*), and summer (*A. auripila*). The emergence timing of other members of the *A. auripila* group show no seasonal isolation between sister taxa, although they may be phenologically isolated on a finer scale (within a season), given the short life spans and weak flight abilities of adult flies (Jones et al. 1983; Gagné 1989). This hypothesis could be tested by monitoring the emergence timing of bivoltine sister taxa such as *A. barbata* and *A. villosa*.

Phenology has been shown to be important in mediating reductions in gene flow leading to speciation or host race formation in many other insect taxa, including *Rhagoletis* flies (Feder and Filchak 1999), *Eurosta* flies (Craig et al. 1993), *Enchenopa* treehoppers (Wood et al. 1990), *Magicicada* cicadas (Cooley et al. 2003), and *Blepharoneura* flies (Condon and Steck 1997). These parallel patterns suggest that temporal isolation may be an important process favoring speciation in phytophagous insects.

Phenological divergence may be facilitated by shifts to competition-free space, in that the insects that have shifted to a new plant part are expected to be released from the strong competition that typifies many gall-inducing species (Denno et al. 1995; Craig et al. 2000; Inbar et al. 2004). The prolonged diapause of the gall midge *Dasineura rachiphaga* is thought to be a mechanism that evolved in the context of selection for reduced intraspecific competition for limiting oviposition sites (Prévost 1990). Similarly, Cook et al. (2002) showed that speciation of *Andricus* gall wasps is more commonly associated with shifts to a novel part of the same host plant than with shifts between different host-plant species, and they suggested that intraspecific competition for oviposition sites has facilitated within-host divergence. In *Chiastocheta* flies inhabiting *Trollius* species, Després et al. (2002) demonstrated that diversification has involved both host shifts and radiation within a host, and the within-host diversification may be a result of competition for oviposition or feeding sites, favoring temporal shifts in oviposition timing and shifts to different larval food resources (Ferdy et al. 2002).

The proximate mechanism of sympatric shifts in host-plant parts may involve a combination of mistakes in oviposition site and variation in the developmental schedules of different plant parts. Insects sometimes lay eggs on unfamiliar host plants or host plant parts; such ovipositional mistakes have been documented for Lepidoptera (Feldman and Haber 1998), Coleoptera (Fox et al., in press), and Diptera (Gratton and Welter 1998), including many Cecidomyiidae (Larsson and Strong 1992; Larsson and Ekbom 1995). When a female oviposits on a plant tissue type other than her natal type (i.e., flower instead of leaf), the eggs in the new tissue type may break diapause later or earlier as a result of differences in the developmental schedule of the different plant tissue types (Linkosalo 2000; Mahoro 2002), and this may translate to the temporal isolation of adults. This hypothesis could be tested with the *Asphondylia* midges on *L. tridentata* by enforcing oviposition on nonnatal host-plant parts (i.e., leaf–stem) and recording changes in emergence timing.

Divergence under allopatry

Colonization of a new plant part could also occur in an allopatric population, resulting in a single species inducing galls on multiple parts of a single host plant. The ability to gall the original part of the host plant may, in theory, be subsequently lost, or the colonizing species may go locally extinct, and differentiation could then occur due to drift and selection in allopatry. Upon secondary contact, we would be left with two sympatric species using different niches on the same host plant. Speciation on the same plant part could also result from allopatric isolation. In this scenario reproductive isolation and ecological divergence might develop as a product of isolation through both selection resulting from different ecological conditions (climate, plant genotype, parasitoids, and composition of the galling community) and differentiation due to genetic drift. Upon secondary contact we would have two ecologically diverged species (e.g., phenologically isolated) on the same plant part. In a third scenario, reproductive isolation could develop in allopatry purely due to genetic drift, and ecological divergence of the resulting species could occur as a result of subsequent interspecific competition.

The host plant of the *A. auripila* group is the dominant shrub throughout an immense area, the southwestern deserts of North America (Hunter et al. 2001). *Larrea tridentata* was isolated in refugia during the major North American glaciations (Hunter et al. 2001), and speciation may have occurred in this manner in refugia during glacial periods. However, under any of the above allopatry hypotheses it is not clear why the ability to gall the original plant part would be lost, or why such progenitor populations would go extinct; moreover, most of the radiation on *L. tridentata* appears to be considerably older than the glaciation cycles starting in the Pleistocene. These allopatry hypotheses could be addressed further through comparative phylogeographic analyses of sister-taxa inducing galls on different plant parts.

ECOLOGICAL ADAPTATION TO SPECIFIC PLANT PARTS

Adaptive changes in insect morphological characters following host shifts have been documented only rarely, despite the central importance of morphological adaptations in insect diversification (Moran 1986; Carroll et al. 1997; Groman and Pellmyr 2000). In this study, we have documented adaptive changes in an ecologically important morphological character, ovipositor length, within the context of radiation on a single host-plant species. Our independent contrast analyses, which account for both uncertainty in the phylogeny and uncertainty in the reconstructions of ancestral galling position states, demonstrate that *Asphondylia* species inhabiting *L. tridentata* show substantially larger changes in ovipositor length following ecological shifts (shifts to new parts of a host plant) relative to the amount of change when no ecological shift has taken place. By contrast, wing length, a trait not predicted to be adaptive in the context of exploitation of different plant parts, shows no significant relation with ecological shifts. The finding that ovipositor length changed more than wing length in response to ecological shifts is consistent with the hypothesis that selection for host-plant part associated morphological differences is driving changes in *Asphondylia* ovipositor lengths.

The morphological basis of adaptation to different host-plant parts in these species is simple: *Asphondylia* species inhabiting different parts of *L. tridentata* deposit their eggs into strikingly different tissue types (stems, leaves, buds, and flowers) that differ markedly in hardness, thickness, and depth to plant vasculature. Thus, the shorter ovipositor of leaf galling species may facilitate the placement of eggs in thinner softer leaf tissue, whereas longer ovipositors of stem, bud, and flower galling species allow egg placement deeper into host-plant tissues. These findings suggest that strong divergent selection on ovipositor length accompanies evolutionary shifts in host-plant part, which would be expected to drive postzygotic isolation; this hypothesis could be tested further via measuring oviposition depths in different plant tissues, and through experimental manipulation of oviposition sites.

Conclusions

Our study provides strong evidence that some clades of *Asphondylia* gall midges have radiated in situ on their host plant *L. tridentata*. This diversification was apparently driven by the ability of these insects to partition the plant ecologically, via two mechanisms that facilitate the evolution of reproductive isolation: shifts to new plant parts and changes in phenology. Evidence from other host-specific phytophagous insects that can use different parts of the same plant species (e.g., Condon and Steck 1997; Cook et al. 2002; Després et al. 2002), and from host-specific parasites (e.g., Simková et al. 2004), suggests that within-host ecological divergence may be a common mechanism of speciation that promotes the extraordinarily high species diversity found in many groups of parasites and plant-feeding insects.

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Appendix

Table A1. Genbank accession numbers for *Asphondylia* samples used in this study. COI refers to cytochrome oxidase subunit I, ITS-2 refers to internal transcribed spacer region 2, Wg refers to wingless, and EF-1 refers to elongation factor 1 alpha.

Species	Genbank accession number(s)			
	COI	ITS-2	Wg	EF-1
Asphondylia Apicata	EF189965	–	–	–
A. apicata	EF189966	–	–	–
A. rosetta	EF189967	EF189921	EF189943	EF189998
A. rosetta	EF189968	EF189922	EF189944	EF189999
A. florae	EF189969	EF189923	EF189945	EF190000
A. florae	EF189970	EF189924	EF189946	EF190001
A. auripila	EF189973	EF189927	EF189949	EF190004
A. auripila	EF189974	EF189928	EF189950	EF190005
A. foliosa	EF189971	EF189925	EF189947	EF190002
A. foliosa	EF189972	EF189926	EF189948	EF190003
A. resinosa	EF189975	EF189929	EF189951	EF190006
A. resinosa	EF189976	EF189930	EF189952	EF190007
A. barbata	EF189977	EF189931	EF189953	EF190008
A. barbata	EF189978	EF189932	EF189954	EF190009
A. clavata	EF189979	EF189933	EF189955	EF190010
A. clavata	EF189980	EF189934	EF189956	EF190011
A. fabalis	EF189985	EF189939	EF189961	–
A. fabalis	EF189986	–	–	–
A. pilosa	EF189981	EF189935	EF189957	EF190012
A. pilosa	EF189982	EF189936	EF189958	EF190013
A. silicula	EF189987	EF189940	–	EF190015
A. silicula	EF189988	–	–	–
A. villosa	EF189983	EF189937	EF189959	EF190014
A. villosa	EF189984	EF189938	EF189960	–
A. digitata	EF189989	–	–	–
A. bullata	EF189990	–	–	EF190016
A. bullata	EF189991	–	–	–
A. caudices	EF189992	EF189941	EF189962	–
A. atriplicis	EF189993	EF189942	EF189963	EF190017
A. neomexicana	EF189994	–	–	–
A. bigeloviabrassicoides	EF189995	–	EF189964	EF190018
A. spp.	EF189996	–	–	EF190019
A. websteri	EF189997	–	–	–
A. conglomerata	AB115566	–	–	–

Table A2. Collection locations for *Asphondylia* samples used in this study. NA refers to coordinates not available.

Species	Location	Collection location	
		Latitude	Longitude
Asphondylia apicata	Arizona	32.85419	−112.76898
A. apicata	Arizona	32.85419	−112.76898
A. rosetta	Arizona	33.66552	−114.00259
A. rosetta	Arizona	35.62776	−114.42500
A. florae	Arizona	32.10646	−110.02626
A. florae	Arizona	32.04849	−111.39339
A. auripila	New Mexico	32.22744	−108.95309
A. auripila	Arizona	32.19672	−112.46421
A. foliosa	Arizona	33.43421	−112.58794
A. foliosa	Arizona	32.19672	−112.46421
A. resinosa	Arizona	33.79714	−112.13309
A. resinosa	Arizona	34.05390	−112.14478
A. barbata	Arizona	32.17640	−112.26275
A. barbata	Arizona	34.61367	−111.86295
A. clavata	Arizona	32.04849	−111.39339
A. clavata	Arizona	32.08436	−110.81089
A. fabalis	Arizona	33.40855	−112.39408
A. fabalis	Arizona	33.40855	−112.39408
A. pilosa	Arizona	33.79716	−112.13789
A. pilosa	Arizona	32.46565	−112.87441
A. silicula	Texas	31.06663	−104.21716
A. silicula	Arizona	32.27415	−110.95036
A. villosa	Arizona	31.96300	−110.80246
A. villosa	Arizona	31.96300	−110.80246
A. digitata	Arizona	32.06105	−110.77532
A. bullata	Texas	31.06663	−104.21716
A. bullata	Texas	31.06663	−104.21716
A. caudices	California	34.92229	−117.27702
A. atriplicis	Arizona	32.75440	−110.64789
A. neomexicana	Arizona	32.75440	−110.64789
A. bigeloviabrassicoides	British Columbia	49.23960	−119.40010
A. spp.	California	32.63629	−116.11862
A. websteri	Arizona	NA	NA
A. conglomerata	Israel	NA	NA