

Altica litigata (Coleoptera: Chrysomelidae: Galerucinae): a DNA approach to species verification¹

Tracie M. Jenkins², Tyler D. Eaton³, and Z. Chen⁴

Abstract. The life cycle from egg to adult of the herbivorous *Altica litigata* flea beetle has been described on plants in the genus *Oenothera* (Onagraceae). Adult *A. litigata* have been reported to feed on primrose, *Oenothera* species as well as on crape myrtles, *Lagerstroemia* species (Lythraceae). Only the adult life stage has been reported on *Lagerstroemia* species. Since *Altica* adult beetles are morphologically difficult to distinguish, host species-specificity for *Oenothera* species and *Lagerstroemia* species has been used to identify *A. litigata* adults in the field. Thus, cytochrome oxidase subunit I (COI) sequence was used to test the hypothesis that adult flea beetles collected on *Oenothera* species and *Lagerstroemia* species and characterized from morphology were *A. litigata* as expected. We discuss the implications of this work.

Keywords. *Altica litigata*; flea beetle; COI sequence

1. Introduction

The flea beetle, *Altica litigata* Fall, is a nursery and landscape pest. Adult beetles, which are metallic blue to green, feed in aggregate on the leaves of indigenous plants in the Onagraceae and important non-indigenous ornamental plants in the Lythraceae (Pettis & Braman 2007). Reproductive phases are optimal on plants of the genus *Oenothera* (Pettis *et al.* 2004) and temperature dependent (Pettis & Braman 2007). Although change in host specificity in herbivorous beetles appears to be labile (Farrell & Sequeira 2004), crape myrtles (Lythraceae: *Lagerstroemia* spp.)

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2 The University of Georgia, Dept. of Entomology, Griffin Campus, Griffin, GA 30223, USA, jenkinst@uga.edu

3 The University of Georgia, Dept. of Entomology, Griffin Campus, Griffin, GA 30223, USA, eaton@uga.edu

4 The University of Georgia, Dept of Crop and Soil Sciences, Griffin Campus, Griffin, GA 30223, USA, zchen@uga.edu

have not been shown to serve as developmental hosts for *A. litigata* (Pettis *et al.* 2004).

Cutleaf evening primroses and other cultivated *Oenothera* counterparts often grow in and around production nurseries. *Altica litigata* have thus been collected from wild herbaceous hosts in the Onagraceae and Lythraceae prior to and during pest outbreaks on crape myrtle. It has, therefore, been postulated that *A. litigata* migrate from these wild herbaceous hosts to crape myrtles (Pettis & Braman 2007).

Taxonomic verification in the genus *Altica* is difficult because many species are morphologically indistinguishable. Even the male aedeagus is often an unreliable character when trying to distinguish species (Laroche *et al.* 1996). DNA markers could provide verification of morphological species and establish a one-one correlation between morphology and DNA. The mitochondrial DNA (mtDNA) cytochrome oxidase subunit I (COI) gene sequence, particularly the 5' 'Folmer region', has been used for phylogeny inference generally (www.barcoding.si.edu; Miller 2007) and specifically in the Galerucinae (Lunt *et al.* 1996; Caterino *et al.* 2000; Clark *et al.* 2001). We hypothesize, therefore, that COI sequence will be species specific and confirm host-plant(s) herbivory.

2. Methods

2.1. Flea beetles

Adult flea beetle specimens were identified from morphological characters and host-plant associations (Table 1). They were collected from host plants in two families, Lythraceae, represented by *Lagerstroemia* spp. and *Lythrum salicaria*, and Onagraceae, represented by *Oenothera laciniata* Hill, *Oenothera speciosa* Nutt, *Oenothera lamarckiana* and *Guara lindheimeri* (Table 1). Collections were identified by three characteristics: plant species (*e.g.*, G for *Guara lindheimeri*, L for *Lagerstroemia* spp., Ls for *Lythrum salicaria*, Ol for *O. laciniata*, Os for *O. speciosa*, and Oa for *O. lamarckiana*); U. S. state from which collected (*e.g.*, GA for Georgia, AL for Alabama, MS for Mississippi, LA for Louisiana and TN for Tennessee); and a number for internal identification. Thus a specimen identified as L-AL-64 (Fig. 1) represents a beetle collected on *Lagerstroemia* spp. in the state of Alabama with an internal identification number of 64. The outgroup species, *Phaedon desotonis* Balsbaugh, was collected from *Coreopsis lanceolata* L. (Asteraceae) in Griffin, Georgia. A sample of collections was preserved in 70% EtOH for morphological identification through the Systematic Entomology Laboratory, USDA, Smithsonian Institute. The rest was preserved in 100% EtOH. Voucher specimens will be deposited in the Georgia Museum of Natural History and referenced in GenBank samples.

Sample*	Host plant	Presumptive species	Coll.	County, State	GenBank
G-GA-2-2	<i>Guara lindheimeri</i>	<i>Altica</i> spp.	23-V-02	Spalding, GA	EU143714
G-GA-8	<i>Guara lindheimeri</i>	<i>Altica litigata</i>	24-IV-01	McDuffie, GA	EU143715
G-GA-9-2	<i>Guara lindheimeri</i>	<i>Altica litigata</i>	24-IV-01	McDuffie, GA	EU142716
L-AL-61	<i>Lagerstroemia</i> 'Potomac'	<i>Altica</i> spp.	10-VII-02	Mobile, AL	EU069475
L-AL-62	<i>Lagerstroemia</i> 'Potomac'	<i>Altica</i> spp.	10-VII-02	Mobile, AL	EU069476
L-AL-63	<i>Lagerstroemia</i> 'Potomac'	<i>Altica</i> spp.	10-VII-02	Mobile, AL	EU069477
L-AL-64	<i>Lagerstroemia</i> 'Potomac'	<i>Lysathia</i> spp.	10-VII-02	Mobile, AL	EU117151
L-AL-66	<i>Lagerstroemia</i> 'Hopi'	<i>Altica</i> spp.	19-VII-02	Madison, AL	EU069478
L-AL-67	<i>Lagerstroemia</i> 'Hopi'	<i>Altica</i> spp.	19-VII-02	Madison, AL	EU069479
L-AL-68	<i>Lagerstroemia</i> 'Hopi'	<i>Lysathia</i> spp.	19-VII-02	Madison, AL	EU117152
L-AL-69	<i>Lagerstroemia</i> 'Hopi'	<i>Lysathia</i> spp.	19-VII-02	Madison, AL	EU117153
L-GA-10	<i>Lagerstroemia</i> spp.	<i>Altica</i> spp.	29-VI-02	Spalding, AL	EU069466
L-GA-13	<i>Lagerstroemia</i> spp.	<i>Altica</i> spp.	29-VI-02	Spalding, AL	EU069467
L-GA-13-3	<i>Lagerstroemia</i> spp.	<i>Altica</i> spp.	21-V-02	Spalding, AL	EU143713
L-GA-88	<i>Lagerstroemia</i> spp.	<i>Lysathia</i> spp.	01-VI-02	Spalding, AL	EU117154
L-GA-89	<i>Lagerstroemia</i> spp.	<i>Lysathia</i> spp.	01-VI-02	Spalding, AL	EU117155
L-GA-90	<i>Lagerstroemia</i> spp.	<i>Lysathia</i> spp.	01-VI-02	Spalding, AL	EU117156
L-GA-92	<i>Lagerstroemia</i> spp.	<i>Altica</i> spp.	01-VI-02	Spalding, AL	EU069481
L-LA-55	<i>Lagerstroemia</i> 'Red Rocket'	<i>Altica</i> spp.	19-VII-02	St. Tammany, LA	EU069469
L-LA-55B	<i>Lagerstroemia</i> 'Red Rocket'	<i>Altica</i> spp.	19-VII-02	St. Tammany, LA	EU069470
L-LA-55C	<i>Lagerstroemia</i> 'Red Rocket'	<i>Lysathia</i> spp.	19-VII-02	St. Tammany, LA	EU117157
L-LA-56	<i>Lagerstroemia</i> 'Red Rocket'	<i>Altica</i> spp.	19-VII-02	St. Tammany, LA	EU069471
L-LA-57	<i>Lagerstroemia</i> 'Red Rocket'	<i>Altica</i> spp.	19-VII-02	St. Tammany, LA	EU069472
L-MS-11	<i>Lagerstroemia</i> spp.	<i>Lysathia</i> spp.	27-V-03	Pearl River, MS	EU117150
L-MS-59	<i>Lagerstroemia</i> 'Country Red'	<i>Altica</i> spp.	28-VI-02	Stone, MS	EU069473
L-MS-60	<i>Lagerstroemia</i> 'Country Red'	<i>Altica</i> spp.	28-VI-02	Stone, MS	EU069474
LS-TN-1	<i>Lythrum salicaria</i>	<i>Lysathia</i> spp.	25-V-00	Unicoi, TN	EU143722
LS-TN-1B	<i>Lythrum salicaria</i>	<i>Lysathia</i> spp.	25-V-00	Unicoi, TN	EU069464
OL-GA-26	<i>Oenothera laciniata</i>	<i>Altica litigata</i>	17-VII-02	Pike, GA	EU069483
OL-GA-27	<i>Oenothera laciniata</i>	<i>Altica litigata</i>	17-V-02	Pike, GA	EU069484
OL-GA-85	<i>Oenothera laciniata</i>	<i>Altica litigata</i>	30-V-02	Decatur, GA	EU069492
OL-GA-86	<i>Oenothera laciniata</i>	<i>Altica litigata</i>	30-V-02	Decatur, GA	EU069493
OL-GA-87	<i>Oenothera laciniata</i>	<i>Altica litigata</i>	30-V-02	Decatur, GA	EU069495
OA-GA-12-2	<i>Oenothera lamarckiana</i>	<i>Lysathia</i> spp.	9-V-02	Spalding, GA	EU143717

Table 1. Collection data.

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Sample*	Host plant	Presumptive species	Coll.	County, State	GenBank
OA-GA-13-1	<i>Oenothera lamarckiana</i>	<i>Altica</i> spp.	9-V-02	Spalding, GA	EU143718
OA-GA-15-2	<i>Oenothera lamarckiana</i>	<i>Altica litigata</i>	9-V-02	Spalding, GA	EU143719
OA-GA-16-4	<i>Oenothera lamarckiana</i>	<i>Altica litigata</i>	9-V-02	Spalding, GA	EU143720
OA-GA-20-1	<i>Oenothera lamarckiana</i>	<i>Altica litigata</i>	9-V-02	Spalding, GA	EU143721
OS-GA-28	<i>Oenothera speciosa</i>	<i>Altica litigata</i>	02-VI-02	Clarke, GA	EU069485
OS-GA-29	<i>Oenothera speciosa</i>	<i>Altica litigata</i>	02-VI-02	Clarke, GA	EU069486
OS-GA-33	<i>Oenothera speciosa</i>	<i>Altica litigata</i>	02-VI-02	Clarke, GA	EU069487
OS-GA-34	<i>Oenothera speciosa</i>	<i>Altica litigata</i>	02-VI-02	Clarke, GA	EU069488
OS-GA-73	<i>Oenothera speciosa</i>	<i>Altica litigata</i>	03-VI-02	Clarke, GA	EU069489
OS-GA-74	<i>Oenothera speciosa</i>	<i>Altica litigata</i>	03-VI-02	Clarke, GA	EU069490
OS-GA-75	<i>Oenothera speciosa</i>	<i>Altica litigata</i>	03-VI-02	Clarke, GA	EU069491
Pdesotonis	<i>Coreopsis lanceolata</i>	<i>Phaedon desotonis</i>	03-VII-07	Spalding, GA	EU143712

* Represents a maternal haplotype and refers to, in order: host plant or plant on which collected (L = *Lagerstroemia* spp., Ls = *Lythrum salicaria*, Ol = *Oenothera laciniata*, Os = *Oenothera speciosa*, Oa = *Oenothera lamarckiana*, G = *Guara lindheimeri*); U. S. state from which collected (AL = Alabama, GA = Georgia, LA = Louisiana, MS = Mississippi, TN = Tennessee); a number for internal tracking.

Table 1 [CONTINUED].

2.2. DNA extraction, PCR, and sequencing

DNA was extracted from individual flea beetles preserved in 100% EtOH from each host plant at each site with the E.Z.N.A. Mollusc DNA kit (Omega Bio-Tek, Inc., Doraville, GA) or DNeasy Blood & Tissue Kit (QIAGEN Inc., Valencia, CA).

The COI (~772-bp) was amplified and sequenced in both directions from each sample (Table 1) with primers C1J2195 (5' - TTGATT(CT)TTTGGTCA (CT)CC(AT)GAAGT - 3') and TL2N3014 (5' - TC(CT)A(AT)TGCA(CT)TAATCT GCCATATT - 3') (Simon *et al.* 1994).

PCR was performed in a standard 25- μ l reaction with 5-20 ng of total genomic DNA. The reaction for the COI amplifications had 1 pmol of each primer, 2.0 mM MgCl₂, 1.0 mM dNTPs, and 0.06 U/ μ l *Taq* DNA polymerase. Amplification was accomplished in a Perkin-Elmer Gene Amp PCR system 9600 or 9700 (Applied Biosystems, Foster City, CA). The procedure included a pre-cycle denaturation at 94 °C for 2 min., a post-cycle extension at 72 °C for 5 min, and 25-30 cycles of a standard three-step PCR (94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min). PCR products were further purified using the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA) protocol. All PCR samples from individual beetles were then sent to the Sequencing and Synthesis Facility (SSF) at Integrated Biotechnology Laboratories

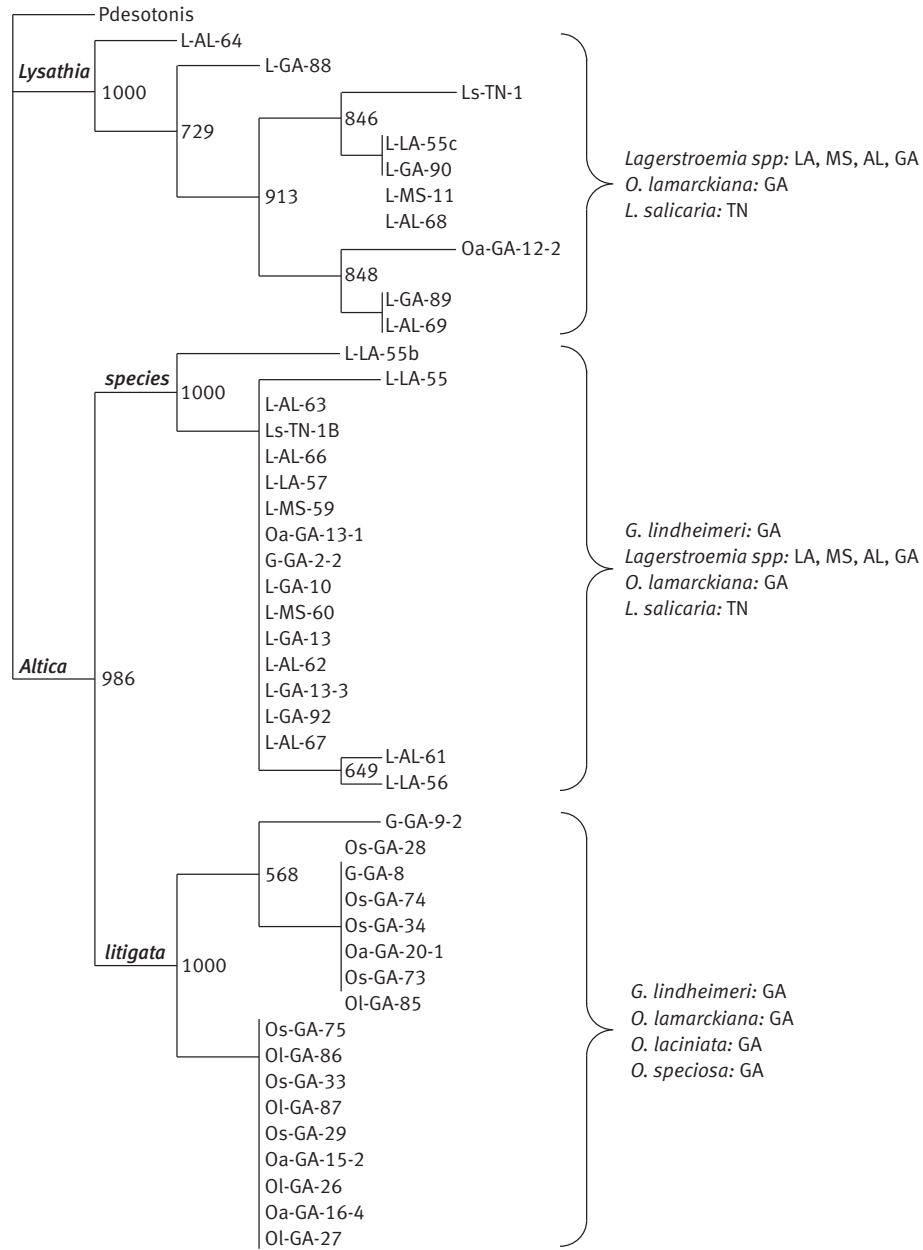


Figure 1. Neighbor-joining phylogenetic tree (Saitou and Nei 1987) of COI sequence created in PHYLIP 3.65 (Felsenstein 1993) with terminals representative of the flea beetle taxa in Table 1. Bootstrap values ($\times 1000$) are indicated at the nodes. All nodes less than or equal to 50% were collapsed. The brackets to the left of the tree outline the *Lysathia* clade and the two *Altica* subclades, *species* and *litigata*. These subclades represent the two presumptive species *Altica* spp. and *Altica litigata*. To the right of each bracketed group is a summary of the hosts and state from which each taxon was collected.

(Athens, GA 30602) or to MWG (4191 Mendenhall Oaks Parkway, Suite 140, High Point, NC 27265) for direct sequencing in both directions.

2.3. Phylogenetic algorithms and analyses

Individual electropherograms were first analyzed and contigs formed using Sequencher 3.1.1 software (Gene Codes Corp., Ann Arbor, MI). All contigs that represented a consensus COI fragment were made into a single consensus sequence identified by host plant, state from which collected, and identification number (Table 1). These sequences were then aligned using the slow and accurate pairwise alignment in CLUSTALW 1.83 (<http://align.genome.jp>) (Thompson *et al.* 1994; Higgins *et al.* 1996) with a multiple alignment gap open penalty of 15 and a gap extension penalty of 6.66. It was formatted for PHYLIP 3.65 (<http://align.genome.jp>) (Felsenstein 1993).

Heuristic searches for the best tree were based on either a distance or character state phylogenetic algorithm (Avice 1994, p. 124; Jenkins *et al.* 2001); and, were accomplished using the following suite of programs in PHYLIP version 3.65 (Felsenstein 1993). Thus conspecific gene flow was estimated with maximum likelihood (ML) using DNAML slow and accurate analysis with a transition/transversion ratio of 2, no global rearrangements, and jumbled once; unweighted maximum parsimony (MP) using DNAPARS (tree not shown), and neighbor-joining (NJ) (Saitou & Nei 1987) using NEIGHBOR (Fig. 1) Genetic distances were calculated with DNADIST according to the Kimura 2-parameter model of sequence evolution. Consensus trees were determined in CONSENSE using the majority rule (extended) model. Character state polarities (Avice 1994, p. 125) were accomplished by rooting all trees, which were generated in TREEVIEW version 3.2 (Page 1996). Two outgroups, *Phaedon desotonis* and *Lysathia* spp., were used in order to test for minimal ingroup monophyly (Smith 1994).

2.4. Population structure

An analysis of molecular variance (AMOVA) was generated with ARLEQUIN version 3.0 on the COI data set to test the hypothesis of spatial panmixia and determine the hierarchical population structure. *Altica* collections were pooled into two groups according to Fig. 1 (species and *litigata*). The number of usable loci for distance computation was 817 with an allowed level of missing data of 0.05. The partitioning of COI genetic variation within and between the groups was determined by performing 10,000 permutations using the default settings. ARLEQUIN was also used to calculate the pairwise genetic distances (F_{ST}), the significance of which was determined by performing 10,000 permutations among the individuals between groups in Fig. 1 using the default settings. The gene flow (Nm) between groups was estimated according to the formula of Wright (1951), $Nm = (1 - F_{ST}) / 4 F_{ST}$.

Since AMOVA uses a hierarchically partitioned matrix of squared genetic distances to determine by permutation the significance of variance components associated with each level of genetic partitioning, it is analogous to a nested analysis of molecular variance (Yannarell *et al.* 2006)

3. Results and discussion

There was insufficient information from the COI sequence to determine the intraspecific branching sequence (Fig. 1). Nodes $\leq 50\%$ were collapsed.

Analyses of COI sequences using PHYLIP v. 3.65 established reliable hierarchical relationships by forming essentially the same majority-rule consensus tree topologies (Ferris *et al.* 2001) (Fig. 1) (PHYLIP generated trees not shown). Bootstrap analyses resolved the *Altica* clade into two strongly supported subclades (node = 98.6%), species and *litigata*, respectively (Fig. 1). Beetles in *Lysathia* clade and *Altica* species subclade were collected from host species in the Onagraceae and the Lythraceae across four states (Fig. 1). Beetles in the *Altica litigata* subclade were collected from four species in the Onagraceae. A sampling bias may have inadvertently been introduced in the *Altica litigata* subclade, however, since all were collected in GA from *Oenothera* species. Thus, the host choice for this group may be more varied than reflected by this data set.

No significant regional population genetic structure was observed within flea beetle populations (7.46 % variation, $P = 0.0000$), as in spatial panmixia. Genetic differentiation among populations, however, was relatively high (77.61 % variation, $P = 0.0000$) and supports genetic isolation between the two host-specific groups. Exact tests on population differentiation were significant ($P < 0.05$) for every clade pairwise comparison. *Altica* groups were genetically distant from each other ($F_{ST} = 0.92014$, $P < 0.05$). The estimated gene flow (Nm) between these two groups according to the formula of Wright (1951), $Nm = (1 - F_{ST}) / 4 F_{ST}$, was 0.013, $\ll 1.0$.

Adults of *A. litigata* were reported to feed on the leaves of *Oenothera* and *Lagerstroemia* (Pettis & Braman 2007). We, therefore, assumed that *A. litigata* was collected from these plants based on past field experience, morphological identification, the literature (Pettis *et al.* 2004), and host-plant associations. Phylogeny analyses of the congruent COI as in other studies of the Chrysomelidae (Gómez-Zurita *et al.* 2007), partitioned species in this case into two *Altica* subclades. COI sequences representing collections from *Oenothera* spp. were submitted to GenBank as *A. litigata* Fall because a verified complete life cycle had been described on these hosts (Pettis *et al.* 2004; Pettis & Braman 2007). Feeding behavior on *Lagerstroemia* spp. may, therefore, represent a relatively recent sympatric host-specific adaptation of *A. litigata* lineage. It may also represent a new *Altica* species such as the newly described *A. copelandi* (Ciegler 2006); or, one of the other *Altica* species known to occur on *Oenothera* (Ciegler 2006). Phylogeny analysis of COII gene sequence from a small subset representing both beetles collected from both host types was congruent with the COI host-specific phylogeny (unpublished data). Furthermore,

when COI sequences from GenBank of 10 different *Altica* species (phylogeny not shown) were added to the data set, the COI topology regarding our samples did not change. To determine, however, if flea beetles collected on *Lagerstroemia* spp. are the evolutionary sister taxon to *A. litigata* a broad survey of *Altica* species is needed, which has begun in our lab.

Flea beetles such as *Altica*, which are morphologically similar, have often been distinguished by their host preference even though host plant association has been shown to be relatively labile in the Chrysomelidae (Farrell & Sequeira 2004). *Lagerstroemia* spp. has long been considered an opportunistic host for *A. litigata* adults (Pettis *et al.* 2004; Pettis & Braman 2007). The *Altica* collected from crape myrtles, therefore, were assumed to be adult *A. litigata* based on established taxonomic criteria and host-specificity. The life cycle of *A. litigata* occurs on plants in the family Onagraceae. Phylogenetic results showed, however, that these beetles fed on several plant species within two families (Fig. 1 & Table 1).

The COI sequence was species-specific and showed that *Altica* spp. are capable of feeding on several host plants. Only plants in the genus *Oenothera* were host to *A. litigata*. But until more collections are done in more geographic regions, a sampling bias cannot be ruled out.

This study confirmed the value of a morphology-based molecular *Altica* taxonomy founded in evolutionary and phylogenetic theory (Vogler & Monaghan 2007). Recent research also showed the importance of this one-to-one correlation between morphology and DNA (Ruhl 2009). The aedeagus of *A. litigata* (Fall, 1910) and *A. foliacea* (LeConte, 1858) appear to have the same morphology (Ruhl 2009; L. LeSage, Ontario, Canada, personal communication). A study, therefore, to verify taxonomy by examining DNA markers as well as scanning electron micrograph (SEM) morphology of *A. litigata* and *A. foliacea* voucher specimens is being conducted (Jenkins, unpublished data).

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