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A Phylogenetic Analysis of the Round-headed Katydids (Tettigoniidae: *Amblycorypha*)

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Abstract

The evolution of song complexity in relation to speciation in the round-headed katydids (*Amblycorypha*) has yet to be studied. This research aims to generate a dataset of comparable genetic markers among the four identified species within the *Amblycorypha rotundifolia* complex and several other undescribed song morphs (probable species) within the complex to generate a robust phylogeny of the group. The study aims to present a phylogenetic estimate of the *Amblycorypha rotundifolia* complex using sequences from 18S, 5.8S, and 28S rDNA, and the internal transcribed spacers (ITS) 1 and 2. The four identified species are *A. rotundifolia, A. alexanderi, A. bartrami,* and *A. parvipennis*. Although all eastern species within the *rotundifolia* complex are morphologically identical, they are readily distinguishable by their individual calling songs. Differences in mating signals are extremely important for locating a mate and for correct pair formation. Thus it is hypothesized that new song morphs are undescribed species. Using standard molecular techniques the nucleotide sequences of the ribosomal DNA cistron ITS1-5.8S-ITS2 (Figure 1) were compared for all samples. A species in a different genus, *Scudderia furcata*, was used as an outgroup. Preliminary data shows greater interspecific variation than intraspecific, providing method confirmation. PCR products from numerous individuals representative of all four complexes, plus outgroups, await cloning and sequencing.

1. Introduction

Acoustic signals in katydids are produced by stridulation. This involves rubbing a scraper across the stridulatory file on the wings to produce a variety of simple to more complex calls. The evolution of acoustic signal complexity is driven by predator avoidance and mate attraction, with these two forces often in direct opposition to one another.[1] Male katydids in the genus *Amblycorypha* exhibit complex acoustic signals when calling for mates.[2] Signal complexity in this genus is highly variable among species, and ranges from simple, repeated single sound units to the songs of the virtuoso katydids, which produce multiple sound units in various syntaxes that may not repeat for more than 50 seconds. The *rotundifolia* complex within *Amblycorypha* currently has four identified species that are flightless.[3] Recordings obtained of males from several populations of what were believed to be *A. alexanderi* and *A. rotundifolia* showed significant differences in song complexity indicating new and unidentified species of *Amblycorypha*.

Within the katydids (Tettigoniidae), groups that mimic leaves or twigs tend to have the greatest species diversity; a correlation that may be due to protection from predation. The phaneropterine katydids exhibit the most diversity with more than 2000 species worldwide.[4] Their extensive diversity is probably tied to their leaf mimicry and unique pair forming mating system that involves duetting. Phaneropterine katydids in the genus *Amblycorypha* show large variation in song complexity.[3] There are three species complexes within the genus. Species in the

oblongifolia complex have simple songs, species in the *rotundifolia* complex have simple or multi-component songs, and species in the *uhleri* complex have the most complex songs composed of four distinct parts.[5] The *rotundifolia* complex studied here can have either simple or multi-component songs. The number of species within the *rotundifolia* complex is currently undetermined because several new song morphs have recently been discovered. We see in *Amblycorypha*, as with other Orthoptera (crickets, katydids and grasshoppers), strong reproductive isolation based on differences in the temporal patterns of the songs. This suggests that calling patterns create reproductive isolation, therefore possible speciation events.[6] We hypothesize that the new song morphs may be positively correlated with speciation events within the *Amblycorypha*

To determine the phylogenetic relationship among different taxa, scientists sequence and compare stretches of the genome that encode for the ribosomal subunits used in protein synthesis.[7] This technique has been used for understanding family-level relationships within the Orthoptera.[8] The DNA coding for the ribosomal subunits are typically highly conserved, therefore differences in the sequences provide information about relationships of taxa that diverged in the distant past. The internal transcribed spacers (ITS1 and ITS2) are tandem repeats of a DNA sequence that occur between the genes that code for the ribosomal subunits.[9] While the genes coding for the ribosomal subunits are relatively conserved, the internal transcribed spacers exhibit more variation and are therefore important for examining relationships of taxa that have diverged relatively recently, e.g. species level relationships. The ITS1-2, with the interior 5.8S region, has been used by numerous other studies to compare genetic variability and relatedness.[10] Similar studies using ITS1-2 have been conducted examining *Phasmatinae*,[11] *Blattodea*,[12] *Orthoptera*[10,13]. Using the ITS1-5.8S-ITS2 cistron as a comparative tool, my study aims to determine the relationship among the song morphs within the *rotudifolia* complex. In addition, these data will be important in understanding the relationships among the different species complexes and evolution of signal complexity in the *Amblycorypha*.

2. Methods

2.1 Katydid collection

Katydids were collected in 21 counties in 8 states along the southeastern seaboard of the United States during 2006-2012. The hind femur from each sample was removed and frozen at -80°C for long-term storage.

2.2 DNA extraction

The Qiagen DNeasy Kit was used for DNA extraction. The proximal half of a frozen katydid femur was removed with a sterile scalpel blade, placed on a sterile microscope slide, cut into small pieces and placed into a sterile 1.5 mL microtube. The tissue was homogenized with a sterile p-1000 tip after the addition of 180 uL of Buffer ATL, disrupting the tissue structure. To disrupt protein structure, 20 uL of proteinase K was added to the suspension, vortexed, and incubated at 56°C for 45 minutes. The sample was again vortexed for 15 seconds, 200 uL of Buffer AL added, and vortexed. To clean up the DNA extract, 200 uL of 100% EtOH was added to the sample and vortex mixed again. The mixture was added to a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 minute. The flow-through was discarded. With a new collection tube, 500 uL of Buffer AW1 was added to the column and centrifuged at 8000 rpm for 1 minute; flow-through discarded. With a new collection tube, 500 uL of Buffer AW2 was added to the column and centrifuged at 14000 rpm for 3 minutes; flow-through discarded. The spin column was placed into a sterile 1.5 ml microtube, 200 uL of elution buffer (AE) added (used to move the DNA extract through the filter), incubated at room temperature for 1 minute, and centrifuged for 1 minute at 8000 rpm. Two 100 uL aliquots were made: one stored at -20°C and the other stored at -80°C. A Nanodrop spectrometer was used to evaluate the quantity and quality of the DNA extraction.

2.3 DNA amplification

The ribosomal DNA internal transcribed spacers (ITS1 and ITS2) and the 5.8S region were amplified with primers White 1 and White 4, respectively. These primers anneal to conserved regions of flanking 18S and 28S genes (Figure 1).[9] The polymerase chain reaction (PCR) was used to amplify the ITS1 and ITS2 regions and the 5.8s

ribosomal gene. The rRNA-encoding genes include both conserved and non-conserved regions that can be used to recover phylogenetic relationships at multiple levels. Thermal cycler conditions used were as follows: initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation (95°C, 1 minute), annealing (51°C, 1 minute), and extension (72°C, 1 minute and 45 seconds) and a final extension at 72°C for 10 minutes. Infinity hold of 4°C. Products were viewed using gel electrophoresis (1% agarose) and ethidium bromide (EtBr) to confirm the presence of one band at approximately 1100 base pairs, which was determined from preliminary research.[14] (Figure 2).

We amplified over 100 samples including 66 individuals in the *rotundifolia* complex (12 *rotundifolia*, 8 *alexanderi*, 15 *bartrami*, 4 *parvipennis*, and 38 undescribed), 15 individuals in the *oblongifolia* complex (4 *oblongifolia*, 6 *carinata*, and 5 *floridana*), 18 individuals in the *uhleri* complex (2 *cajuni*, 8 *longinicta*, and 8 *arenicola*), and specimens from two other katydid genera, *Atlanticus* and *Scudderia.*

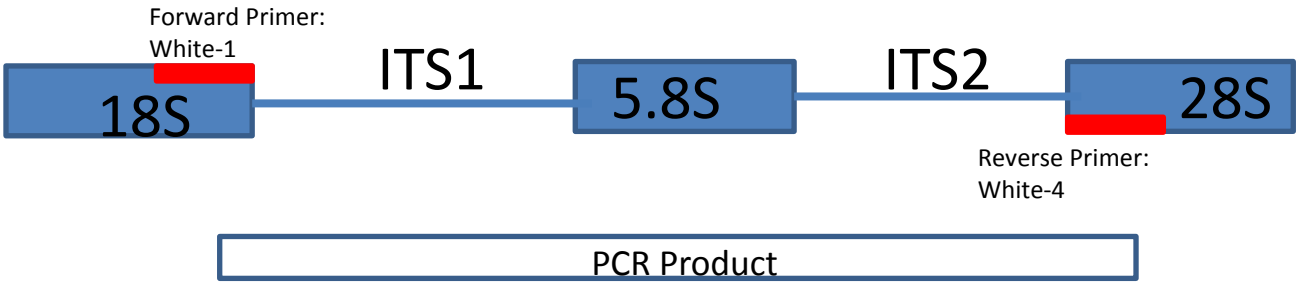

Figure 1. Ribosomal DNA cistron.

Figure 1 Ribosomal DNA cistron containing the regions 18S, internal transcribed spacer 1 (ITS1), 5.8S, internal transcribed spacer 2 (ITS2), and 28S. The primers (red bands) are White-1 and White-4 and correspond to sequences found at the ends of 18S and 28S, respectively. The 18S, 5.8S and 28S rDNA units are highly conserved, whereas ITS1 and ITS2 are hypervariable.

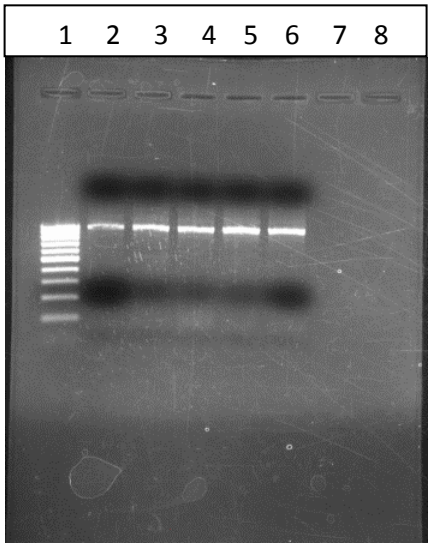

Figure 2. Agarose gel with PCR product.

Figure 2 Agarose gel (1%) stained with ethidium bromide (EtBr), run 16 Aug 2012. Shows desired single bands at ~1100 bp for all five samples loaded. From left to right: (1) 1kbp ladder, (2) MS_093_12, (3) MS_094_12, (4) MS_095_12, (5) MS_096_12, (6) MS_097_12, (7) no sample, (8) no sample.

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2.4 DNA cloning and sequencing

A cloning reaction, using Invitrogen TOPO-TA cloning kit, was set up in the following order: 4uL of fresh PCR product, 1 uL salt solution, and 1 uL TOPO vector. The reaction was mixed gently and incubated at room temperature for 20 minutes. This reaction was either used immediately in the subsequent steps, or frozen at -20°C for use the following day.

After an incubation period of 20 minutes at room temperature, 2 uL of TOPO cloning reaction was added to thawed competent *E.coli* cells and mixed gently. To facilitate uptake of the plasmid into the *E. coli* competent cells, the reaction was incubated on ice for 30 minutes, heat-shocked for 30 seconds at 42°C without shaking, and immediately transferred to ice. Off of ice, 250 uL of room temperature SOC medium was added to stabilize the competent cells, the tube capped tightly, and shaken horizontally at 200 rpm at 37°C for 1 hour. Each transformant was spread at two different volumes; usually one with 10% of available reaction mix and the other with the remaining mix, on pre-warmed ampicillin selective plates and incubated overnight at 37°C. Up to 10 colonies were picked for analysis. The resulting plasmid DNA samples were purified using Promega, PureYield Plasmid Miniprep system.

BLASTN, a program used to search nucleotide databases using nucleotide query results, was run with data sequenced from *Scudderia furcata*, which served as the outgroup.

3. Results

The results of the BLASTN search confirms our PCR strategy by showing that the 5.8s region is conserved across broad taxonomic groups within the insects including grasshoppers (*Chorthippus* and *Oxya*), roaches (*Periplaneta*, *Parcoblatta* and *Blatella*) and wasps (*Trichogramma* and *Dilophotopsis*), and that there is large variation in both ITS1 and ITS2 (Figure 3).

The nucleotide sequences isolated from the ITS1, ITS2 and 5.8S regions were subjected to traditional approaches for estimating phylogenetic relationships among members of the *Amblycorypha*. Outgroups for comparisons included members of *Scudderia*, a genus in the same subfamily as *Amblycorypha*. Currently we have data from two pairs of cryptic species (*A. carinata* & *A. floridana* and A. *rotundifolia* & *A. alexanderi)*. The sequences are varied enough to distinguish both cryptic pairs. Differences of 5-6% occurred between sequences of *Amblycorypha* and *Scudderia*, the closest outgroup (Table 1). The DNA sequences (~1100 bp) for ITS1 differed on average by 1.5% of the base pairs (0.5% between *A. rotundifolia* and *A. alexanderi* ; 2.8% between *A. floridana* and A. *carinata*) and for ITS2 differed by about 2% of the base pairs (2.1% between *A. rotundifolia* and *A. alexanderi* ; 3.2% between *A. floridana* and A. *carinata*). Those differences were greater than the 0.5% differences for within species comparisons (Figure 4).

Significantly more data are in the process of being generated. Approximately 100 samples have been extracted, subjected to the PCR, and are currently in storage awaiting fresh competent cells for cloning. Once these samples have been sequenced and the relationships among all of the groups determined, we will have a phylogeny that will provide information on the evolution of signal complexity in this genus.

Table 1. Pairwise distance calculation modeled on the number of nucleotide differences between the ITS1-5.8S-ITS2 cistron (seen in Figure 1).

Individual	**Species, Sex**	1	2	3	4	5	6	7
1	*A. oblongifolia*, female							
2	*A. oblongifolia*, female	0.005						
3	*A. oblongifolia*, male	0.005	0.004					
4	*A. carinata*, female	0.009	0.008	0.008				
5	*A. rotundifolia*, male	0.008	0.007	0.007	0.012			
6	*A. alexanderii*, male	0.014	0.013	0.013	0.015	0.012		
7	*A. floridana*, male	0.027	0.026	0.026	0.031	0.025	0.026	
8	*S. furcata*, male	0.051	0.047	0.047	0.053	0.048	0.047	0.061

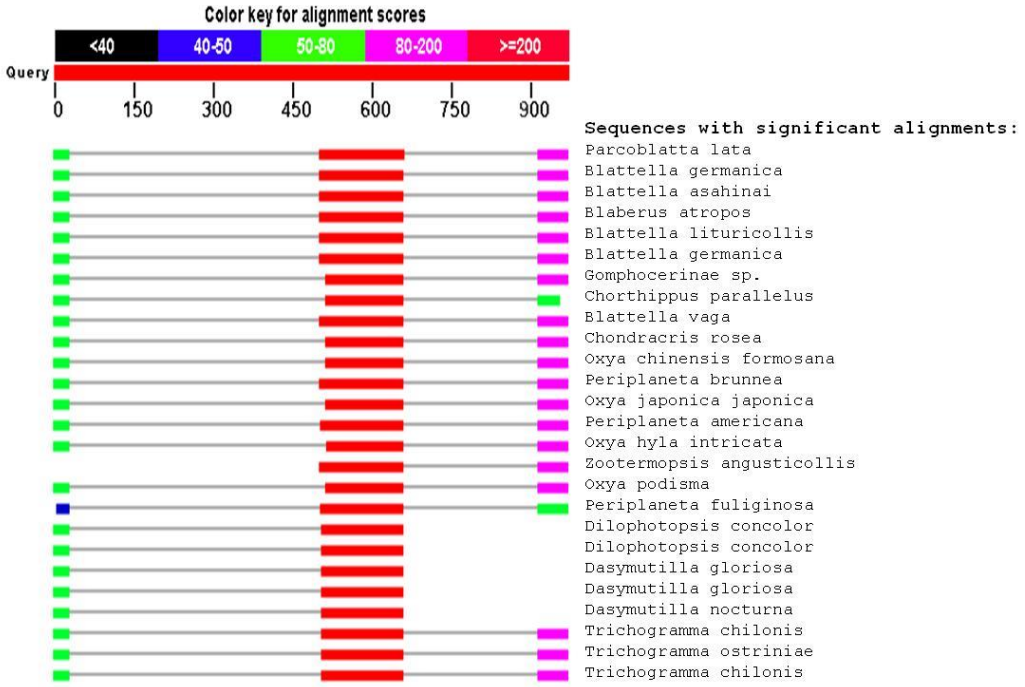

Figure 3. BLASTN comparison

Figure 3 BLASTN sequence comparison for *Scudderia furcata*, with each line representing alignment with the species listed on the right. Alignment scores are indicated by colored bar at the top. From left to right: the left most section (mostly green) identifies a portion of 18S region, the grey shows ITS1, the red shows 5.8S, the second grey section shows ITS2, and the right most section (pink and green) are alignments within the 28S region. Alignments were found between *S. furcata* and numerous other species including *Blattella sp.* (cockroaches), *Dilophotopsis sp.* (velvet ants) and *Trichogramma sp.* (stingless wasps). The almost perfect alignment and identical matching across the 5.8S region (red) confirms our PCR strategy that uses 5.8S as a sequencing checkpoint.

	ITS1	ITS2
Amblycorypha rotundifolia		
Amblycorypha alexanderi	0.5%	2.1%
Amblycorypha floridana		
Amblycorypha carinata	2.8%	3.2%
Amblycorypha **(interspecies)**	1.5%	2.1%
Amblycorypha oblongifolia **(intraspecies)**	0.50%	0.54%

Figure 4. Inter- and intra-specific comparisons of *Amblycorypha*.

Figure 4 Percent differences in number of nucleotides in ITS1 and ITS2 for cryptic species pairs (*A. rotundifolia* vs *A. alexanderi; A. floridana* vs *A. carinata*), interspecific average differences within *Amblycorypha*, and intraspecific differences within *A. oblongifolia*.

4. Discussion

4.1 Related studies

The rDNA ITS1-2 region has been used to compare variability and relatedness among arthropod taxa by numerous other studies.[10] Genetic data on Phasmatinae have been generated that support the grouping of individuals based on their geography over their morphology,[11] meaning that less variation within groups was observed when individuals were grouped based on geographical location rather than morphological characteristics. This is of particular interest to our study of song morphs residing in close, yet distinct geographical locations, as it lends credence to our hypothesis that different song morphs, found in discrete locations, have undergone speciation. Studies particular to Orthoptera have been conducted using ITS1-2 to infer phylogenetic relationships. These studies have used both mitochondrial and nuclear genetic data, and found both to be precise and reliable (mDNA and nDNA report similar results over repeated trials).[10] Cryptic (indistinguishable morphologically) arthropods including *Sphaeropthalma* (velvet ant), *Aphelinus varipes* (parasitoid wasp), and *Aceria tosichella* (wheat curl mite) have been genetically investigated using the ITS1-2 cistron with great success.[15-17] Geographical differences in temperature and precipitation appear to play a role in speciation of some cryptic species,[15] a correlation which may be useful to this study when comparing ITS1 and ITS2 genetic results with the geographical locations where specimens were collected. *Trichuris*, a cryptic genus of roundworms, showed variability in ITS1 and ITS2 sequences among species of slightly less than 1%,[18] lower than the variability seen among the cryptic *Amblycorypha* and therefore providing confidence that differences of greater than 1% are indicative of speciation.

Within the ITS1-5.8S-ITS2 cistron, the 5.8S rDNA region is highly conserved across many unrelated insects.[19] This provides a sequence alignment checkpoint when comparing the more variable sequences of ITS1 and ITS2. The

data generated from these sequences could be useful in distinguishing cryptic species that have already been sequenced. From data generated thus far, individuals from the same complex appear to be more similar genetically than individuals compared between complexes (Figure 3). *S. furcata*, an individual from an entirely different genus, shows the highest genetic difference when compared to the other specimens. Sequence differences are fairly large across species (up to 2.1%), while sequence differences are small within species (around 0.5%) (Figure 4). These data provide preliminary comparisons and strongly confirm our methodology. As more data are collected, I hope to gain insight into how genetic variation is related to song calling patterns in *Amblycorypha*.

4.2 Future directions

In the future, three to five clones for each PCR reaction will be sequenced by Davis Sequencing Services (Davis, CA). Sequence alignment will be performed using the multiple alignment program Clustal X[20] guided by predicted secondary structure homologies for ITS sequences. Sequence and phylogenetic analyses are to be performed with MEGA version 4.0[21] and PAUP* version 4.0,[22] respectively. These analyses will examine differences in the DNA sequences for each of the genes and determine the evolutionary relationships among the species.

Once the phylogenetic analysis is completed, corresponding mating songs can be mapped with their appropriate species. The resulting phylogeny can then be used to answer questions about signal and song complexity with regard to evolution. Future research should look at multiple individuals from different song morphs occurring both within and among populations.

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