Insights Into Flea Beetle (Coleoptera: Chrysomelidae: Galerucinae) Host Specificity From Concordant Mitochondrial and Nuclear DNA Phylogenies

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ABSTRACT Flea beetles in the genus *Altica* are herbivorous, urban agricultural pests that are morphologically difficult to distinguish. Host plant associations, therefore, have been used as an important species character in field studies. Indigenous weeds in the Onagraceae, genus *Oenothera*, are known to serve as developmental hosts for the flea beetle *Altica litigata* Fall. Although host plant specificity in herbivorous beetles is labile and adult *A. litigata* have been reported to aggregately feed on plants in the nonindigenous Lythraceae, genus *Lagerstroemia*, there is no evidence that these ornamental trees serve as developmental hosts. Because adult *A. litigata* feed on host plants from species in two plant genera, this study was designed to test two hypotheses. The first hypothesis that was tested was whether adult flea beetles collected from primrose and crape myrtle plants across four ecoregions are phenotypically (morphology) and genotypically (genotype) *A. litigata*. The second hypothesis that was tested was whether two unlinked loci, cytochrome oxidase subunit I and internal transcribed spacer, are phylogenetically concordant for flea beetle species. If so, they could be used to determine the intraspecific geographic history of *A. litigata* collected from *Oenothera* and *Lagerstroemia* species. We discuss how these markers, in conjunction with morphology and host plant feeding behavior, can not only help to validate morphologically difficult taxa but also can illuminate herbivore-plant genetic structure through phylogeny analyses.

KEY WORDS *Altica litigata*, flea beetle, mitochondrial DNA, internal transcribed spacer, herbivore

Altica litigata Fall, 1910 (Coleoptera: Chrysomelidae) is a nursery and landscape pest that feeds on plants in the Lythraceae and Onagraceae families (McKenney et al. 2003, Pettis et al. 2004) and normally produces two to three generations each year (LeSage 1995). The metallic blue-to-green adults have well-developed metathoracic legs that are used to jump and scatter quickly from plants when disturbed. Adults are aggregate feeders whose sudden and unpredictable outbreaks can result in dramatic defoliation of crape myrtles (Lythraceae: *Lagerstroemia*) in late spring and early summer when they feed (Pettis et al. 2004, Cabrera et al. 2008).

A. litigata larval development is known to occur on primrose (*Oenothera* spp.), but it has not been reported on crape myrtles (Pettis et al. 2004, McKenney et al. 2003). Adult *A. litigata* have been collected from weedy primrose species and other cultivated *Oenothera* counterparts, which may grow in and around production nurseries (Pettis et al. 2004). Adults also have been collected from these weedy plants before and during pest outbreaks on crape myrtle (Pettis et al. 2004). Although intraspecific population genetic studies have to date not been done, adult *A. litigata* are believed to migrate from wild herbaceous hosts in the Onagraceae and Lythraceae families to crape myrtles (Pettis et al. 2004).

Classification of beetles in the genus *Altica* is difficult because many species are morphologically indistinguishable when comparing gross anatomy or microscopic characters such as the male aedaegus (Phillips 1979). However, beetles may differ in host plant choice. Thus, DNA markers could provide unequivocal identification and/or verification of morphological species, host plant specificity, and intraspecific population genetic structure. A better understanding of intraspecific herbivore–host history also could provide predictive information with implications for pest management.

The mitochondrial DNA (mtDNA) genome is essentially a single copy genome (Avise 1994) that evolves faster than nuclear coding regions; rates of 2.3% per million years to 1.5% per million years have

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been reported for coleopteran mtDNA cytochrome oxidase subunit I (COI) sequences (Bell et al. 2007, Kölsch and Pedersen 2008). The mitochondrial COI gene sequence, particularly the 5′ "Folmer region," has become the standard maternal marker used for phylogeny inference generally (www.barcoding. si.edu; Miller 2007) and the Galerucinae subfamily particularly (Caterino et al. 2000, Clark et al. 2001), thus facilitating comparison across studies. The nuclear internal transcribed spacer (ITS) regions have been used successfully for phylogenetic analyses of insect species and genus relationships (Coleman 2003; Jenkins et al. 2007, 2001). The ITS of the rDNA array is composed of ribosomal genes 18S rRNA, 5.8S rRNA, and 28S rRNA that are evolutionarily conserved, and two internal transcribed spacers (ITS1, between the 18S rRNA and 5.8S rRNA; and ITS2, between the 5.8S rRNA and 28S rRNA) that evolve faster (Navajas et al. 1999).

Conceptually genealogical phylogenetic concordance is, according to Bernardi et al. (1993), of two types: gene-gene and gene-geography. Gene-gene phylogenetic concordance refers to phylogenetic topology agreement between unlinked loci. Thus, the overall purpose of this study is twofold. First was to test the hypothesis that adult flea beetles collected from primrose and crape myrtle plants across four ecoregions will group into a single *A. litigata* clade. Second was to test the hypothesis that the unlinked loci, COI and ITS, are phylogenetically concordant across all flea beetle collections.

Methods

Flea Beetle Collection. Adult flea beetle specimens were collected from host plants in two families, Lythraceae, represented by *Lagerstroemia* spp., and Onagraceae, represented by *Oenothera laciniata* Hill and *Oenothera speciosa* Nutt. Collections were from four ecoregions, interior plateau (IP), piedmont (P), ridge and valley (RV), and southern coastal plains (SCP), which included four southeastern states in the United States (Table 1). *A. litigata* were identified from the field by the authors. D. Boyd, Jr., also sent 75 samples of presumptive *A. litigata* collected from *Lagerstroemia* spp. to a Chrysomelidae taxonomist and received an email report that all 75 were identified as *A. litigata.* Samples of flea beetles presumed to be *Lysathia ludoviciana* (Fall 1910) were collected from crape myrtle and also sent to the same Chrysomelidae taxonomist by D. Boyd, Jr. These samples were reported to be *Lysathia* species because the male genitalia morphology did not conform to *L. ludoviciana.* For use as an outgroup species in phylogeny analysis, *Phaedon desotonis* Balsbaugh, 1983, was collected from *Coreopsis lanceolata* L. (Asteraceae) in Griffin, GA (Table 1), and identified from morphology. All flea beetles were collected live. A sample of collections was preserved in 70% ethanol (EtOH) for morphological identification. The rest was preserved in 100% EtOH for DNA extraction.

DNA Extraction, Polymerase Chain Reaction (PCR), and Sequencing. DNA was extracted from 86 individual flea beetles (Table 1) according to Jenkins et al. (2007).

Two unlinked loci, mitochondrial and nuclear, were sequenced per flea beetle in an effort to improve the power of the phylogenetic analyses and minimize genome-dependent processes that may influence sequence evolution. The COI gene ($\approx$800 bp) was amplified and sequenced in both directions for each sample (Table 1) with primers C1J2195 (5′-TTGAT T(CT)TTTGGTCA (CT)CC(AT)GAAGT-3′) and TL2N3014 (5′-TC(CT)A(AT)TGCA(CT)TAATCT GCCATATT-3′) (Simon et al. 1994).

The ITS array (ITS1, 5.8S, and ITS2) was amplified and sequenced in both directions. The fragment was initially amplified with *Drosophila* primers CS249 (5′-TCGTAACAAGGTTTCCG-3′) and CS250 (5′-GTT (A/T)GTTTCTTTTCCTC-3′) (Schlőtterer and Tautz 1994). These sequences were then spot verified with three sets of primers. The first set of primers FBTFwd (5′-CCAAGAAAAATAACAAAT-3) and FBTRev (5′-AGACATACAGATTTACGC-3′) amplified an internal fragment ($\approx$650 bp) that contained the 5.8S rDNA and were anchored in the ITS1 and ITS2 regions, respectively. The second set of primers paired FBTRev with FB18SFwd (5′-CGCTACACT GAAGGAAT-3′) and amplified a fragment that was $\approx$1220 bp and were anchored in the ITS2 regions and 18S rDNA respectively. The third set of primers FBT2Fwd (5′-TACCAAGAAAAATAACAAAT-3′) and TDE28SRev (5′-CCGTTCAAGAAGGACT-3′) amplified a fragment that was $\approx$970 bp and were anchored in the ITS1 and 28S rDNA regions, respectively. Primers FB18SFwd and TDE28SRev were designed from aligned Coleoptera sequences from GenBank.

PCR was performed in a standard 25-μl reaction with 5–20 ng of total genomic DNA. The reaction for the COI amplifications had 1 pmol of each primer, 2.0 mM MgCl$_2$, 1.0 mM dNTPs, and 0.06 U/μl TaqDNA polymerase. Amplification was accomplished in a PerkinElmer Gene Amp PCR system 9600 or 9700 (Applied Biosystems, Foster City, CA). The procedure included a precycle denaturation at 94°C for 2 min, a postcycle 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). The ITS fragment was first amplified using primers CS249 and CS250 according to Schlőtterer and Tautz (1994) and included 35 cycles of a standard three-step PCR (94°C for 1 min, 57°C for 1 min, 72°C for 1 min). The internal ITS fragments were amplified with primers FBTFwd and FBTRev and also included 35 cycles of a standard three-step PCR (94°C for 1 min, 48°C for 1 min, 72°C for 1 min) with a precycle denaturation at 94°C for 2 min and a postcycle extension at 72°C for 2 min. Primers FB18SFwd and FBTRev had an annealing temperature of 58°C. Primers FBT2Fwd and TDE28SRev had an annealing temperature of 54°C. All PCR fragments were treated with exonuclease I (10U/μl) and shrimp alkaline phosphatase (1 U/μl) (Jenkins et al. 2001).

Table 1. Collection data with species verification based on phylogeny analyses (see to Fig. 2): Subclade I represents *A. litigata* and Subclade II represents *Altica* spp.

Sample[a,b]	Host plant	Beetle species	L[c]	Date	Collection site	Eco[d]	Collected by
L-d-AL-61 (J)	*Lagerstroemia Potomac*	*Altica* spp.	d	10-VII-2002	Twin Oaks Nur, Wilmer, AL, Mobile Co.	SCP	DW Boyd, Jr.
L-d-AL-62 (A)	*Lagerstroemia Potomac*	*Altica* spp.	d	10-VII-2002	Twin Oaks Nur, Wilmer, AL, Mobile Co.	SCP	DW Boyd, Jr.
L-d-AL-63 (L)	*Lagerstroemia Potomac*	*Altica* spp.	d	10-VII-2002	Twin Oaks Nur, Wilmer, AL, Mobile Co.	SCP	DW Boyd, Jr.
L-d-AL-66 (D)	*Lagerstroemia Hopi*	*Altica* spp.	d	19-VII-2002	Byers Nur., Huntsville, AL, Madison	IP	C Pounders
L-d-AL-67 (B)	*Lagerstroemia Hopi*	*Altica* spp.	d	19-VII-2002	Byers Nur., Huntsville, AL, Madison Co.	IP	C Pounders
L-d-GA-10 (A)	*Lagerstroemia* spp.	*Altica* spp.	d	29-VI-2002	UGA Garden, Griffin, GA, Spalding Co.	P	GV Pettis
L-d-GA-13 (E)	*Lagerstroemia* spp.	*Altica* spp.	d	29-VI-2002	UGA, Griffin, GA, Spalding Co.	P	SK Braman
L-d-GA-49	*Lagerstroemia* spp.	*Altica* spp.	d	31-V-2001	Wight's Nursury, Cairo, GA, Grady Co.	RV	GV Pettis
L-d-GA-91	*Lagerstroemia* spp.	*Altica* spp.	d	01-VI-2002	UGA, Griffin Campus, Spalding Co.	P	GV Pettis
L-d-GA-92	*Lagerstroemia* spp.	*Altica* spp.	d	01-VI-2002	UGA, Griffin Campus, Spalding Co.	P	GV Pettis
L-d-GA-93	*Lagerstroemia* spp.	*Altica* spp.	d	01-VI-2002	UGA, Griffin Campus, Spalding Co.	P	GV Pettis
L-d-LA-8	*Lagerstroemia* Red Rocket	*Altica* spp.	d	19-VII-2002	Zelenka Nur, St. Tammany Parish, LA,	SCP	DW Boyd, Jr.
L-d-LA-55	*Lagerstroemia* Red Rocket	*Altica* spp.	d	19-VII-2002	Zelenka Nur, St. Tammany Parish, LA,	SCP	DW Boyd, Jr.
L-d-LA-55b	*Lagerstroemia* Red Rocket	*Altica* spp.	d	19-VII-2002	Zelenka Nur, St. Tammany Parish, LA,	SCP	DW Boyd, Jr.
L-d-LA-56	*Lagerstroemia* Red Rocket	*Altica* spp.	d	19-VII-2002	Zelenka Nur, St. Tammany Parish, LA,	SCP	DW Boyd, Jr.
L-d-LA-57	*Lagerstroemia* Red Rocket	*Altica* spp.	d	19-VII-2002	Zelenka Nur, St. Tammany Parish, LA,	SCP	DW Boyd, Jr.
L-d-MS-59	*Lagerstroemia* Country Red	*Altica* spp.	d	28-VI-2002	Green Forrest Nur., Parkinson, MS, Stone Co.	SCP	DW Boyd, Jr
L-d-MS-60	*Lagerstroemia* Country Red	*Altica* spp.	d	28-VI-2002	Green Forrest Nur., Parkinson, MS, Stone Co.	SCP	DW Boyd, Jr.
L-l-AL-64	*Lagerstroemia Potomac*	*Altica litigata*	l	10-VII-2002	Twin Oaks Nur, Wilmer, AL, Mobile Co.	SCP	DW Boyd, Jr.
L-l-AL-68	*Lagerstroemia Hopi*	*Lysathia* spp.	l	19-VII-2002	Byers Nur., Huntsville, AL, Madison Co.	IP	C Pounders
L-l-AL-69	*Lagerstroemia Hopi*	*Lysathia* spp.	l	19-VII-2002	Byers Nur., Huntsville, AL, Madison Co.	IP	C Pounders
L-l-GA-88	*Lagerstroemia* spp	*Lysathia* spp.	l	01-VI-2002	UGA, Griffin, GA, Spalding Co.	P	GV Pettis
L-l-GA-89	*Lagerstroemia* spp.	*Lysathia* spp.	l	01-VI-2002	UGA, Griffin, GA, Spalding Co.	P	GV Pettis
L-l-GA-90	*Lagerstroemia* spp.	*Lysathia* spp.	l	01-VI-2002	UGA, Griffin, GA, Spalding Co.	P	GV Pettis
L-l-LA-55c	*Lagerstroemia* Red Rocket	*Lysathia* spp.	l	19-VII-2002	Zelenka Nur, St. Tammany Parish, LA,	SCP	DW Boyd, Jr.
L-l-MS-11	*Lagerstroemia* spp.	*Lysathia* spp.	l	27-V-03	Poplarville, MS, Pearl River Co	SCP	DW Boyd, Jr.
Ol-d-GA-9	*Oenothera laciniata*	*Altica litigata*	d	17-VII-2002	Middle GA Nur, Meansville, Pike Co.	P	GV Pettis
Ol-d-GA-26	*Oenothera laciniata*	*Atlica litigata*	d	17-VII-2002	Middle GA Nur, Meansville, Pike Co.	P	GV Pettis
Ol-d-GA-27	*Oenothera laciniata*	*Altica litigata*	d	17-V-2002	Middle GA Nur, Meansville, Pike Co.	P	GV Pettis
Os-d-GA-28	*Oenothera speciosa*	*Altica litigata*	d	02-VI-2002	UGA, Athens, GA, Clark Co.	P	GV Pettis
Os-d-GA-29	*Oenothera speciosa*	*Altica litigata*	d	02-VI-2002	UGA, Athens, GA, Clark Co.	P	GV Pettis
Os-d-GA-33	*Oenothera speciosa*	*Altica litigata*	d	02-VI-2002	UGA, Athens, GA, Clark Co.	P	GV Pettis
Os-d-GA-34	*Oenothera speciosa*	*Altica litigata*	d	02-VI-2002	UGA, Athens, GA, Clark Co.	P	GV Pettis
Os-d-GA-73	*Oenothera speciosa*	*Altica litigata*	d	03-VI-2002	UGA, Life Sci. Bldg. Athens, GA, Clarke Co.	P	GV Pettis
Os-d-GA-74	*Oenothera speciosa*	*Altica litigata*	d	03-VI-2002	UGA, Life Sci. Bldg. Athens, GA, Clarke Co.	P	GV Pettis
Os-d-GA-75	*Oenothera speciosa*	*Altica litigata*	d	03-VI-2002	UGA, Life Sci. Bldg. Athens, GA Clarke Co.	P	GV Pettis
Ol-d-GA-85	*Oenothera laciniata*	*Altica litigata*	d	30-V-2002	Attapulgus, GA, Decatur Co.	RV	GD Buntin
Ol-d-GA-86	*Oenothera laciniata*	*Altica litigata*	d	30-V-2002	Attapulgus, GA, Decatur Co.	RV	GD Buntin
Ol-d-GA-87	*Oenothera laciniata*	*Altica litigata*	d	30-V-2002	Attapulgus, GA, Decatur Co.	RV	GD Buntin
Pdesotonis	*Corepsis lanceolata*	*Phaedon desotonis*		03-VII-2007	Griffin, GA, Spalding Co.	P	SK Braman

[a] Strain identification represents a maternal consensus sequence or haplotype (refer to text) and refers to, in order, host plant (L, *Lagerstroemia*; Ol, *Oenothera laciniate*; Os, *Oenothera speciosa*), leg morphology (d, dark legs; l, light legs), U.S. state site from which collected (AL, Alabama; GA, Georgia; LA, Louisiana; MS, Mississippi). Strains L-d-55b, L-l-LA-55c, L-d-LA-8, Ol-d-GA-9, Os-d-GA-34, and Os-d-GA-75 do not represent a consensus sequence, but a single sequence.

[b] mtDNA haplotypes (see Fig. 2).

[c] L refers to the leg morphology, which was either dark (d) or light (l).

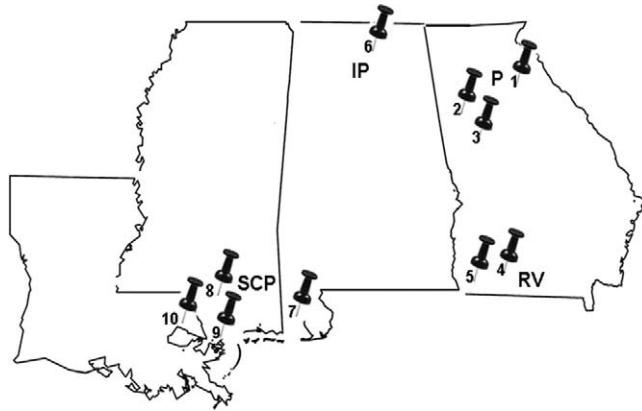[d] Eco refers to the ecoregions from which samples were taken (see text).

Fig. 1. Collection sites across the states of Georgia (GA), Alabama (AL), Mississippi (MS), and Louisiana (LA) (refer to Table 1). Collection sites are represented as follows: 1–5 in GA represent Clark Co., Spalding Co., Pike Co., Grady Co., and Decatur Co., respectively; 6 and 7 represent Madison Co. and Mobile Co. respectively; 8 and 9 represent Stone Co. and Pearl River Co., respectively; and 10 represents Tammany Parish. Ecoregions are represented as in Table 1.

PCR products were further purified according to Jenkins et al. (2007). All PCR samples from individual beetles were then sent to the Sequencing and Synthesis Facility (SSF) at Integrated Biotechnology Laboratories (Athens, GA) or to MWG (High Point, NC) for direct sequencing in both directions.

Phylogenetic Algorithms and Analyses. Individual electropherograms were first analyzed and contigs formed using Sequencher 3.1.1 software (Gene Codes Corp., Ann Arbor, MI) for both COI and ITS sequences. All contigs that represented a consensus COI or ITS fragment were made into a single consensus sequence. Each sequence was identified by plant from which collected (L for *Lagerstroemia* spp., Ol for *Oenothera laciniata,* or Os for *Oenothera speciosa*), leg color morphology (d for dark and l for light), state from which collected (GA, AL, MS, and LA), and identification number for laboratory use (Table 1). These COI and ITS 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 opening penalty of 15 and a gap extension penalty of 6.66. It was formatted for PHYLIP 3.65 (http://align.genome.jp) (Felsenstein 1993) and PAUP* (Swofford 1996). Rooted trees (Fig. 1) were generated with TREEVIEW version 3.2 (Page 1996). Character state polarities (Avise 1994, p. 125) and a test for minimal ingroup monophyly (Smith 1994, p. 26) for *Altica* were accomplished by rooting all gene trees (COI and ITS), which were generated in TREEVIEW (Page 1996), with *P. desotonis* and including samples of *Lysathia* spp.

Heuristic searches for the best tree were done with PHYLIP 3.65 (Felsenstein 1993) for COI and ITS sequences. Conspecific gene flow was estimated using three methods: maximum likelihood (ML) by using DNAML slow and accurate analysis with a transition/transversion ratio of 2, no global rearrangements, and jumbled once; unweighted maximum parsimony (MP) by using DNAPARS default

settings; and neighbor-joining (NJ) (Saitou and Nei 1987) by using NEIGHBOR default settings and 1,000 multiple data sets. Genetic distances were calculated with the DNADIST program of PHYLIP (Felsenstein 1993) according to the Kimura two-parameter model of sequence evolution and 1,000 replicates. Consensus trees were determined in CONSENSE by using the majority rule (extended) model. ML analysis of the COI dataset was also done in PAUP* 4.0b10 (Swofford 1996). All GenBank accession numbers are listed in Table 1.

Node support estimates for majority-rule consensus NJ, MP, and ML trees (PHYLIP version 3.65, CONSENSE) for COI and ITS sequence were assessed by two statistical nonparametric resampling protocols for COI: bootstrap, which tests clade stability to differential weighting by resampling with replacement (Felsenstein 1985); and jackknife, which tests clade resolution when less data are present through resampling without replacement. SEQBOOT (PHYLIP version 3.65) generated 1,000 nonparametric bootstrap and jackknife pseudoreplicates. The ML bootstrap and jackknife analyses in PAUP* for COI data included 100 resamplings and used the default likelihood parameter settings (HKY85 six-parameter model of nucleotide substitution, empirical base frequencies with the exception of the transition/transversion ratio, which was set to 1.399331:1). Clade node support in Fig. 2 was based on PAUP* resampling.

Population Structure. Maternal hierarchical structure was determined with an analysis of molecular variance (AMOVA) generated with ARLEQUIN version 3.0 (Excoffier et al. 2005, http://lgb.unige.charlequin) on the COI data set. Collections were pooled into three groups according to Fig. 2 (group I, *Altica* Subclade I; group 2, *Lysathia*; group 3, *Altica* Subclade II). The number of usable loci for distance computation was 817, and the allowed level of missing data was 0.05. The partitioning of COI genetic variation within and among the three groups and between *Altica* Subclade I (group 1) and *Altica* Subclade II
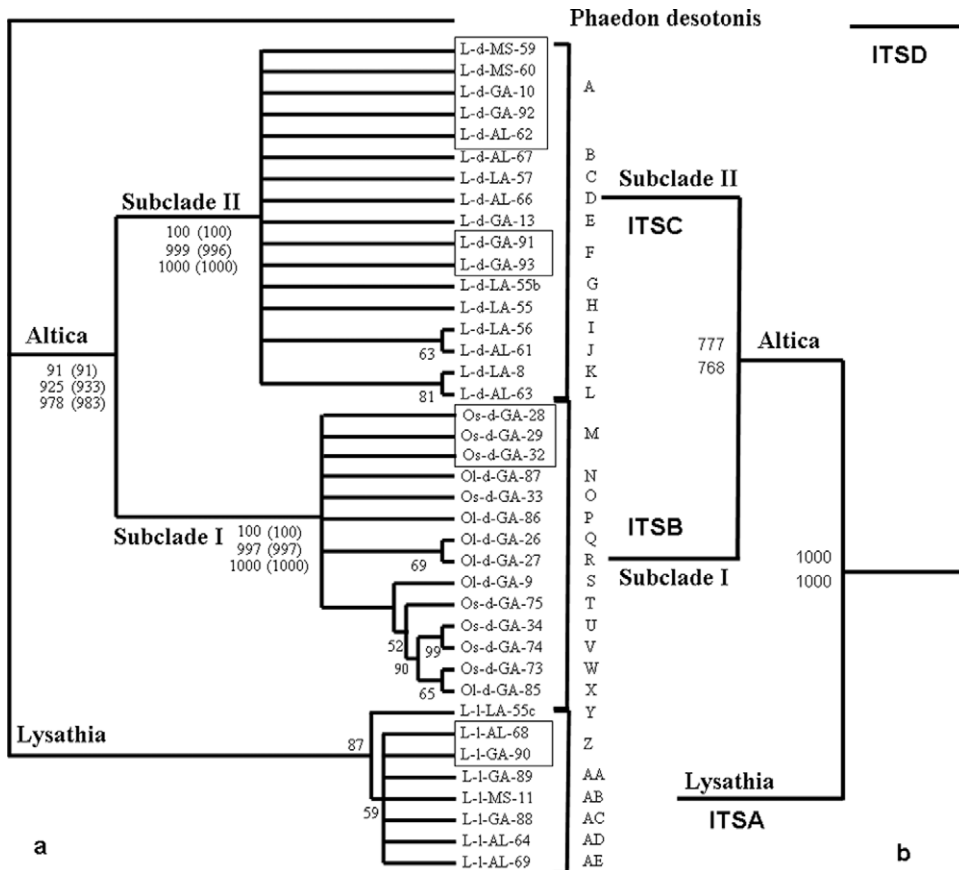
Fig. 2. Phylogenetic concensus trees representing (a) COI maternal haplotype (A through AE) (Table 1) and (b) ITS haplotypes ITSA, ITSB, ITSC, and ITSD (refer to text). All node support estimates (≥50%) for COI haplotypes are represented by numbers generated by bootstrap (left) and jackknife (right in parentheses) nonparametric resampling protocols (refer to text). The top number and branch support represents ML estimates generated in PAUP* 4.0b (Swofford 2000); the middle and bottom numbers represent ML and NJ analyses, respectively, generated from 1,000 pseudoreplicates in PHYLIP 3.65 (Felsenstein 1993). Clade nodes refer to PAUP* bootstrapped replicates (refer to text). Node support for b also was generated by bootstrap, 1,000×, with the top number representing ML and the bottom number representing NJ. *P. desotonis* was the outgroup species for both a and b. Boxed area in a represents a single haplotype (refer to text).

(group 3) specifically was then determined with 99% probability by performing 16,000 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 1,000 permutations among the individuals between groups in Fig. 2 by using the default settings. Gene flow (Nm) between groups was estimated according to the formula of Wright (1951), $Nm = (1 - F_{ST})/4 F_{ST}$.

Results

Genetic Diversity. Adult beetles collected from primroses and crape myrtles were all identified as *A. litigata* based on morphology and host plant association. Adults collected from primroses, however, differed genetically from adults collected from crape myrtles (Fig. 2). Because the life cycle of *A. litigata* is known for primrose, and because genetic identity should corroborate morphology, life cycle, and ecol-

ogy, those beetles collected on primroses were designated *A. litigata* (Subclade I) and those collected on crape myrtles were designated *Altica* spp. (Subclade II) (Fig. 2). David Boyd, Jr., sent representatives from samples of light-legged beetles presumed to be *L. ludoviciana* and collected on *Lagerstroemia* spp. in Alabama for taxonomic identification. He received an email that the "male genitalia of true *L. ludoviciana* [are] slightly different" from our specimens, which, in 2002, were not known from Alabama. Thus, the light-legged specimens were identified as "*Lysathia* spp. close to *L. ludoviciana.*" Furthermore, the email informed D. Boyd, Jr. that "the known hosts of *L. ludoviciana* are *Myriophyllum, Ludwigia,* and *Oenothera,* [and] the range covers from Georgia to Florida, west to Texas, [and the] West Indies." The adult beetles identified as *Lysathia* spp. in this study were found only on *Lagerstroemia* spp. (Table 1; Fig. 2) and were collected from Georgia, Alabama, Mississippi, and Louisiana.

Table 2. GenBank numbers (see Fig. 2)

Clades/subclades	Strain	COI haplotype	GenBank no.	ITS haplotype	GenBank no.
Lysathia	L-l-LA-55c	Y	EU117157	ITSA	EU682397
	L-l-AL-68	Z	EU117152	ITSA	
	L-l-GA-90	Z	EU117156	ITSA	
	L-l-GA-89	AA	EU117155	ITSA	
	L-l-MS-11	AB	EU117150	ITSA	
	L-l-GA-88	AC	EU117154	ITSA	
	L-l-AL-64	AD	EU117151	ITSA	
	L-l-AL-69	AE	EU117153	ITSA	
Altica					
Subclade I	Os-d-GA-28	M	EU069485	ITSB	EU682395
	Os-d-GA-29	M	EU069486	ITSB	
	Ol-d-GA-87	N	EU069495	ITSB	
	Os-d-GA-33	O	EU069487	ITSB	
	Ol-d-GA-86	P	EUO69493	ITSB	
	Ol-d-GA-26	Q	EU069483	ITSB	
	Ol-d-GA-27	R	EU069484	ITSB	
	Ol-d-GA-9	S	EU069494	ITSB	
	Os-d-GA-75	T	EU069491	ITSB	
	Os-d-GA-34	U	EU069488	ITSB	
	Os-d-GA-74	V	EU069490	ITSB	
	Os-d-GA-73	W	EU069489	ITSB	
	Ol-d-GA-85	X	EU069492	ITSB	
Subclade II	L-d-GA-49	A	EU069468	ITSC	EU682396
	L-d-MS-59	A	EU069473	ITSC	
	L-d-MS-60	A	EU069474	ITSC	
	L-d-GA-10	A	EU069466	ITSC	
	L-d-GA-92	A	EU069481	ITSC	
	L-d-AL-62	A	EU069476	ITSC	
	L-d-AL-67	B	EU069479	ITSC	
	L-d-LA-57	C	EU069472	ITSC	
	L-d-AL-66	D	EU069478	ITSC	
	L-d-GA-13	E	EU069467	ITSC	
	L-d-GA-91	F	EU069480	ITSC	
	L-d-GA-93	F	EU069482	ITSC	
	L-d-LA-55b	G	EU069470	ITSC	
	L-d-LA-55	H	EU069469	ITSC	
	L-d-LA-56	I	EU069471	ITSC	
	L-d-AL-61	J	EU069475	ITSC	
	L-d-LA-8	K	EU069465	ITSC	
	L-d-AL-63	L	EU069477	ITSC	
Pdesotonis			EU143712	ITSD	EU682398

Thirty-one COI haplotypes (Fig. 2), not including the *P. desotonis*, were observed (Fig. 2). Haplotypes that were the same across collections included A (collected across three states, all ecoregions), F (P ecoregion), M (P ecoregion), and Z (IP and P ecoregions) (Fig. 2).

ITS sequences were homogenized and clade-specific and interclade variable (Fig. 2). *Lysathia* spp., designated as haplotype ITSA, was ≈1169 bp. Altica Subclade I, designated as haplotype ITSB, was ≈1148 bp. Altica Subclade II, designated as haplotype ITSC, was ≈1205 bp (Table 2; Fig. 2). The outgroup, *P. desotonis*, was a fourth haplotype designated ITSD and was ≈1490 bp (Table 2; Fig. 2). ITS haplotypes were congruent with COI clades (Fig. 2).

Host Plant Maternal Structure. Multiple maternal lines from multiple species infested the same plant (Fig. 2). A *Lagerstroemia* cultivar in Tammany Parish, LA (Table 1; Fig. 2) had two *Altica* haplotypes, H and G, and one *Lysathia* spp. haplotype, Y. Three *Lysathia* spp. haplotypes, AA, AC, and Z were collected from a crape myrtle in Georgia. Haplotypes X, P, and N were collected from an *O. laciniata* in the RV ecoregion

(Table 1). Three *Altica* haplotypes were collected from an *O. speciosa* in the P ecoregion: M, O, and U (Figs. 1 and 2). A *Lagerstroemia* cultivar had five *Altica* haplotypes, K, H, G, I, and C. Haplotype A was found on *Lagerstroemia* spp. cultivars in three states. A single *O. laciniata* in Georgia had three haplotypes, Q, R, and S. An *O. speciosa* primrose in Georgia had Altica haplotypes W, V, and T.

Phylogeny and Host Specificity. Analyses of COI sequences by using PAUP version 4.0b and PHYLIP version 3.65 established reliable hierarchical relationships because essentially the same majority-rule consensus tree topologies were formed (Fig. 2) (MP trees not shown). Bootstrap and jackknife analyses produced three stable and resolved clades with strong node support (≥91%) (Fig. 2): Lysathia clade, Altica Subclade I, and Altica Subclade II (Table 2; Fig. 2). Beetles with dark legs, identified as Altica, formed the larger Altica clade and beetles with light legs grouped into clade Lysathia as expected. The two Altica clades were host-specific. Beetles of Altica Subclade I fed on *Oenothera* spp. and beetles of Altica Subclade II fed on *Lagerstroemia* spp. (node support ≥91%) (Fig. 2).

Altica Subclade I also had a three-base-pair deletion at the 5′ end that was not found in the COI sequence of beetles in Altica Subclade II. Polytomies dominated all COI clades. These branches, which represented unresolved relationships, indicated that the COI sequence did not provide enough information for intraspecific separation.

Population Structure. No significant regional population genetic structure was observed within flea beetle populations (Fig. 2) (7.46% variation, $P = 0.0000$), as in spatial panmixia. Genetic differentiation among populations, however (Fig. 2), was relatively high (77.61% variation, $P = 0.0000$) and supports genetic isolation between Altica Subclade I and Altica Subclade II. Exact tests on population differentiation were significant ($P < 0.05$) for every clade pairwise comparison. F_{st} pairwise differences >0.25 suggested high genetic differentiation (Wright 1951). The *Lysathia* population was genetically distant from Altica Subclade I population and Altica Subclade II population because $F_{st} = 0.94401$ ($P < 0.05$) and 0.88950 (P < 0.05), respectively. Altica Subclade I and Altica Subclade II were also 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, which is considerably <1.0.

Discussion

Altica **Species.** Adults of *A. litigata* were reported to feed on the leaves of *Oenothera* and *Lagerstroemia* (Pettis and Braman 2007). Many collections were made in areas where *Oenothera* and *Lagerstroemia* species domains overlapped (Table 1). We assumed that *A. litigata* was collected from these plants based on past field experience, morphological identification, the literature (McKenney et al. 2003, Pettis et al. 2004), and host plant associations. Phylogeny analyses of the congruent COI and ITS sequences (Fig. 2), as in other studies of the Chrysomelidae (Gómez-Zurita et al. 2007), partitioned species in this case into two Altica clades. COI sequences representing collections from Subclade I were submitted to GenBank as *A. litigata* Fall (Table 2) because they were collected from *Oenothera* spp. on which a complete life cycle is known (McKenney et al. 2003, Pettis and Braman 2007). Subclade II submitted as *Altica* spp. *A. litigata* has long been documented on *Oenothera* spp. (Pettis et al. 2004). Therefore, feeding behavior on *Lagerstroemia* spp. may, represent a relatively recent sympatric host-specific adaptation of *A. litigata* lineage. It also may represent a new *Altica* species, such as recently described *A. copelandi* (Ciegler 2006) or other *Altica* species known to occur on *Oenothera* (Ciegler 2006). Phylogeny analysis of COII gene sequence from a small subset representing both clades was congruent with the COI host-specific phylogeny (unpublished data).

Flea beetles thought to represent *L. ludoviciana* could not be unequivocally identified using only morphological characters. They were submitted to GenBank as *Lysathia* spp. Thus, if morphological and molecular characters are to be species-specific, then COI and ITS sequence haplotypes must be generated from morphologically unequivocal *L. ludoviciana* for there to be a DNA–morphology species correlation.

ITS Cluster. ITS regions evolve relatively rapidly (Oliverio et al. 2002), which is why the sequence has been used to resolve interspecific relationships (Beltrame-Botellho et al. 2005). Although species-specific, the ITS sequence in this study, as in other studies, lacked the power for intraspecific discrimination. This phenomenon could have its genesis in a recent geographical expansion because of extensive spatial and temporal gene flow (Szalanski and Owens 2003).

Intraspecific ITS homogenization, which has been observed in studies of the chrysomeloid (Szalanski and Owens 2003) and other insects (Jenkins et al. 2001, 2007), likely resulted from the processes of concerted evolution (Dover 1982), gene conversion and the mechanisms of DNA replication and repair, gene amplification and unequal crossing over during meiosis I (Dover et al. 1993, Elder and Turner 1995, Beebe et al. 2000). Once ITS homogenization became fixed, it would be maintained through selection, drift or molecular drive (Dover 1982, Schlötterer and Tautz 1994, Beebe et al. 2000).

The fast evolving ITS2 also may have potential for deep phylogeny as well as biogenesis research. It has been used for low-level phylogenetic studies (Selig et al. 2007), but recent research suggests the ITS2 has value as a general phylogenetic marker because of a conserved folding pattern across the eukaryotes (Coleman and Vacquier 2002, Wolf et al. 2005, Selig et al. 2007). This conserved structure could be maintained by compensatory mutations (Tillier and Collins 1998, McCutchan et al. 2004) that result in sexual incompatibility (Müller et al. 2007) and ultimately speciation. The sequence consists of four domains of secondary structure that rise from a preserved central core (Morgan and Blair 1998, Gómez-Zurita et al. 2007). These homologous core regions, as studies have indicated (Wesson et al. 1992), may function in the process of ribosomal biogenesis (Hlinka et al. 2002, Coleman 2003). Because rRNA is necessary for translation, homogenization may ensure intraspecific compatibility with all molecular components of the translational process (Averbeck and Eickbush 2005). Thus, an rRNA biogenetic process that depends on sequence and structure conservation can provide a strong species-specific DNA marker, particularly when concordant with a mitochondrial marker. Such analyses, therefore, can be used to validate identifications of adult beetles that are morphologically difficult to distinguish.

Phylogeny and Host Plant Structure. The life cycle of *A. litigata* occurs on plants in the family Onagraceae. Phylogenetic results (Fig. 2) showed, with high probability (node support between 91 and 98%), that the *Altica* in Subclade I and the *Altica* in

Subclade II were not the same species. The *Altica* samples in Subclade II were collected from *Lagerstroemia* spp., which has been considered an opportunistic host for *A. litigata* adults (Pettis et al. 2004, Pettis and Braman 2007). Flea beetles, because of their morphological similarity, have often been distinguished by their host preference even though host plant association has been shown to be relatively labile in the Chrysomelidae (Farrell and Sequeira 2004). The *Altica* adults collected from crape myrtles, therefore, were assumed to be adult *A. litigata.* Thus, DNA markers would be advantageous in population genetic studies of *Altica* spp. for verification of morphological taxonomy.

Interspecific infestations of *Altica* and *Lysathia* species were often found on a single host plant (Table 1; Fig. 2). This phenomenon also was observed in a field study with *Lagerstroemia* spp. (D.W.B., unpublished data) in which *A. litigata* and *Lysathia* spp. were observed. The field observations did not employ genetic markers to confirm and/or to differentiate between and among species. Although we do know that at least two adult species, representing two genera, were feeding together on these crape myrtles, we do not know with certainty the actual number of species feeding on these plants, nor do we have DNA verification that all the *Altica* were *A. litigata.* DNA markers would have provided greater insights into host-specific population structure.

Flea beetle larvae were observed to survive after oviposition on nonhost plants (Xue and Yang 2007). These flea beetles adapted behaviorally, possibly through minor genetic mutations (Jermy 1983), to differences in plant chemistry and phenology. Furthermore, when nonhost plant density was relatively high compared with host plant density, flea beetles such as *Altica* oviposited on nonhost plants (Xue and Yang 2007), although performance on the atypical host plant was poor (Xue and Yang 2007). This could be because of, as suggested for the flea beetle *Phyllotreta nemorum* L. (Breuker et al. 2006), negative pleiotropic effects of a dominant autosomal resistance (R) gene mutation, or selection for R-genes (Nielsen and DeJong 2005). Other studies have shown that inheritance of R alleles on autosomes and both sex chromosomes facilitated the survival of P. nemorum populations on atypical host plants. Selection may favor beetles with coadapted gene complexes (Orr and Coyne 1992).

Host plant adaptation and/or evolution of host range expansion require genetic-based physiological and behavioral adjustments (An et al. 2007). If the flea beetles in this study have host-shifted or are in the process of doing so, as expected for sympatric speciation (Futuyma et al. 1995, Craig et al. 2001), then mating behavior linked to host plant fidelity could ultimately result in mating isolation and speciation (Joy and Crespi 2006).

This study confirmed the concordance between the COI and ITS unlinked loci. It showed that these markers can not only validate the morphological taxonomy of *Altica* spp. through the concordance of gene-gene phylogenetic analyses but also can illuminate host-specific intra- and interspecific genetic structure.

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