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## Short Communication

## Compensatory base changes illuminate morphologically difficult taxonomy

Michael W. Ruhl<sup>a</sup>, Matthias Wolf<sup>b</sup>, Tracie M. Jenkins<sup>a,\*</sup><sup>a</sup> University of Georgia, Griffin Campus, Department of Entomology, Griffin, GA 30223, USA<sup>b</sup> Department of Bioinformatics, Biocenter, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany

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## ABSTRACT

Compensatory base changes (CBCs) in the ribosomal RNA (rRNA) internal transcribed spacer 2 (ITS2) secondary structures have been used to successfully verify the taxonomy of closely related species. CBCs have never been used to distinguish morphologically indistinct species. Under the hypothesis that CBCs will differentiate species in higher eukaryotes, novel software for CBC analysis was applied to morphologically indistinguishable insect species in the genus *Altica*. The analysis was species-specific for sympatric *Altica* beetles collected across four ecoregions and concordant with scanning electron microscopy data. This research shows that mining for CBCs in ITS2 rRNA secondary structures is an effective method for eukaryotic taxon analysis.

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## 1. Introduction

Compensatory base changes (CBCs) are mutations that occur in both nucleotides of a paired structural position while retaining the paired nucleotide bond. A hemi-CBC (hCBC) is a mutation of a single nucleotide in a paired structural position while maintaining the nucleotide bond. Analysis of these CBCs in the ribosomal RNA (rRNA) internal transcribed spacer 2 (ITS2) secondary structure has been used to successfully verify the taxonomy of closely related species. These analyses have been performed primarily in plants and fungi (Muller et al., 2007) and have never been used to distinguish morphologically indistinct species in eukaryotes. This study, therefore, tests the hypothesis that a CBC analysis would differentiate species in a morphologically equivocal insect taxon, the beetle genus *Altica*.

The Evening Primrose Beetle (*Altica litigata*) (Coleoptera: Chrysomelidae) is an established landscape and nursery pest that has been reported to aggregately feed on Crape Myrtle (non-indigenous North American ornamental in the family Lythraceae) and Primrose (indigenous North American ornamental in the family Onagraceae) plant families (Pettis and Braman, 2007). The entire life cycle of *A. litigata* has been described on Primrose. Only adult beetles have been found on Crape Myrtle (*Lagerstroemia* spp.). These are assumed to migrate from Primrose to Crape Myrtle in

late spring/early summer to feed (Pettis et al., 2004). Recent sequence data contradicted this assumption and revealed that flea beetles collected from Crape Myrtle and Primrose were not the same *Altica* species (Jenkins et al., 2009a; L. LeSage, Ontario Canada, personal communication). A definitive morphological identification necessitates, therefore, examination of the type specimen.

## 1.1. Morphology

Correct insect taxonomy is crucial to understanding the interactions between the insect herbivore and its host plant. Flea beetle species in the genus *Altica* are morphologically indistinct. Accurate species identification has been challenging (Fernandez and Hilker, 2006; Phillips, 1979) and, therefore, host-insect behavior difficult to study, particularly since many *Altica* species are polyphagous with multiple species feeding on the same plant at the same time (Jenkins et al., 2009a).

The adult male aedeagus (intromittent organ) is commonly used to identify species within the genus *Altica* (Laroche et al., 1996; LeSage, 1995). Scanning electron microscope (SEM) data from this study showed that the aedeagus of *A. litigata* and *Altica* sp. were morphologically distinct from each other (Fig. 3). This character, however, has been reported to be unreliable due to intraspecific variation, parthenogenetic reproduction, and unrecognized sibling species (Laroche et al., 1996; Phillips, 1979). A DNA molecular marker that is reliable, inexpensive, and provides relatively fast results would be useful for verification of species, especially in the genus *Altica*.

\* Corresponding author. Address: Department of Entomology, University of Georgia, Griffin Campus, 1109 Experiment Street, Griffin, GA 30223, USA. Fax: +1 011 770 228 7287.

E-mail addresses: [asystole@uga.edu](mailto:asystole@uga.edu) (M.W. Ruhl), [matthias.wolf@biozentrum.uni-wuerzburg.de](mailto:matthias.wolf@biozentrum.uni-wuerzburg.de) (M. Wolf), [jenkinst@uga.edu](mailto:jenkinst@uga.edu) (T.M. Jenkins).

### 1.2. ITS2 structure

The ITS2 intergenic sequence is located between the 5.8S and 28S ribosomal genes. It can evolve 30× slower (Caccone et al., 2004) than mitochondrial DNA (mtDNA) and has been shown to be an important part of ribogenesis (Joseph et al., 1999). Despite variability in ITS2 sequence and length, its rRNA secondary transcript structure contains a highly conserved core (Coleman, 2003; Schultz et al., 2005). This highly conserved core is common to all eukaryotes and should be useful for evaluating the relationships of closely related organisms (Coleman, 2007, 2009; Schultz et al., 2006). Moreover, a CBC between two closely related species may infer that they can no longer successfully intercross (Muller et al., 2007).

### 1.3. Sequence homogenization

Jenkins et al. (2009a) showed that for species in three beetle genera (*Altica*, *Lysathia*, and *Phaedon*) the rDNA sequence array (18S partial, ITS1, 5.8S, ITS2, and 28S partial) was not only species-specific but homogenized within a species. Could this homogenization of the rDNA array translate into a CBC within one of the conserved helices of the ITS2 secondary transcript structure between *A. litigata* and *Altica* sp., as in Muller et al. (2007)? The purpose of our research is to answer this question.

## 2. Materials and methods

### 2.1. 5.8S–28S rRNA interaction and HMM-based ITS2 annotation

All ITS2 sequences from Jenkins et al. (2009a,b), unique to this study, or accessed from GenBank (Table 1) were annotated by identifying a 25 nucleotide interaction of the 5' 5.8S rDNA subunit end with 25 nucleotides of the 28S rDNA subunit 3' end (Keller et al., 2009). Sequences without this interaction were discarded as they are either incomplete, contain errors, or lacked the required 25 nucleotides on each end for the interaction to occur.

The National Center for Biotechnology Information (NCBI) database (Benson et al., 2008) was queried for sequences containing the 5.8S (partial or complete), internal transcribed spacer 2 (ITS2 complete), and 28S (partial or complete). All sequences from the Family Chrysomelidae meeting this criterion (Table 1) were analyzed for the 5.8S–28S rRNA gene interaction. Sequences that had the interaction were included in this study.

**Table 1**

ITS2 species data (Coleoptera: Chrysomelidae: Galerucinae) including GenBank No., species, tribe and presence or absence of helix IV. GQ325432, GQ325433, EU682395, EU682396, EU682397 sequence each represent a consensus haplotype since the ITS2 is homogenized within species (refer to text).

GenBank No.	Species <sup>a</sup>	Tribe	Helix IV	Total CBCs between <i>A. litigata</i> & species listed
GQ325432	<i>Altica corni</i>	Alticini	No	4
GQ325433	<i>Altica chalybea</i>	Alticini	No	3
EU682395	<i>Altica litigata</i>	Alticini	No	0
EU682396	<i>Altica</i> species	Alticini	No	4
EU682397	<i>Lysathia ludoviciana</i>	Alticini	No	6
EU110868	<i>Psylliodes crambicola</i>	Alticini	No	5
AY116107	<i>Lema</i> sp.	Lemini	Yes	8
AF278565	<i>Diabrotica virgifera zea</i>	Luperini	No	15
AF278564	<i>Diabrotica virgifera virgifera</i>	Luperini	No	17
AJ622022	<i>Timarcha rugulosa</i>	Timarchini	No	3

<sup>a</sup> Species used in this study accessed from GenBank 8/2008 to 6/2009.

### 2.2. DNA extraction, polymerase chain reaction (PCR), and sequencing

A total of 129 individual *Altica* samples were analyzed for ITS2 sequences. Specimens from previous studies totaled 119 (Jenkins et al., 2009a,b). New DNA samples were extracted from an additional 10 individuals according to Jenkins et al. (2007). These include three *A. litigata* collected in Tifton, GA by Will Hudson, four *Altica corni* collected in Quebec, Canada by Laurent LeSage, two *Altica chalybea* museum specimens collected in Quebec, Canada and mounted by Laurent LeSage, and one *Altica chalybea* collected in Griffin, GA by Tyler D. Eaton.

ITS2 fragments were amplified according to Jenkins et al. (2007) using novel primers anchored in the 5.8S (FB5.8SFW: CTGACCA CTCCTGGCT) and 28S rDNA region (FB28SREV: GGTAGTCTCA CCTGCTCTG).

Amplified ITS2 DNA fragments were sequenced in both directions by the Sequencing and Synthesis Facility (SSF) at MWG (High Point, NC). Sequences were verified for accuracy using Sequencher v.3.1.1 (Gene Codes Corp., Ann Arbor, MI) software. *A. litigata* ITS2 rDNA fragments from this study were 100% identical to the ITS2 rDNA fragments in Jenkins et al. (2009a) and represented a single consensus sequence. New sequences of *A. corni*, four contigs, and *A. chalybea*, three contigs, were merged into species-specific consensus sequences.

### 2.3. Helix IV query

GenBank was queried with the search string “Coleoptera Internal Transcribed Spacer 2 Complete” on June 22, 2009. The query produced 709 sequences. Each sequence was individually analyzed to verify it contained a complete ITS2 fragment utilizing the “Annotate” feature (default settings) on the ITS2 website (<http://its2.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/index.pl?annotator>). If neither the 5.8S rDNA nor 28S rDNA ends were identified, the annotate process was repeated with the “Analyze reverse complement (exclusively)” option enabled. Each complete ITS2 fragment was then folded using RNAstructure (Mathews et al., 2004) (default settings at 37 °C) with default settings and analyzed for the presence of helix IV. The purpose of analyzing all Coleoptera was to establish whether the reduction of helix IV, seen in *Altica*, was an order or family level characteristic.

### 2.4. Homology modeling

ITS2 rDNA secondary structures of *A. litigata* (GenBank No. EU682395) were folded using RNAstructure (Mathews et al., 2004) (default settings at 37 °C). All obtained secondary structures, energetically optimal and suboptimal ones, all against all, were tested for homology modeling (Schultz et al., 2006; Selig et al., 2008; Wolf et al., 2005a) with the ‘model’ program on the ITS2 website (refer to Section 2.3). *A. litigata* was chosen for the homology model because it had a close genetic relationship with our unknown beetle.

Homology modeling using *A. litigata* template structures was performed on all ITS2 sequences in Table 1 (set to “Identity” matrix with a threshold value >50%, gap costs: gap open 15, gap extension 2). Sequences with structure information were saved in extended FASTA format. Mean helix variance among *Altica* sequences was calculated (standard deviation (SD) calculated using the formula:

$SD = \sqrt{[\sum(S - M)^2 / (n - 1)]}$ . Species outside the genus *Altica* (Table 1) were chosen at random and utilized as outgroups in ITS2 and CBC tree construction.

### 2.5. 4SALE alignment and CBC analysis

The ITS2 sequences with homologous structures were synchronously aligned using 4SALE (Seibel et al., 2006, 2008). CBCAnalyzer (Wolf et al., 2005b) was used to confirm initial results produced by the CBC Matrix feature in 4SALE. An analysis of hemi-CBCs was done using CBCAnalyzer version 1.1. The alignment with structural information was exported. The resultant filename was changed to a filename with the extension “.xfasta” (Schultz and Wolf, 2009).

### 2.6. Phylogenetic analysis using ProfDistS

The alignment output file, with the ‘.xfasta’ extension, from 4SALE was imported into ProfDistS (Friedrich et al., 2005; Wolf et al., 2008). “RNA/DNA structure Profile Neighbor-Joining” was selected from the “Run” menu (Bootstraps = 1000, Distance Correction Model = General Time Reversible, Ratematrix Q = Q\_ITS2.txt (an ITS2 specific substitution model included as a supplemental file in ProfDistS)). The resultant tree file with node strengths were viewed in ProfDistS and then visualized and reproduced in Dendroscope (Huson et al., 2007) (annotated in Microsoft PowerPoint).

### 2.7. Morphological characterization

Each species’ aedeagus was photographed by scanning electron microscopy (SEM) using the Zeiss 1450EP variable pressure SEM (Carl Zeiss MicroImaging, Inc. One Zeiss Drive, Thornwood, NY 10594, and Oxford Instruments X-Ray Technology, Inc., 275 Technology Circle, Scotts Valley, CA 95066). Specimens were first photographed in variable pressure mode and then coated in a 2-micron layer of gold using the SPI Module Sputter Coater (Structure Probe, Inc., 569 East Gay Street, West Chester, PA 19380). Coated specimens were photographed in high vacuum (2600 Pa) mode.

The length and width of each aedeagus was digitally measured from seven *A. litigata* and six *Altica* species using the measurement tool within the Zeiss 1450EP SEM operating program. The mean length and width was calculated for each species and standard deviations were calculated per Section 2.4.

## 3. Results

### 3.1. 5.8S–28S rRNA interaction and HMM-based ITS2 annotation

The presence of a 5.8S–28S rRNA gene interaction is evidenced by the ability to produce an HMM-based ITS2 annotation (not shown). Since ITS2 sequence was homogenized across all individuals within a species, annotated ITS2 sequences were deposited in GenBank under the accession numbers *A. corni* (GQ325432), *Altica chalybea* (GQ325433), *A. litigata*, haplotype ITSB (EU682395), *Altica* sp. haplotype ITSC (EU682396), and *Lysathia ludoviciana*, haplotype ITSA (EU682397).

### 3.2. DNA extraction, polymerase chain reaction (PCR), and sequencing

The primer pair FB5.8SFW and FB28SREV effectively amplified the entire ITS2 sequence with a minimum of 25 nucleotides from the 5’ 5.8S rDNA and 25 nucleotides from the 3’ 28S rDNA regions.

### 3.3. Helix IV query

Of 709 GenBank sequences matching the query, all of which were analyzed for the presence of helix IV, only nine contained helix IV. Of the nine, six sequences were Chrysomelid beetles, two were Curculionid beetles, and one was Hymenopteran (a parasitoid

wasp of *Altica*). One of the Chrysomelids, *Lema* sp. (AY116107), containing helix IV is used in this study (Table 1).

### 3.4. Homology modeling

The most parsimonious *A. litigata* template structure, determined by the homology modeling program (Schultz et al., 2006; Selig et al., 2008; Wolf et al., 2005a), was used for homology modeling. This structure was the fourth lowest energy structure with dG-79.10. Homology modeling produced a high mean structural identity of 98.41667% (SD = 2.90637%) considering all helices of the genera *Altica* in this study. Even when the homology modeling of all Chrysomelid species (Table 1) in this study were considered, there was a high mean structural identity of 88.35714% (SD = 13.95552%) observed.

### 3.5. 4SALE alignment and CBC analysis

Aligned sequences produced a phylogenetic tree (Fig. 1) that separated the *Altica* beetle species in concordance to the phylogenetic tree shown in Jenkins et al. (2009a). It also separated all Chrysomelid species used in this study according to genera (Fig. 1, Table 1). As hypothesized, CBC analysis (Fig. 2) using novel software (Keller et al., 2009; Seibel et al., 2008) revealed the presence of CBCs between *A. litigata* and *Altica* sp. that distinguishes *Altica* species.

CBC Analysis located one CBC in the third helix and three additional hemi-CBCs, also in the third helix, between *A. litigata* and *Altica* sp. (Fig. 2). CBCs were also present between all four *Altica* species represented in the study (Table 1). In this study, only *Lema* sp. of the Lemini tribe had helix IV (Table 1).

### 3.6. Phylogenetic analysis using ProfDistS

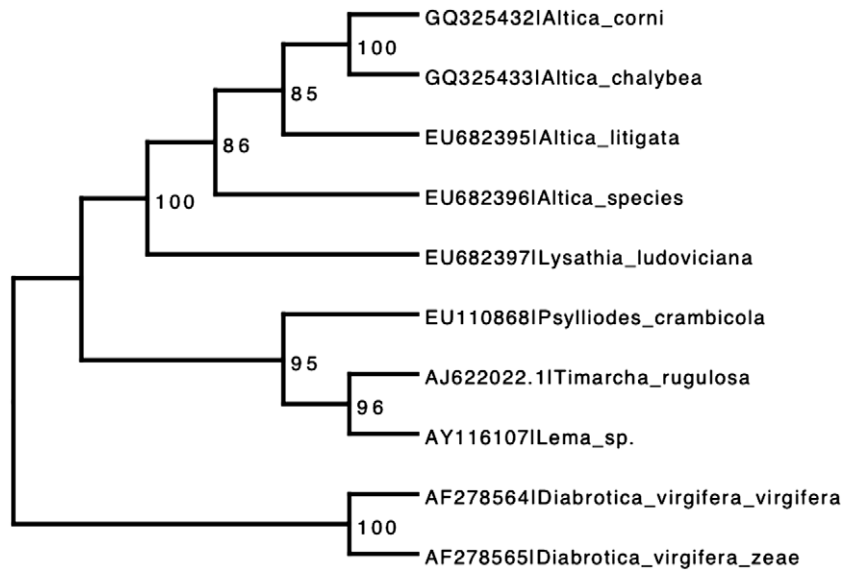
Tree topology from the phylogenetic analysis using synchronous ITS2 secondary structure and sequence separated the *Altica* clade from other genera in this study with a bootstrap value of 86. A well-supported node strength (bootstrap = 85) also separated the species *A. litigata*, *A. chalybea* and *A. corni* from *Altica* sp. (Fig. 1).

### 3.7. Morphological characterization

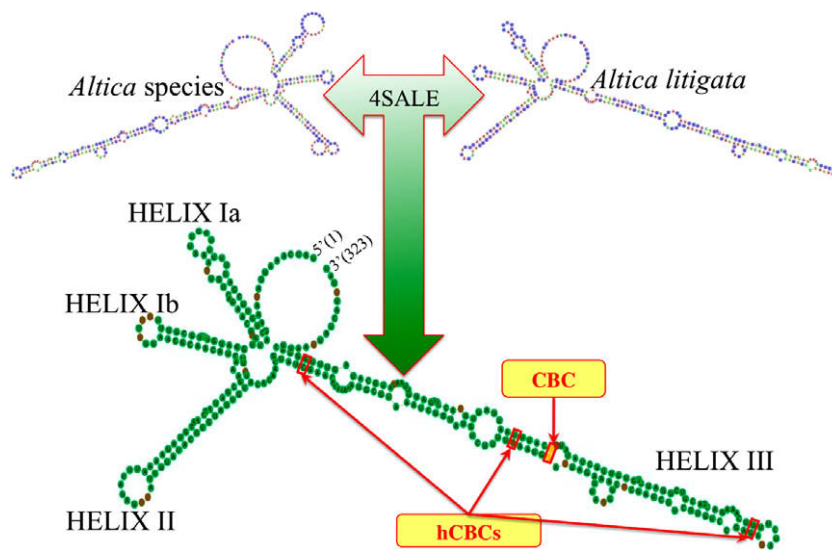
The aedeagus of *A. litigata* was distinctly different in shape, length, and width in comparison to *Altica* sp. (Fig. 3). Mean (M) length of *A. litigata* was  $M = 1.81329$  mm (SD = 0.07695 mm) and width  $M = 375.61429$   $\mu$ m (SD = 15.50607  $\mu$ m). Mean length of *Altica* sp.  $M = 1.26833$  mm (SD = 0.03549 mm) and width  $M = 305.51667$   $\mu$ m (SD = 10.30602  $\mu$ m).

## 4. Discussion

It is important to note that the CBC criterion usually works in only one direction, for example, if there is a CBC then there are two species; if there is no CBC there still could be two species. Muller et al. (2007) noted intraspecific CBCs might occur in 5.25% of samples within a CBC analysis. This may occur when many individuals from a diverse population are studied. In this study, 129 individuals were examined and no intraspecific variation was seen. All specimens from a single species were observed to have no sequence variation regardless of geographic origin. The homogenization (Jenkins et al., 2009a) of the ITS array in *Altica* translated into one complete CBC as well as three hCBCs within the conserved helix III (Fig. 2) of the ITS2 secondary transcript structure. This result was also concordant with the ITS2 sequence/structure phylogeny



**Fig. 1.** Profile Neighbor-Joining (PNJ) tree obtained by ProfDistS, synchronously calculated on ITS2 sequence and secondary structure information, using an ITS2 specific general time reversible (GTR) substitution model. Bootstrap support values from 1000 pseudo-replicates.

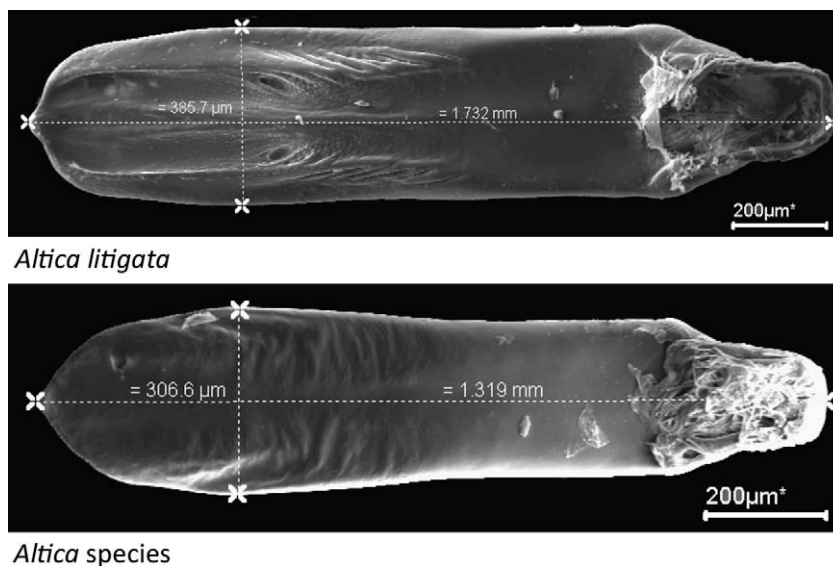


**Fig. 2.** Analysis showing presence of compensatory base changes (CBCs) in conserved helix III of the internal transcribed spacer 2 (ITS2) rDNA secondary transcript structure. Lower structure is the ITS2 consensus structure of *Altica litigata* and *Altica species*. Green areas represent highly conserved base pairs. One significant CBC was found in the conserved helix III. Three additional hemi-CBCs were found within the conserved helix III, thus, providing further evidence for distinguishing species.

(Fig. 1) and the SEM morphological data sets (Table 2), which attest to the strength of the CBC analysis.

The ITS2 sequences with their homology-based structure must be synchronously aligned if ITS2 CBC analysis is to be effectively applied. When ITS2 sequence alone is used, as in DNA barcoding of ITS2 sequences, nucleotide changes in conserved pairing positions that comprise a CBC are unaccounted for. Therefore, no assumption can be made of the biological relationship between species analyzed with DNA barcoding. However, a CBC analysis between species may illuminate the biological relationship. It is also important to understand that the ITS2 sequence contains more variability than its associated secondary transcript structure. Therefore, a CBC analysis within the ITS2 structure should contain less intraspecific variation than DNA barcoding while providing increased confidence in delimiting biological species.

The ITS2 helix IV was not observed in most of the beetles in this study (Table 1). This does not appear to be an Order or Family level characteristic as the helix IV was observed in a few species within Chrysomelidae including three from the tribe Alticini, one from the tribe Lemini (used in this study), and two from the tribe Bruchini. The etiology of the fourth helix reduction is not clear, nor is the role, if any, of helix IV in biogenesis. What is clear is that biogenesis still occurs. Interestingly most beetles without a helix IV in the rDNA secondary transcript structure were observed to have additional helices located between helices III and I. Four helices were therefore spatially maintained within the structure. This tendency toward four helices may have implications for the binding of proteins integral to ribosome biogenesis. The region of helix IV could be a target site for natural selection. As the adaptive needs of a beetle change natural selection may act within the area of helix IV to facilitate a change in the direction of a more defined helix. This



**Fig. 3.** Male intromittent organ (aedeagus). Scanning electron microscopy (SEM) examination revealed morphological differences (shape, mean length, and mean width) between *Altica litigata* and *Altica* species. Shown are representative samples from that study (unpublished data). Both aedeagus in this figure, and those listed in Table 2, were taken from specimens identified as *Altica litigata* by external morphology.

**Table 2**

Aedeagus<sup>a</sup> length (mm) and width (μm) morphometrics for *Altica litigata* and *Altica* species (Refer to Fig. 3).

<i>Altica litigata</i>		<i>Altica</i> species	
Length (mm)	Width (μm)	Length (mm)	Width (μm)
1.787	367.5	1.319	306.6
1.949	373.7	1.228	320.7
1.872	349.3	1.269	306.5
1.831	383.7	1.253	306.5
1.744	397.9	1.240	288.3
1.732	385.7	1.301	304.5
1.778	371.5		

<sup>a</sup> The aedeagus from *Altica litigata* and *Altica* species were digitally measured from seven *A. litigata* and six *Altica* sp. specimens using the measurement tool within the Zeiss 1450EP SEM operating program (Refer to text).

change may serve to more efficiently recruit non-ribosomal protein factors. Like previous work which suggest a framework for studying ribosomal biogenesis in yeast (Côté et al., 2002), this research may offer a framework from which to study ribosomal biogenesis in insects.

The significance of this study to species determination is three-fold. First, CBC analysis is capable of distinguishing morphologically indistinguishable eukaryotes. The CBC process is relatively quick (with known ITS2 sequences it takes less than two hours), inexpensive and the tools are available online for free. SEM morphological analysis of the aedeagus from 13 specimens (two species) cost<sup>1</sup> approximately \$480 USD. The same project at UGA commercial rates would have cost approximately \$1550 USD.

In contrast, the total cost for a CBC analysis of the same 13 specimens was approximately \$315<sup>2</sup>. Also, since processing DNA for DNA barcoding is essentially the same (DNA Barcoding, 2006), the cost difference between DNA barcoding and CBC analysis is mar-

ginal. However, DNA barcoding is not as reliable when using a single genetic locus (Spooner, 2009). Analyzing multiple loci for DNA barcoding is therefore more costly than CBC analysis. Therefore, the ability to use a single locus to identify species, as shown in this study, could represent significant cost savings.

Secondly, this study showed the value of CBC analyses for insects: within species, between species, and between genera. CBC analysis was species-specific. It differentiated two morphologically indistinguishable species sympatric across four ecoregions.<sup>3</sup> This research further demonstrates the possible universality of CBC analysis in eukaryotic organisms.

Lastly, based on this study and sequence data from Jenkins et al. (2009a,b), it is clear that, although the gross morphology is indistinguishable, *A. litigata* and *Altica* sp. are separate species that were easily differentiated by CBC analysis.

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<sup>1</sup> Incurred cost estimate based on non-UGA rate for academic purposes. Fees are accurate as of 6/29/2009 as noted by the University of Georgia Center for Advanced Ultrastructural Research lab (<http://www.uga.edu/caur/equipment/fees.htm>).

<sup>2</sup> Technician time at \$20/h. Four technician hours to process DNA, \$15/specimen for DNA extraction, PCR (including primers), and sequencing, and two technician hours to perform computerized CBC analysis.

<sup>3</sup> Collections from four ecoregions (Southeastern Plains, Southern Coastal Plains, Interior Plateau, and Piedmont) across four US southern states (Louisiana, Alabama, Mississippi, and Georgia) (Jenkins et al., 2007; 2009a,b). Ecoregion map is available from <ftp://ftp.epa.gov/wed/ecoregions/us/useco.pdf>. We also added 4 *Altica litigata* specimens from *Oenothera* bordering a Pecan orchard in Tifton, GA (a Southeastern Plains ecoregion).

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