Geographic variation in the calling songs and genetics of Bartram's round-winged katydid *Amblycorypha bartrami* (Tettigoniidae, Phaneropterinae) reveal new species

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Abstract

Previous work on Bartram's round-winged katydid, *Amblycorypha bartrami* Walker, found inconsistencies in song variation across the species' range. Individuals of purported populations of *A. bartrami* from sandhills across the southeastern US were collected, recorded, and their genes were sequenced to better understand their population structure and evolution. Significant differences in songs, morphology, and genetics were found among populations from Alabama (AL), Georgia (GA), North Carolina (NC), and South Carolina (SC), and they differed from those of individuals collected from the type locality in Florida (FL). Males from all populations produced songs composed of a series of similar syllables, but they differed in the rates at which syllables were produced as a function of temperature. At temperatures of 25 °C, the calling songs of males from populations in northern AL and GA were found to have the highest syllable rates, those from SC had the lowest rates, and those from NC were found to produce songs with doublet syllables at rates that were intermediate between those of males from FL and those of AL and GA. These song differences formed the basis for cluster analyses and principal component analyses, which showed significant clustering and differences in song spectra and morphology among the song morphs. A Bayesian multi-locus, multi-species coalescent analysis found significant divergences from a panmictic population for the song morphs. Populations from GA and AL are closely related to those of *A. bartrami* in FL, whereas populations from NC and SC are closely related to each other and differ from the other three. Large river systems may have been important in isolating these populations of flightless katydids. Based on the results of our analyses of songs, morphology, and genetics, three new species of round-winged katydids from the southeastern coastal plain and piedmont are described.

Keywords

Introduction

The round-headed katydids of North America (*Amblycorypha* Stål, 1873) consist of three species groups—*oblongifolia*, *rotundifolia*, and *uhleri*—that differ in morphology and size (Rehn and Hebard 1914, Walker 2004). Walker et al. (2003) reviewed the *rotundifolia* complex and described two species, *Amblycorypha bartrami* Walker, 2003 and *A. alexanderi* Walker, 2003, based on differences in their calling songs and ecology. All three species in the complex from the eastern United States are cryptic, with calling song being the only useful character for distinguishing between *A. rotundifolia*, *A. alexanderi*, and *A. bartrami*. Bartram's round-winged katydid, *A. bartrami*, occurs primarily in xeric longleaf pine and turkey oak habitats in the southeastern United States. During Walker et al.'s (2003) research, it became apparent that populations of supposed *A. bartrami* near Aiken, South Carolina differed significantly in calling songs from typical *A. bartrami* from Florida. The specimens were designated *A. nr bartrami* at the time. Other populations (e.g., in North Carolina) also exhibited song anomalies that indicated more thorough investigations were needed. Two of us (TGF and JDS) undertook a broader examination of *A. bartrami* across its range, including collecting DNA and using molecular data to understand the population structure and evolution within this species. Because the song rates of *A. nr bartrami* in South Carolina are similar to those of *A. parvipennis*, whose populations are all west of the Mississippi River, we also include data from populations of *A. parvipennis* in Arkansas and Missouri.

In this paper, we describe the variation in calling song, morphology, and genetics of populations of purported *A. bartrami*. We present the first molecular phylogenetic data from widespread populations in the *rotundifolia* complex, which show significant

divergence among them. Members of the *rotundifolia* group, including *A. bartrami*, are flightless, which probably influences gene flow among populations. Therefore, we discuss the phylogeography of *A. bartrami* and how our genetic results relate to isolation and spatial population structure, particularly concerning river drainages and fragmentation of the longleaf pine habitat. Clustering analyses across populations also detected previously unidentified population differences in song and morphology. The significant genetic, acoustical, and morphological variation we discovered reveal new species that were, at one time, considered *Amblycorypha bartrami*.

Materials and methods

Fieldwork.—Fieldwork occurred mostly at night, and katydids were collected by listening for and finding males as they called or by searching vegetation for males and females using headlights. In some cases, males and females were collected during the day using sweep nets in areas and from vegetation likely to harbor katydids. Katydids were housed in 10 × 10 × 10 cm cages (either clear plastic or screened) with ad libitum water and food (apple, lettuce, oats, or a dry high-protein artificial diet; Gwynne 1988). For some individuals, we removed a hind leg that was stored at -80°C for DNA extraction and sequencing (see below). Collection sites were typical *A. bartrami* habitats of longleaf pine, turkey oak sandhills distributed throughout the southeastern US, including Alabama (AL) (3♂: 1♀, Cleburne Co.), Florida (FL) (4♂: 1♀, Liberty Co.), Georgia (GA) (4♂: 4♀, Gordon Co.), North Carolina (NC) (9♂: 1♀, Richmond Co.), and South Carolina (SC) (4♂: 3♀, Aiken Co. [SCA]; 6♂: 8♀, Edgefield Co. [SCE]; 6♂: 4♀, Georgetown Co. [SCG]). Collection sites for *A. parvipennis* include Arkansas (AR) (5♂: 3♀, Faulkner Co.) and Missouri (MO) (3♂: 0♀, Shannon Co.). Because the songs of GA and AL specimens were found to have similar features and females from each population duetted with males from each population, their data were combined in many analyses and were designated GAL.

Acoustic recordings and analyses.—Calling songs of free-ranging males in the field or caged males in the laboratory were recorded with Sennheiser ME66 shotgun microphones and either a Tascam DAP-1 DAT recorder or a Marantz PMD-670 solid-state recorder. The sampling rate for the digital recordings was either 44 or 48 kHz. Time and frequency characteristics of the calling songs were determined with Audacity 2.3 or using the seewave package in R (Sueur et al. 2008, Sueur 2018, R Core Team 2020, RStudio team 2020). To reduce noise and echoes, laboratory recordings were made with microphones 0.5 m from the caged males with the substrate between the male and microphone covered with Sonex acoustic foam.

The songs of *A. bartrami* are relatively uniform, and a complete cycle of wing movement (syllable = phonatome, Baker, and Chesmore 2020) is indicated by repeated patterns in the time waveforms of the songs (Walker et al. 2003). Syllable rates of katydids vary with temperature, and these relationships differ among species, making syllable rate a distinguishing character (Walker 1975). For consistency, one of us (TGF) measured syllable rates during the sustained portion of calling songs (see also Walker et al. 2003). When possible, the rates were based on 10 syllables. However, in some recordings, the sustained series had fewer than 10 syllables at consistent rates. In those cases, the rates were determined based on 4–8 (typically 5) syllables. We also included recordings from previous work in our analyses (Shaw et al. 1990, Walker 2003).

Spectral variation among populations was also examined at two different temporal levels: 30 s of calling song and for individual syllables. Because spectra can be influenced by the recording environment, we used songs with high signal-to-noise ratios ($\bar{x} \pm SE = 43 \pm 1.4$ dB). Before spectral analyses, we removed low-frequency noise from the recordings using a finite impulse response bandpass filter (5 kHz–22 kHz, hanning window length = 512). The average spectra of each recording were normalized to probability mass functions during each discrete Fourier transform (DFT: window length = 2048 with 0% overlap), and Kolmogorov–Smirnov (K–S) distances (Gasc et al. 2013) or relative frequency dissimilarities (Deecke and Janik 2006) were computed between each pair of recordings. The K–S distances are the maximum difference between the cumulative probability mass functions of each spectrum in the pair. Relative frequency dissimilarity is a percentage based on the sum of the ratio of minimums and maximums across all frequencies in the two spectra. Only single recordings from each male were used (30 s recordings—GAL: 4♂, FL: 3♂, NC: 4♂, SC: 6♂, *A. parvipennis*: 5♂; single syllables—GAL: 4♂, FL: 3♂, NC: 5♂, SC: 8♂, *A. parvipennis*: 5♂). A hierarchical cluster analysis (hclust function in R) was performed on the distance/dissimilarity matrices to produce a dendrogram showing the relationships among the individuals' songs or syllables (Sueur 2018). To test for differences among populations, we used distance-based redundancy analysis (db-RDA, ade4 package in R) and a principal coordinate analysis (PCoA, ade4 package in R) on the distance/dissimilarity matrices with population as a factor. We then ran Monte Carlo simulations (N =10000) to test for significant clustering by population under the H_o of the db-RDA output (Sueur 2018).

Morphological measurements.—To test for differences in morphological characters among populations, we positioned preserved, pinned museum specimens so that digital images (11Mpix) could be taken of their dorsal and lateral aspects. In each photo, a scale in the same focal plane as the structures to be measured allowed calibrated measurements to be made with ImageJ software (Schneider and Rasband 2012). Measures (to the nearest 0.1 mm) included pronotal length along the midline (PrnL), maximal pronotal width (PrnW), tegminal length (TegL) and width (TegW), hindwing exposure (HwEx), femur and tibia lengths of the hindleg (FemL and TibL, respectively), and for females, ovipositor length (OviL). See also Walker et al. (2003). Measurements for each character were analyzed using ANOVA to test for differences among populations. We also used principal component analysis (PCA, ade4 package in R) on the matrix of morphological measures with population as a factor and conducted Monte Carlo simulations (N = 10000) to test for significant clustering by population under the H_o of the PCA output.

DNA extraction and sequences.—Genomic DNA was extracted from the proximal portion of the frozen femur of individuals from field populations of purported *A. bartrami* (AL (2♂: 1♀, Cleburne Co.), FL (1♂: 1♀, Liberty Co.), GA (1♂: 2♀, Gordon Co.), NC (4♂: 0♀, Richmond Co.), *A. nr bartrami* SC (0♂: 1♀, Aiken Co.; 1♂: 0♀, Edgefield Co.; 2♂: 2♀, Georgetown Co.) and for *A. parvipennis* in AR (2♂: 1♀, Faulkner Co.) and MO (2♂: 0♀, Shannon Co.). We used the standard protocol for the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA) and stored the gDNA extracts at either -20°C or -80°C until they were used for PCR amplification and sequencing.

Because reliance on a single barcoding gene might cause problems in phylogenetic analyses (Moulton et al. 2010), massively

parallel sequencing was performed to simultaneously interrogate regions of mitochondrial, and nuclear DNA for analysis. In particular, we sequenced the cytochrome oxidase subunit I (COI, 658 bp) mitochondrial gene and a large region of nuclear ribosomal DNA (rDNA) that included portions of 28S and 18S rDNA as well as the entire region of 5.8S rDNA and two internal-transcribed spacers ITS1 and ITS2 (~3700 bp). The COI gene is a barcoding gene that has short divergence times and has been used extensively in molecular systematics (Hebert et al. 2003). Ribosomal genes (28S, 5.8S, and 18S) are relatively conserved with little change over long periods of time, whereas the internal transcribed spacers are more labile and thus have been used successfully to distinguish cryptic species in some taxa (Li and Wilkerson 2005, Li et al. 2010). Additionally, we sequenced three nuclear genes, histone 3 (HIS), tubulin-alpha I (TUB), and wingless genes (WNG), that have been used in tettigoniid phylogenies (Mugleston et al. 2013).

PCR amplification.—Published primer pairs were used to amplify the regions of interest (Table 1). The Roche FastStart High Fidelity PCR System (Millipore Sigma, St. Louis, MO) was used for all amplifications. PCR amplification for the ~3700 bp rDNA region used conserved primers LR7 and NS19b with an initial denaturation at 95°C for 2 min followed by 35 cycles of 60 sec at 95°C, 60 sec at 50°C, and 5 min at 68°C plus an additional 20 seconds each successive cycle. The final PCR extension was 7 min at 72°C. PCR reactions (50 µL total volume) contained final reagent concentrations of 2.5 U of Roche FastStart High Fidelity enzyme blend, 1.8 mM $MgCl_2$, 0.4 µM each forward and reverse primer, 4% DMSO, and 0.2 mM each dNTP. Reverse touchdown amplification of COI used LCO1490 and HCO2198 primers and had thermal cycling parameters including an initial denaturation of 95°C for 2 min followed by 6 cycles of 30 sec at 94°C, 90 sec at 45°C, and 60 sec at 72°C and an additional 34 cycles of 30 sec at 94°C, 90 sec at 49°C and 60 sec at 72°C with a final extension of 7 min at 72°C. PCR reactions (25 µL total volume) contained final reagent concentrations of 5 U of Roche FastStart High Fidelity enzyme blend, 1.8 mM $MgCl_2$, 0.6 µM each forward and reverse primer, 6.25% DMSO, and 0.2 mM each dNTP. Tubulin-alpha I genes were amplified with 294F1 and 294R1 primers, histone 3 genes with H3 AF and H3 AR primers, and wingless genes with WG550F and WGABRZ primers, respectively. Tubulin-alpha I, histone 3, and wingless genes were amplified in independent PCR

reactions with thermal cycling parameters having an initial denaturation at 95°C for 2 min followed by 35 cycles of 30 sec at 94°C, 30 sec at 50°C, and 50 sec at 72°C with a final 7 min extension at 72°C. PCR reactions (25 µL total volume) contained final reagent concentrations of 5 U of Roche FastStart High Fidelity enzyme blend, 1.8 mM $MgCl_2$, 0.6 µM each forward and reverse primer, 6.25% DMSO, and 0.2 mM each dNTP. Amplicon products were quantified using an Agilent 2100 Bioanalyzer and DNA 1000 kit (Agilent Technologies, Inc., Santa Clara, CA).

Library preparation and massively parallel sequencing (MPS).—PCR products were diluted to a concentration of 0.2 ng/µL and enzymatically fragmented and tagged with MPS sequencing adapters using the Illumina Nextera XT Library Prep kit (Illumina, Inc., San Diego, CA). Limited-cycle PCR was used to add flow cell adapters and multiplexing barcodes to fragmented libraries. Flow cell adapters enable library fragments to anchor to the surface of the solid support where sequencing occurs. Barcodes allow for postsequencing parsing of sample-dependent data, which permits a high degree of multiplexing per sequencing run. Solid-phase reversible immobilization (SPRI) beads were used to purify the prepared libraries via the removal of unincorporated primers and dNTPs that could affect sequencing downstream. Libraries were then normalized to ensure equal representation of each sample, and equal volumes were pooled to create a master library for sequencing. Sequencing was performed on an Illumina MiSeq using a v3 2 × 300 cycle kit (Illumina, Inc., San Diego, CA).

Assembly, validation, and alignment.—Sequence analyses were carried out using Geneious Prime 2020.1.2. NextGen Fastq sequences were first set as paired reads and trimmed using BBDuk with a minimum quality Q30 and a minimum length of 20. These reads were then assembled to GenBank (Clark et al. 2016) reference sequences (COI: HQ968170 and ITS/5.8s ribosomal genes: AM888963) of *Scuddera furcata*, another phaneropterine katydid, and two other sequences from members of *Amblycorypha* (tubulin-alpha I: KF571404 and wingless: KU550854.1). Major vote consensus sequences were extracted from these assemblies, inspected for quality, and searched for within NCBI using BLAST (Altschul et al. 1990). All alignments were made using Clustal Omega 1.2.2 with fast clustering, a cluster size of 100, and 3 refinement iterations (Sievers et al. 2011).

Table 1. Primer pairs and annealing temperatures for PCR and expected size for sequences.

Primer	Sequence 5'3'	Anneal (°C)	%GC	Amplicon Size (bp)	Ref
COI Primers					
F LCO1490	GGTCAACAAATCATAAAGATATTGG	59.7	32.0		Folmer et al. 1994
R HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	64.5	34.6	658	Folmer et al. 1994
28S and 18S rDNA Primers					
F LR7	TACTACCACCAAGATCT	53.6	41.2		Vigalys and Hester 1990
R NS19b	CCGGAGAGGGAGCCTGAGAAC	68.9	66.7	~3700	Bruns Lab, UC Berkeley
Histone 3 Primers					
F H3 AF	ATGGCTCGTACCAAGCAGACV	50.0	55.6		Colgan et al. 1998
R H3 AR	ATATCCTTRGGCATRATRGTG	50.0	40.5	~375	Colgan et al. 1998
wingless (wg) Primers					
F WG550F	ATGCGTCAGGARTGYAARTGY	50.0	47.6		Mugleston et al. 2013
R WGABRZ	CACTTNACYTCRCARCACCAR	50.0	50.0	~450	Mugleston et al. 2013
tubulin-alpha I Primers					
F 294F1	GAAACCRGTKGGRCACCAGTC	50.0	59.5		Buckman et al. 2012
R 294R1	GARCCCTACAAYTCYATTCT	50.0	42.5	~350	Buckman et al. 2012

Phylogenetic analysis.—Phylogenetic relationships were inferred using Bayesian analysis in *BEAST2, which uses the Markov chain Monte Carlo (MCMC) process to explore tree space based on posterior probabilities (Bouckaert et al. 2019). We used BEAUTi2 to generate the analysis parameters. We set each gene sequence as a separate partition in the multi-locus, multi-species coalescent analysis and allowed the program to integrate analytical population size. We set the site substitution model for all genes to HKY with frequencies empirically estimated. The analysis was run under a strict clock for each gene partition with priors, using the birth-death model to estimate birth and death rates during the analysis. The number of MCMC iterations was 1.2E8, which was sufficient for the model to reach stationarity after a 20% burn-in. The output of each *BEAST2 run was inspected using Tracer v1.7.2., and the trees were visualized and annotated using Densi-Tree v2.2.7 and TreeAnnotator v2.6.6, respectively. TreeAnnotator produced trees with maximum clade credibility for each gene tree and for the species tree that resulted from the coalescent analysis. We used different random seeds to conduct 5 *BEAST2 analyses to ensure that the random process adequately covered tree space and that the output trees generated were robust. We ran the analyses with all populations separated and with putative 'species' that

were suspected based on differences in syllable rate functions with temperature (see below).

Deposition of specimens, recordings, and sequences.—Unless otherwise indicated, the specimens are currently housed at the University of North Carolina at Asheville (UNCA). The collection, along with types, will be transferred to the Florida State Collection of Arthropods (FSCA), Gainesville, FL. Recordings will be made available through the Macaulay Library of Natural Sounds at the Cornell Lab of Ornithology and Singing Insects of North America (SINA) website. Sequencing data have been uploaded to the National Center for Biotechnology Information (NCBI) under BioProject PRJNA906584.

Results

Song variation.—Figs 1–6 show the temporal structure of calling songs among populations. For all populations, the calling songs are a series of easily quantified, repeated syllables representing a single cycle of wing movement. The calling songs of *A. bartrami* and *A. parvipennis* do not exhibit the extreme song complexity of the virtuoso *Amblycorypha*, which have 4 syllables
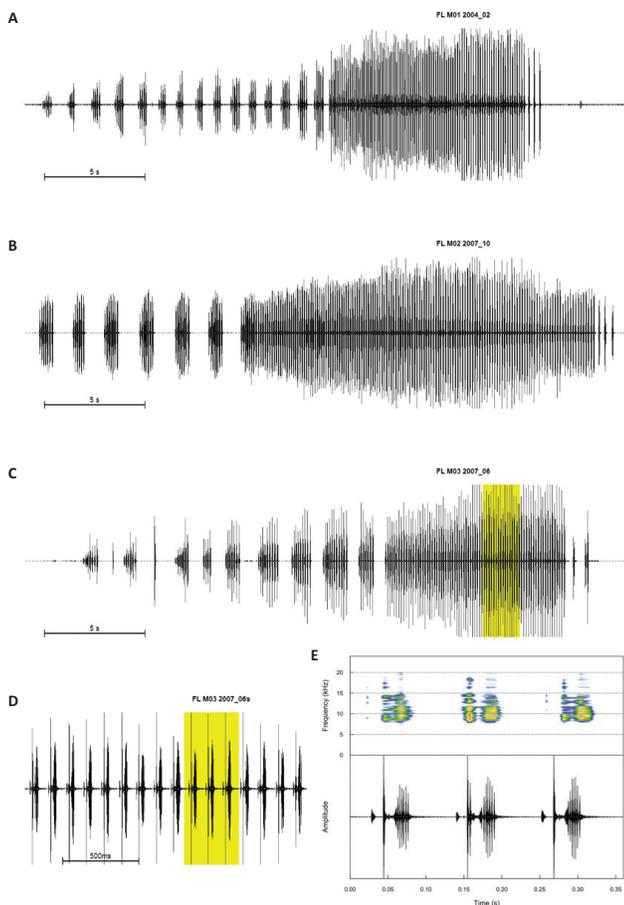

Fig. 1. A–C. Oscillograms (30s) of calling songs of 3 male *A. bartrami* from Liberty Co., Florida. Songs consist of a long duration, sustained main series of (~100) syllables preceded by several (15–20) short-duration series of 1–7 syllables; **D.** Oscillogram showing 16 syllables within yellow highlighted portion of the main series of **C**; **E.** Oscillogram and spectrogram showing the fine temporal structure and frequency content of 3 syllables highlighted in **D**.
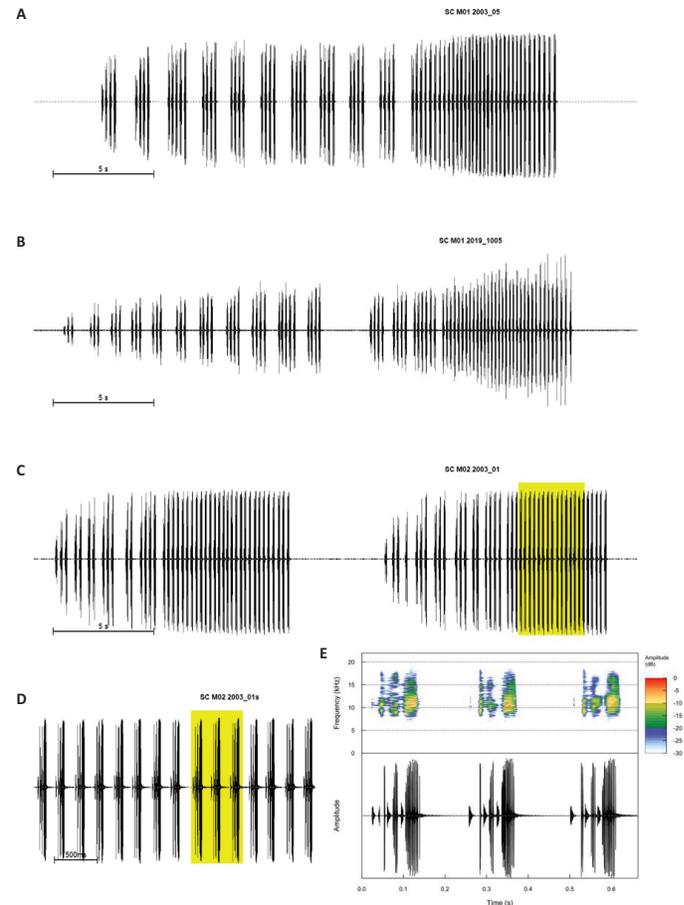
Fig. 2. A–C. Oscillograms (30s) of calling songs of 3 male *A. nr bartrami* from Aiken Co., South Carolina. Songs consist of a long duration, sustained main series of syllables preceded by several shortduration series of 1–5 syllables; **D.** Oscillogram of 15 syllables highlighted in **C**; **E.** Oscillogram and spectrogram showing the fine temporal structure and frequency content of 3 syllables highlighted in **D**.

that may be produced with varying syntaxes (Walker and Dew 1972, Walker 2004). In most cases, the songs of *A. bartrami* consist of a longer duration, sustained (main) series of syllables preceded by 1 to >20 shorter series that typically increase in amplitude. Calling songs vary significantly among the populations in several ways.

Temporal variation.—Songs of males from the Florida panhandle (N=3♂: 9 series) have sustained portions with significantly more syllables ($\bar{x}\pm SE=107\pm22$) than all other populations (GAL: 8±1, N=4♂: 53 series; NC: 17±1, N=8♂: 30 series; SC: 25±2, N=11♂: 112 series; *A. parvipennis*: 24±3 N=6♂: 39 series; Fig. 7). Males from NC nearly always produced syllables in doublets during the sustained main portion of their calling song (Fig. 5E). Of the 517 syllables produced in 30 main series from 8 males, 482 were doublets, 8 (2%) were singlets, and 9 (5%) were triplet syllables.

Series that precede the main series of calling songs have, on average, 5–6 syllables for males from FL, whereas those in songs of males from other populations have fewer (GAL: 3–8; NC: 2–3; SC: 2–4; *A. parvipennis*: 1–2; Fig. 7). Males of *A. parvipennis* rarely (13%, 5 of the 39 series from 6 males) produce syllables preceding the main series of their calling songs (Fig. 7). See Suppl. material 3.

Syllable rate variation.—Based on the relationships of syllable rates with temperature (Fig. 8, Suppl. material 2), there are at least 4 different song types across the populations we sampled. Males from northern GA and northern AL (GAL) have functions with the greatest slopes (m=0.81, Fig. 8: green) and rates of ~13.1s⁻¹ at 25°C. SC males (*A. nr bartrami*) have the slowest syllable rates at ~5.8s⁻¹ at 25°C (m=0.29, Fig. 8: orange), which is very similar to that of *A. parvipennis* ~5.0s⁻¹ at 25°C (m=0.16, Fig. 8: black). Males from northern FL and the FL panhandle produce syllables at intermediate rates of 10.0s⁻¹ at 25°C (Fig. 8: red). Males from western AL had songs with syllable rates similar to those of FL males (Fig. 8: pink). Because NC males produced songs with syllable doublets, two rates were calculated. The faster syllable rate, within a doublet (m=0.58, 11.6s⁻¹ at 25°C), falls between rates for songs of FL males and those of GA and AL males whereas the slower rate, between doublets (m=0.17, ~4.0s⁻¹ at 25°C), was slower than the rates of SC *A. nr bartrami* and *A. parvipennis* males.

Syllable variation.—Fig. 9 shows the variation in the fine temporal structure of syllables produced by males from each population. The impulses in each syllable are probably the result of the scraper engaging and releasing a single tooth on the file. Males from FL have a single pulse train followed by a longer
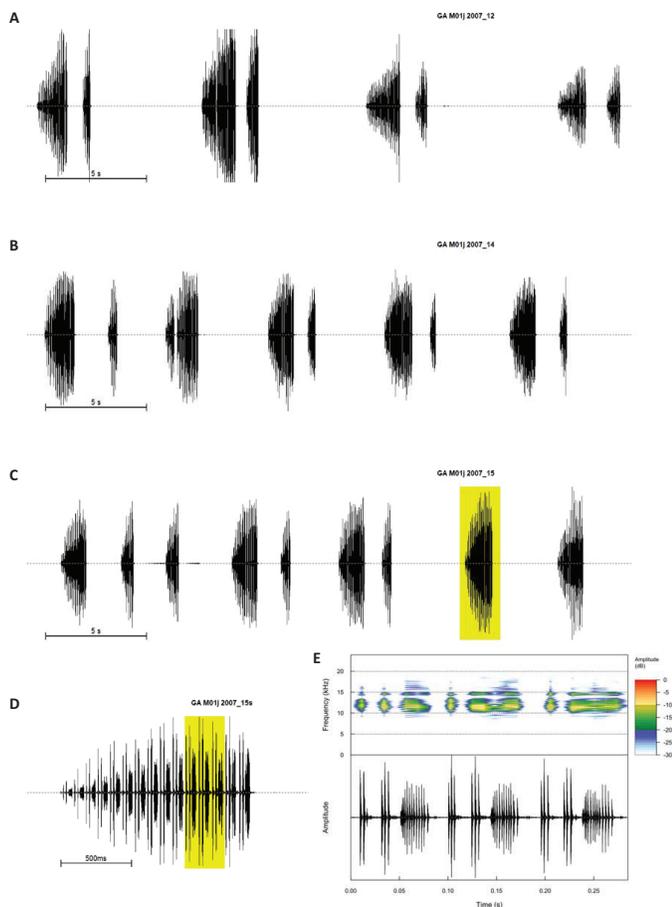

Fig. 3. A–C. Oscillograms (30s) of calling songs of 1 male *A. bartrami* from Gordon Co., Georgia. The long-duration sustained, main series of syllables are rarely preceded by shorter series as found in the songs from other supposed populations of *A. bartrami*; **D.** The yellow highlighted portion of **C**; **E.** Oscillogram and spectrogram of 3 syllables highlighted in **D**.
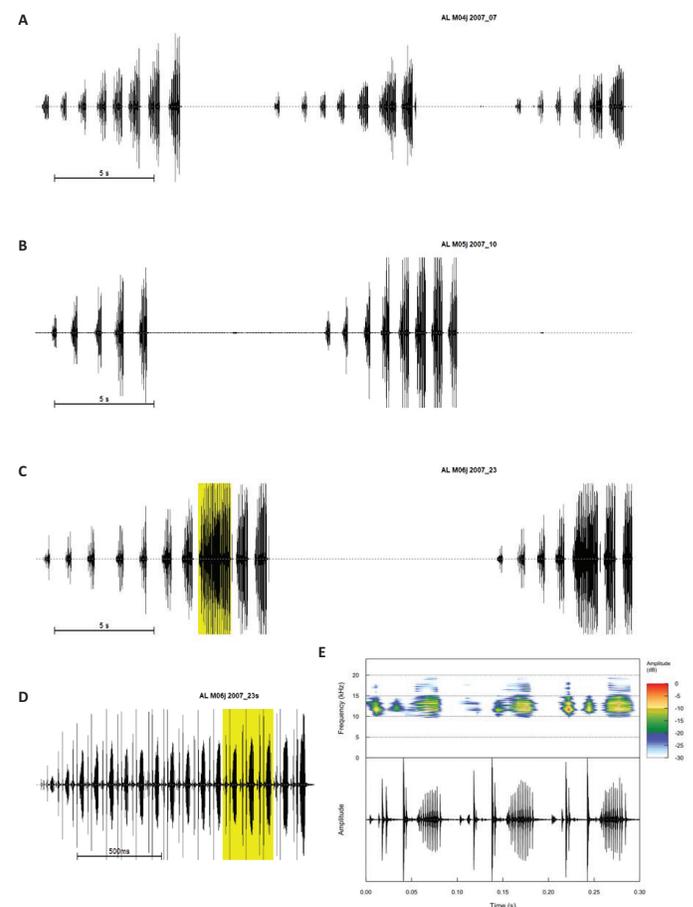
Fig. 4. A–C. Oscillograms (30s) of calling songs of 3 male *A. bartrami* from Cleburne Co., Alabama. Syllable rates of sustained main series are similar to those of males from north Georgia (Figs 3, 7); **D.** Oscillogram of 17 syllables highlighted in **C**; **E.** Yellow highlighted portion of **D** showing detailed temporal structure and spectral composition of 3 syllables.
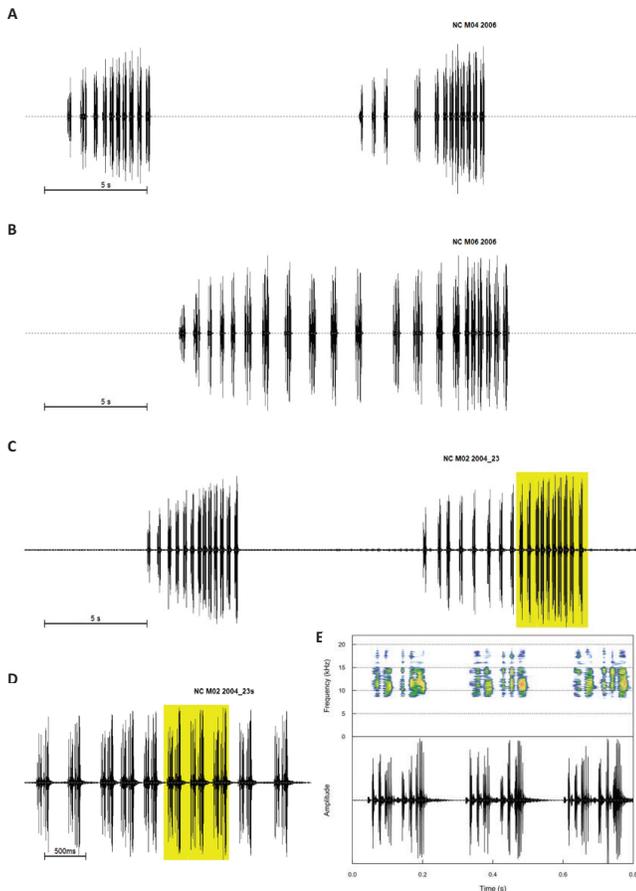
Fig. 5. A–C. Time waveforms (30s) of calling songs for 3 NC *A. bartrami* males. Note the variation in the number of syllables that precede the main sustained train of syllables; **D.** Highlighted portion in **C**; **E.** Oscillogram and spectrogram of highlighted portion of **D** with 6 syllables in 3 doublets. Doublet syllable rates were faster than those of FL *A. bartrami* and slower than those of GA-AL *A. bartrami*. FL, GA, AL, and SC males rarely produced doublet syllables.

Fig. 6. A–C. Oscillograms (30s) of calling songs of 3 male *A. parvipennis*; **D.** Oscillogram of 10 syllables highlighted in **C**; **E.** Fine temporal structure and spectrogram of 3 syllables highlighted in **D**.

terminal pulse train in the syllable. In all other populations, males exhibited two pulse trains made up of 1–5 pulses before the terminal pulse train.

Spectral variation.—The signals produced by males are broadband, with most of the energy between 10–15 kHz (Panel E of Figs 1–6). Hierarchical cluster analyses using K–S distance and relative frequency dissimilarity metrics indicate that significant spectral variation exists between populations of *A. bartrami*, *A. nr bartrami*, and *A. parvipennis* at the level of calling song and syllables (Figs 10, 11, respectively, Suppl. material 4). Although there is much overlap among populations in the dendrograms (Figs 10A, B, 11A, B), principal coordinate analyses calculated on the distance/dissimilarities show significant clustering within populations and differences among populations. Average spectra computed over the entire calling song (30s) differed significantly from random when using population as a factor for both distance metrics (K-S distance, Monte-Carlo test simulation, N=10000, p=0.032, first two components explain 77% of total variance; relative frequency dissimilarity, Monte-Carlo test simulation, N=10000, p=0.018, first two components explain 43% of total variance; Figs 10C, D). The same was found when the average spectra were calculated on smaller time scales associated with single syllables (K-S distance, Monte-Carlo test simulation, N=10000, p<0.008,
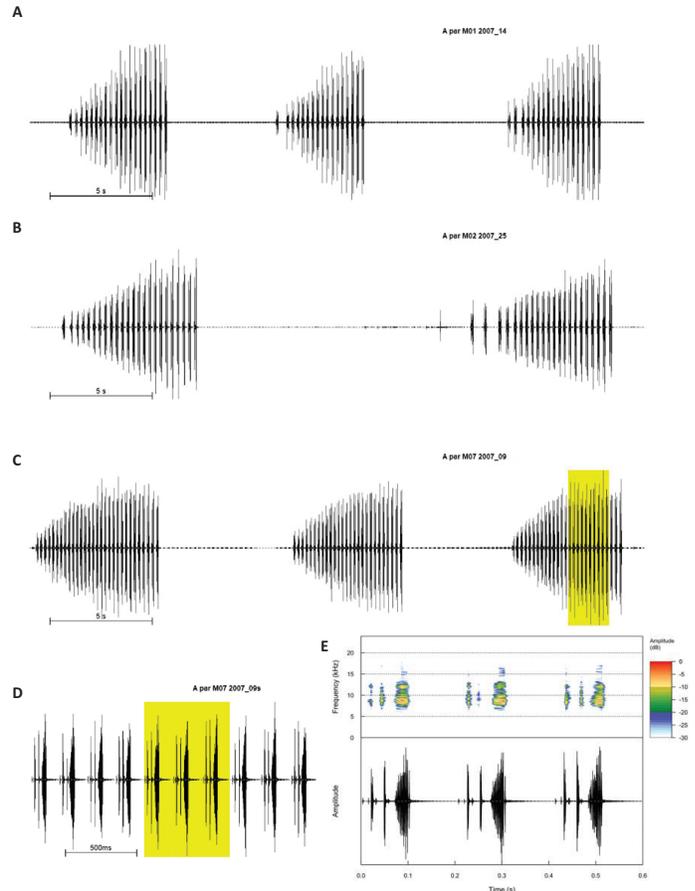
first two components explain 74% of total variance; relative frequency dissimilarity, Monte-Carlo test simulation, N=10000, p<0.002, first two components explain 32% of total variance; Figs 11C, D).

Morphological variation.—Morphological characters differed among some of the populations (Table 2, Suppl. material 1). The only significant differences among females' character measurements were pronotal length (PrnL), where GAL females have significantly shorter PrnL (5.99±0.23 mm) than SC females (6.60±0.07 mm), and respective *A. parvipennis* females (6.96±0.19 mm). The lack of significant differences among any other female morphological measurements may, in part, be due to the small sample sizes associated with many of the populations.

There were many differences in male size among some of the populations. Similar to our findings for female PrnL, GAL males also had significantly shorter PrnL (5.08±0.14 mm) than SC males (6.03±0.09 mm) and *A. parvipennis* males (6.15±0.13 mm). For nearly every morphological measurement (TegL, TegW, FemL, TibL), GAL and *A. parvipennis* were shorter than the other populations (Table 2). GAL males differed significantly from NC males in all measures except HwEx (PrnW: 3.64±0.08 vs 4.32±0.06 mm, respectively; TegL: 25.4±0.70 vs 28.2±0.21 mm, respectively; TegW: 7.83±0.21 vs 9.32±0.18 mm, respectively; FemL: 23.3±0.11 vs 27.3±0.38 mm, respectively; TibL: 25.1±0.20 vs 28.7±0.44 mm, respectively). GAL males had significantly shorter hind femurs (FemL) and hind tibiae (TibL) than that of all other purported *A. bartrami* populations (Table 2).
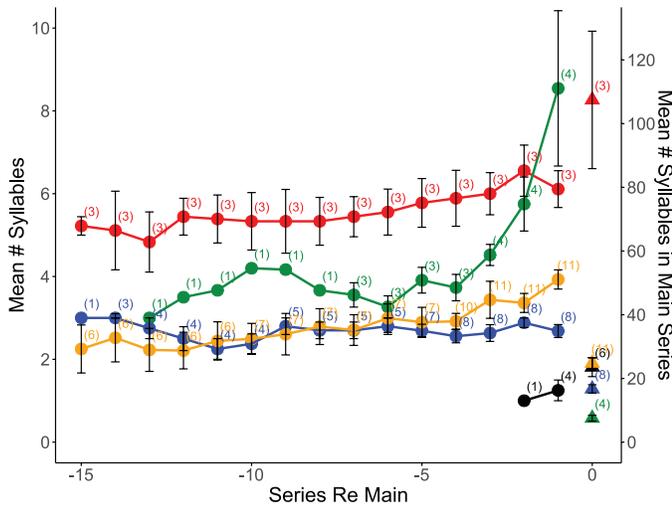
Fig. 7. Mean (±SE) number of syllables as a function of the temporal relationship between series in male calling song from populations of supposed *Amblycorypha bartrami* (GA & AL: green, NC: blue, FL: red, SC: orange) and *A. parvipennis* (black). Triangles (X=0) represent the mean number of syllables in the sustained main series of songs averaged over the number of males shown in parentheses. Circles are the means for each series preceding the main series with the number of males contributing to the average indicated in parentheses.
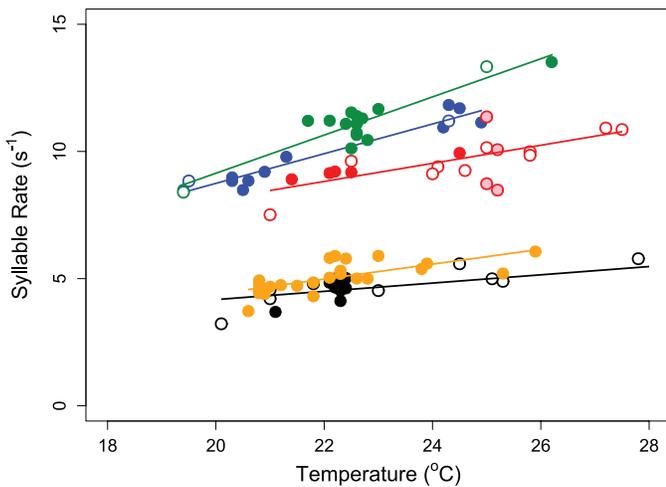

Fig. 8. Syllable rate as a function of temperature for populations of *Amblycorypha bartrami* (GA & AL: green, NC: blue, FL: red, AL: pink, SC: orange) and *A. parvipennis* (black). Solid symbols are recordings from our research and open symbols are recordings from other published work (Walker et al. 2003 for *A. bartrami* and *A. parvipennis*, Shaw et al. 1990 for *A. parvipennis*).

Because the functions of syllable rate and temperature between SC *A. nr bartrami* and *A. parvipennis* are so similar, it is important to compare the morphological traits among them. Male SC *nr bartrami* had significantly longer tegmina (TegL: 26.2±0.44 vs 23.8±0.47 mm, respectively) and significantly longer hindwing exposure (HwEx: 2.74±0.17 vs 0.89±0.34 mm, respectively) than *A. parvipennis* males. Hindwing exposure is one of the key characteristics distinguishing *A. parvipennis* from all eastern members of the *rotundifolia* complex (Rehn and Hebard 1914).

Principal component analysis indicates morphological differences among the supposed populations of *A. bartrami* (Fig. 12). In
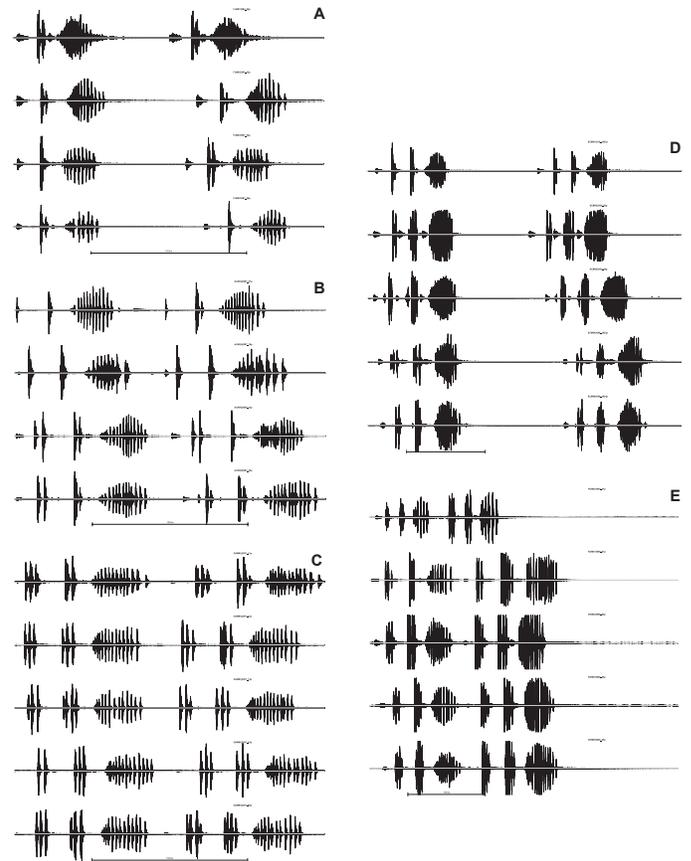

Fig. 9. Syllable variation among populations of *A. bartrami*. **A.** Florida N=3♂; **B.** Alabama N=3♂; **C.** Georgia N=1♂; **D.** South Carolina N=5♂; **E.** North Carolina N=5♂. Syllables consist of brief decaying impulses that are likely the result of the scraper engaging and releasing a single tooth on the file. Males from FL **(A.)** typically have a single pulse train of 1–2 pulses preceding the longer terminal pulse train in the syllable. Males from all other populations have two pulse trains (1–5 pulses) preceding the terminal pulse train. Scale bars: 100ms.

particular, the slope of the ordination in the PCA for GAL males is negative, whereas it is positive for all other populations whose relationships are all parallel (Fig. 12). FL, NC, and SC males tended to be more similar in the averages of their morphological characters. Characters that contributed most to PC1 were TibL (20%), FemL (19%), PrnW (16%), TegL (16%), and TegW (16%), and those that contributed most to PC2 were HwEx (40%), PrnL (28%), and TegL (14%).

Genetic variation.—Once sequences were processed and aligned, the lengths of consensus sequences were COI: 658 bp, H3: 333 bp, ITS1, ITS2, and 5.8S ribosomal genes = ITS3k: 3262 bp, TUB: 341 bp, and WNG: 371 bp. BLAST searches of each gene sequence, except for TUB, invariably matched those of *Amblycorypha* or other members of the Phaneropterinae (COI: all individuals >90% match to COI of *Amblycorypha floridana* [HQ983647.1, HQ983648.1, HQ983649.1], *Amblycorypha oblongifolia* [HQ983655.1, JN294610.1, KM532357.1, KM536809.1, KR144595.1] or *Amblycorypha* sp. [MG466233.1]; H3: all individuals 100% match to histone 3 gene of *Amblycorypha* sp. [KF571154.1]; ITS3k: all individuals >98% match to 28S *Microcentrum rhombifolium* or *Scudderia furcata*; WNG: all individuals

Table 2. Mean (SE, N) of morphological measures (mm) from populations of supposed *Amblycorypha bartrami* and populations of *A. parvipennis*.*

Sex Population	PrnL	PrnW	TegL	TegW	FemL	TibL	OviL
Females							
FL	6.52[ab]	4.33[a]	28.6	9.11	29.6	30.6	9.83
	(NA, 1)	(NA, 1)	(NA, 1)	(NA, 1)	(NA, 1)	(NA, 1)	(NA, 1)
GAL	5.99[b]	3.86[a]	26.0	8.07	25.9	26.6	10.7
	(0.23, 5)	(0.13, 5)	(0.67, 5)	(0.34, 5)	(0.49, 5)	(0.59, 5)	(0.38, 5)
NC	7.05[ab]	4.58[a]	28.0	9.63	29.9	31.0	11.0
	(NA, 1)	(NA, 1)	(NA, 1)	(NA, 1)	(NA, 1)	(NA, 1)	(NA, 1)
SC	6.60[a]	4.12[a]	26.6	8.12	27.6	28.7	9.93
	(0.07, 17)	(0.08, 17)	(0.47, 13)	(0.25, 15)	(0.40, 15)	(0.40, 14)	(0.25, 17)
A. par	6.96[a]	4.52[a]	25.8	7.81	27.1	28.3	9.9
	(0.19, 3)	(0.07, 3)	(0.78, 3)	(0.27, 3)	(0.54, 3)	(0.68, 3)	(0.54, 2)
Males							
FL	5.88[ab]	4.01[ab]	29.7[a]	9.31[ab]	28.0[a]	28.8[a]	
	(0.05, 3)	(0.14, 3)	(0.72, 3)	(0.17, 3)	(0.38, 3)	(0.59, 3)	
GAL	5.08[b]	3.64[b]	25.4[cd]	7.83[b]	23.3[c]	25.1[c]	
	(0.14, 7)	(0.08, 7)	(0.70, 7)	(0.21, 7)	(0.11, 7)	(0.20, 7)	
NC	5.84[ab]	4.32[a]	28.2[ab]	9.32[a]	27.3[ab]	28.7[ab]	
	(0.04, 9)	(0.06, 9)	(0.21, 9)	(0.18, 9)	(0.38, 6)	(0.44, 6)	
SC	6.03[a]	4.00[ab]	26.2[bc]	8.21[b]	27.1[ab]	28.2[ab]	
	(0.09, 17)	(0.09, 17)	(0.44, 17)	(0.17, 17)	(0.42, 15)	(0.34, 15)	
A. par	6.15[a]	3.97[ab]	23.8[d]	7.71[b]	25.5[bc]	26.6[bc]	
	(0.13, 8)	(0.08, 8)	(0.47, 8)	(0.25, 8)	(0.48, 8)	(0.41, 8)	

* Comparisons of means within each sex were made for each morphological trait. Means within a column followed by different letters are significantly different (ANOVA, Tukey honest significant difference posthoc test, P<0.05).

>99% match to WNG *Amblycorypha longinicta* [KU550854.1] or *Amblycorypha* sp. [KF571288.1]. There was no genetic variation in H3 among all samples; therefore, we did not include H3 sequences in any further analyses. For all individuals sequenced, TUB sequences matched tubulin-alpha I sequences of members in the Tettigoniidae, with 11 individuals matching (85–97% identical) sequences in the Phanertopterinae (*Syntechna* and *Trigonocorypha*), 11 individuals matching (79–80% identical) *Lipotactes maculatus* (Lipotactinae), and one individual matching 82% of the tubulin-alpha I sequence of *Kuzicus megaterminatus* (Meconematinae). Because these matches for TUB were so varied, we ran the *BEAST2 analyses with and without TUB sequences included.

Multiple runs of our multispecies coalescent analyses produced identical phylogenetic topologies with only small differences in the posterior probabilities at the nodes. Effective sample sizes (ESS) for every parameter of the models were always over 1500.

Gene trees.—Gene trees based on COI, ITS3k, and WNG sequences were similar to the species trees generated in our analysis (Figs 13, 14). Gene trees using TUB sequences differed substantially from the species trees. The estimated mutation rate of mitochondrial gene COI was about 45X that estimated for ITS3k, about 10X that for TUB, and almost 15X that for WNG.

Our gene tree for COI using the Bayesian coalescent approach showed high support for most populations (AL, GA, NC, SCG, AR *A. parvipennis*, MO *A. parvipennis* with all posterior probabilities >0.97, Fig. 13A). The greatest uncertainty involved the two South Carolina populations (SCA and SCE) where we had data from only single individuals. There was high support for grouping the South Carolina population (Georgetown Co., SCG) with the North Carolina population (posterior probability = 1.0).

Support values for our ribosomal gene trees were variable. The tree supported monophyly of *A. parvipennis* (posterior = 1.0),

grouped the two individuals from South Carolina together (SCA and SCE, posterior probability = 0.98), grouped two of the SCG individuals with all North Carolina individuals (posterior probability = 1.0), grouped all GA individuals with most of the AL individuals, and grouped the two FL individuals (posterior probability = 0.79) (Fig. 13B).

The gene trees for our nuclear sequences (TUB and WNG) differed more from the population/species trees than the COI and ITS3k gene trees. Interestingly, the *A. parvipennis* populations clustered in the middle of both gene trees (Fig. 13C, D).

Species/population trees.—The output of our coalescent analyses (trees with maximum clade credibility) indicated genetic divergences among all populations that we sampled (Fig. 14A). Our phylogenetic analyses showed that AR and MO populations of *A. parvipennis* differed genetically and are more closely related to each other than they are to all supposed *A. bartrami* populations we sampled (posterior probability = 1.0). The population tree indicates (Fig. 14A) that *A. bartrami* populations from AL and FL split more recently and that there was an earlier divergence from populations in GA. Populations of supposed *A. bartrami* in the west (AL, FL, and GA) differ from those in the east (NC and SC). Interestingly, populations from within SC differ from each other genetically although they have identical syllable rates in their songs. South Carolina *A. nr. bartrami* from Georgetown Co., SC were found to be more closely related to NC 'bartrami' than to populations in Edgefield Co. or Aiken Co., SC *A. nr. bartrami*. Note that the NC and Georgetown Co. SC populations are also closer geographically (see *Phylogeography* below).

When the analysis was done with individuals grouped by calling song information (Fig. 14B), song morphs from GAL (GA and AL) diverged from those in FL and differed (posterior probability = 1.0) from the two eastern song morphs NC and SC (posterior
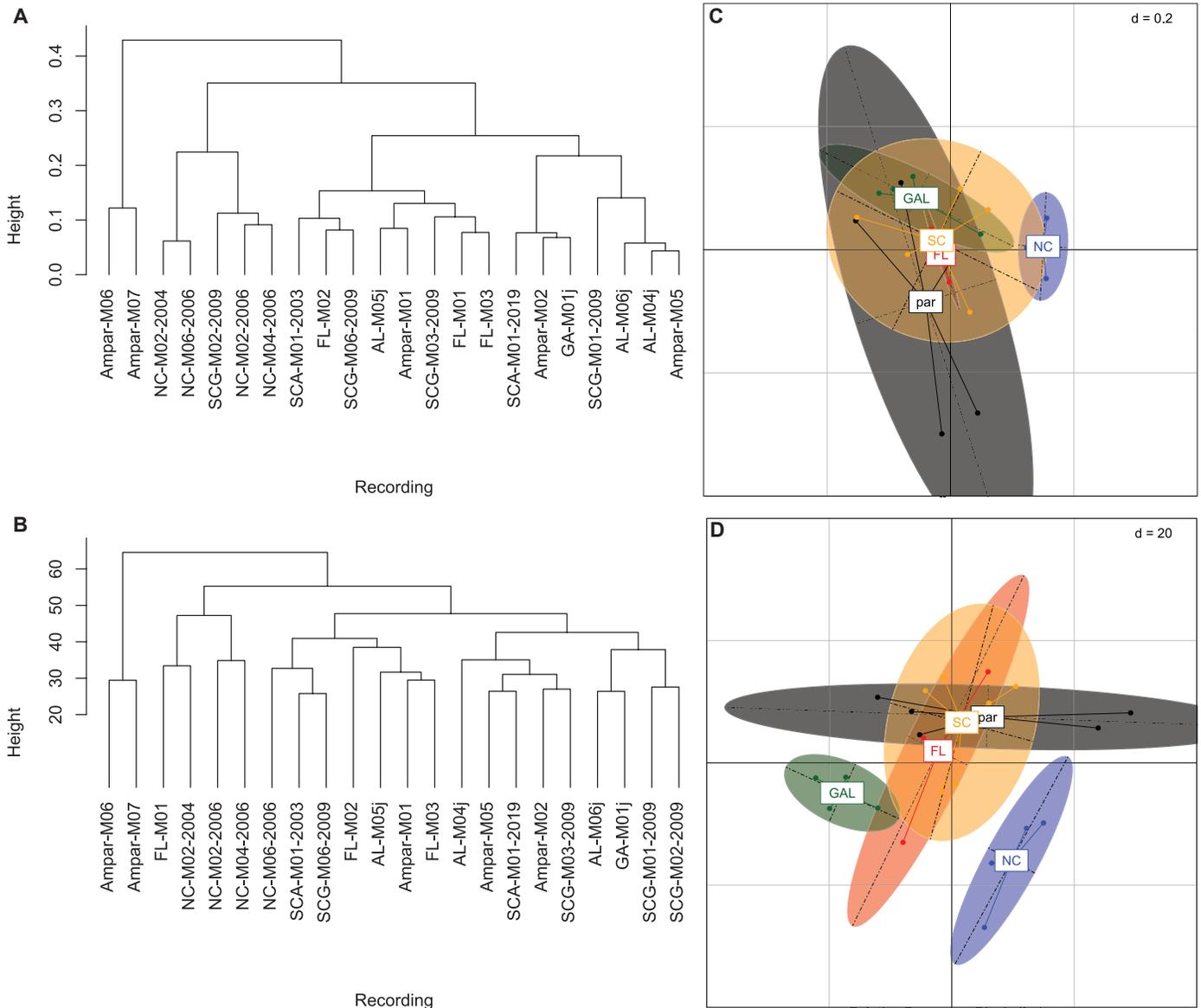
Fig. 10. A, B. Song Dendrograms. The hierarchical cluster analyses are based on average spectra of 30s recordings of individual calling songs of males from populations of *A. bartrami* and *A. parvipennis*; **C, D.** Principal Coordinate Analyses calculated on distance/dissimilarity matrices of each pair of average spectra of the same 30s recordings in **A, B**. Shaded ellipses encompass 95% of observations expected for populations (green: GAL: 4♂; red: FL: 3♂; blue: NC: 4♂; orange: SC: 6♂; black: *A. parvipennis*: 5♂). Clustering by populations differed significantly from H_o (no relationships between ordination axes) for **C** (Kolmogorov-Smirnov Distance, Monte-Carlo test simulation, N=10000, p=0.032, first two components explain 77% of total variance) and **D** (Relative Frequency Dissimilarity, Monte-Carlo test simulation, N=10000, p=0.018, first two components explain 43% of total variance).

probability = 0.77). Data that include genetic information from other members of the *rotundifolia* complex indicate that the purported populations of *A. bartrami* we studied are not monophyletic, as suggested in the phylogenies we present (Forrest unpublished, Sither 2018).

Phylogeography.—The phylogeographic relationships among the populations we studied indicate that proximity of populations is related to the genetic distances among them (Fig. 15). The two populations of *A. parvipennis* were clearly isolated genetically and geographically from the eastern populations we studied. Although individuals from AL populations were closely related to FL populations (Fig. 15A), when the phylogenetic analysis was done factoring in song rate, GA and AL populations coalesced and differed from FL populations (Fig. 15B). Similarly, SCG populations from the coast (Georgetown Co.) clustered with nearby NC populations, whereas they clustered with the other SC counties (SCA, SCE) when taking song rates into account to delineate species in the coalescent model.

Discussion

Amblycorypha in North America have been assigned to three species groups based on morphology (Rehn and Hebard 1914, Walker et al. 2003, Walker 2004). Those in the *oblongifolia* complex are relatively large, those in the *uhleri* group are small, and those in
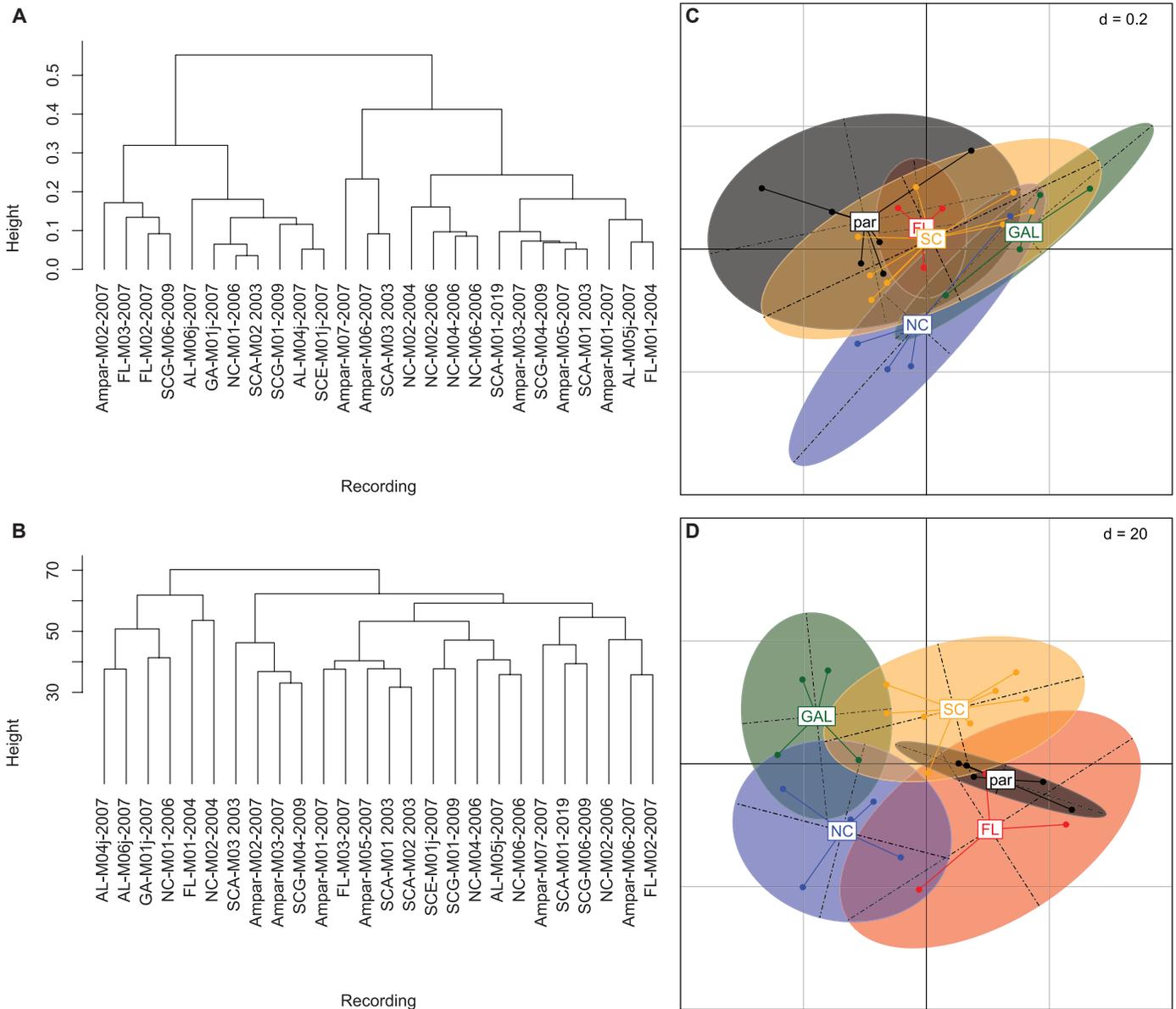
Fig. 11. A, B. Syllable Dendrograms. The hierarchical cluster analyses are based on average spectra of pairs of syllables in male songs from populations of *A. bartrami* and *A. parvipennis*; **C, D.** Principal Coordinate Analyses calculated on distance/dissimilarity matrices of each pair of average spectra of the same recordings in **A, B**. Shaded ellipses encompass 95% of observations expected for populations (green: GAL: 4♂; red: FL: 3♂; blue: NC: 5♂; orange: SC: 8♂; black: *A. parvipennis*: 6♂). Clustering by populations differed significantly from H_o (no relationships between ordination axes) for **C** (Kolmogorov-Smirnov Distance, Monte-Carlo test simulation, N=10000, p<0.008, first two components explain 74% of total variance) and **D** (Relative Frequency Dissimilarity, Monte-Carlo test simulation, N=10000, p<0.002, first two components explain 32% of total variance)).

the *rotundifolia* complex are intermediate in size. Members of the *uhleri* and *oblongifolia* groups can fly, whereas the medium-sized members of the *rotundifolia* group are flightless and individuals move only about 10–15 m per day (Shaw et al. 1981, Cusick 2008).

The populations we studied in this paper are in the *rotundifolia* complex and were originally considered members of *A. bartrami* because of similarities in their calling song (consisting of a series of single syllables with short bouts of syllables leading up to a longer sustained series with a rate of about 10–13 syllables per sec), their morphology, and their habitat (longleaf pine, turkey oak sandhills). Our analyses show that there are significant differences in calling songs, morphology, and genetics between some of these populations.

Song variation.—The songs of *A. bartrami*, *A. nr bartrami*, and *A. parvipennis* are relatively simple and have only a single syllable type. They do not exhibit the extreme song complexity of the virtuoso katydids (Walker et al. 2004), which have 4 syllable types usually produced in sequence but can be quite varied in their order, for example, *A. longinicta* (Walker 2004). In general, phaneropterine songs are extremely diverse with a wide range of complexity that has evolved multiple times (Heller et al. 2015), probably in response to duetting courtship signals and the evolution of countermeasures to eavesdropping by rival males (Heller et al. 2017). Closely related species may have simple songs with a single syllable type, while others have multiple syllables with varying syntax (Heller et al. 2015, ter Hofstede et al. 2020).
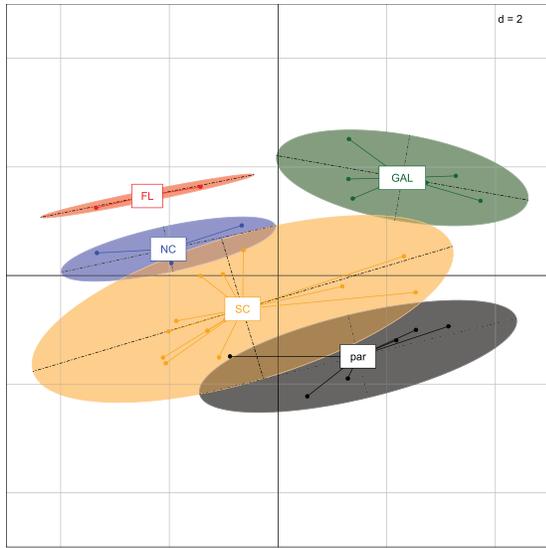
Fig. 12. Principal Components Analysis on six morphological measures of males from supposed populations of *A. bartrami* and populations of *A. parvipennis*. (green: GAL: 7♂; red: FL: 3♂; blue: NC: 6♂; orange: SC: 13♂; black: *A. parvipennis*: 8♂). Shaded ellipses around the means encompass 95% of the expected values for each population. The first two dimensions of the analysis account for 84% of the variation among the morphological measures and clustering of populations showed significant relationships in the ordination of the two dimensions (Monte-Carlo test simulation, N=10000, p<0.0001).

Among cryptic species complexes in the phaneropterines, changes in syllable rates as a function of temperature are often used to recognize species (Walker et al. 2003, Dutta et al. 2017, Heller et al. 2017). In our analysis of the temporal features of the calling songs of supposed members of *A. bartrami*, we found populations whose calls differed in terms of the functions of syllable rate in response to changes in temperature (Fig. 8), differed in the number of syllables and their fine temporal structure (Figs 7, 9, respectively), and differed in the overall call structure (Figs 1–6). Using these temporal differences in calling songs as a clue, we combined data from populations having similar syllable rate functions and analyzed the spectral features of their song and their morphology. Cluster analyses of the spectral features of songs (Figs 10, 11) and morphological measures (Fig. 12) showed significant grouping among the populations we studied. In addition, our phylogenetic analysis using multiple loci in a multispecies coalescent model showed genetic divergence among all populations (Figs 13, 14), suggesting spatial structure and isolation among them (Fig. 15).

Isolation and population spatial structure.—Spatially structured populations may be caused by variation and heterogeneity in the landscape and depend on adaptation to the local environment and variation in the strength of gene flow across that landscape (Revilla and Wiegand 2008, Rettelbach et al. 2016, Pina and Schertzer 2018). Longleaf pine turkey oak sandhills are a stable, fire-adapted fire-climax community (Croker and Boyer 1975, Peet and Allard 1993). One might expect that loss of flight (as in *A. bartrami*) would evolve in stable habitats compared with
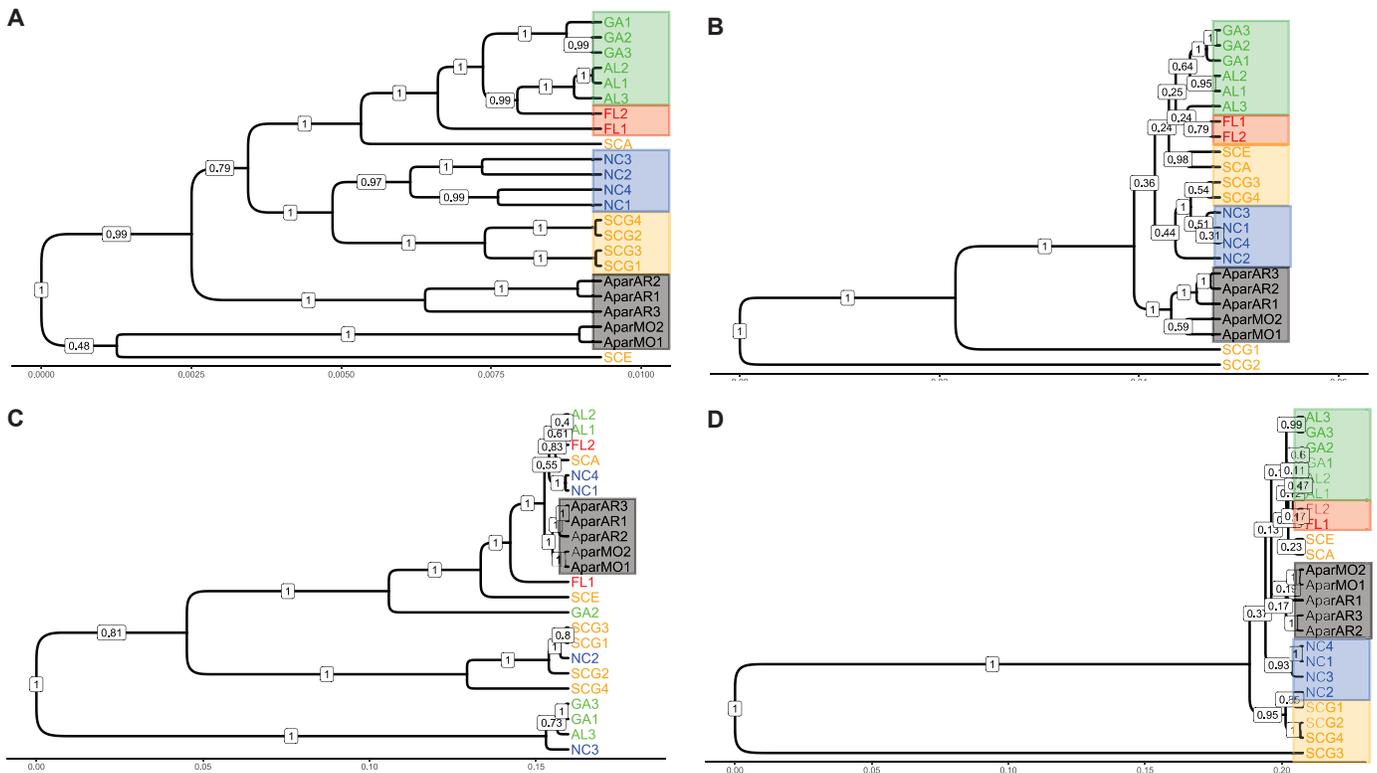

Fig. 13. Gene trees based on Bayesian multi-locus coalescent analyses. Numbers in branches are the Bayesian posterior probabilities from the analysis. Colored rectangles represent song morphs identified by relationships of syllable rate and temperature (green: GAL; red: FL; blue: NC; orange: SC; black: *A. parvipennis*). **A.** Cytochrome Oxidase I, barcoding gene (COI: 658bp); **B.** ITS1, ITS2, and 5.8S ribosomal genes (ITS3k: 3262bp). **C.** Tubulin-alpha I nuclear gene (TUB: 341bp). **D.** Wingless nuclear gene (WNG: 371bp). Phylogenies were plotted using R package ggtree (Yu et al. 2017).
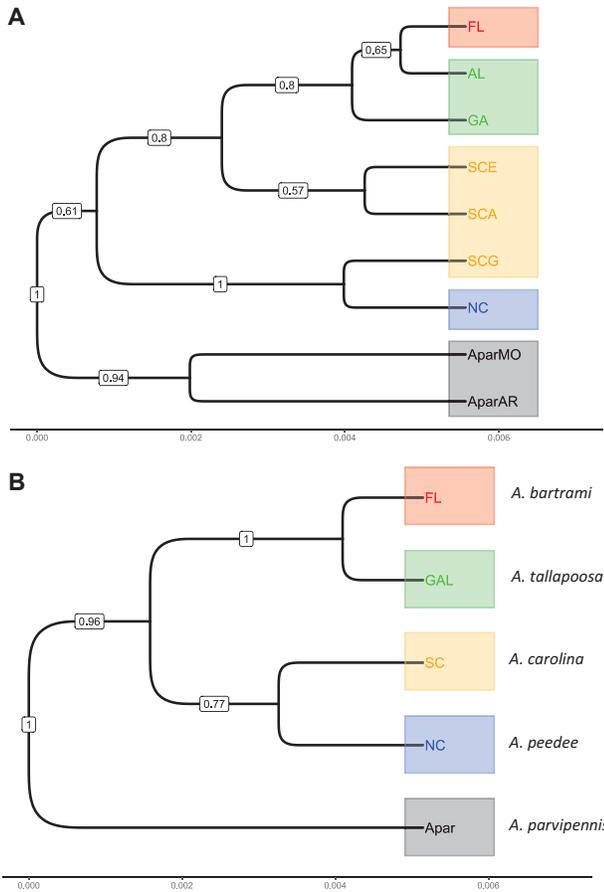
Fig. 14. Phylogenetic relationships (population/species trees) based on multi-locus coalescent analyses for populations of *A. bartrami* and *A. parvipennis*. Branch numbers are the Bayesian posterior probabilities for members in that lineage. Time shown is substitutions per site. Both topologies are robust across several analyses with different random starting points. **A.** Analysis for all populations sampled; **B.** Analysis for putative species (song morphs) based on relationships of syllable rate with temperature. Phylogenies were plotted using R package ggtree (Yu et al. 2017).

higher vagility adapted to more ephemeral and unpredictable environments (Grzywacz et al. 2018). Many ancient and present-day river drainages flowing from the Appalachian Mountains to the Atlantic Ocean and the Gulf of Mexico have, and continue to, fragment the longleaf pine ecosystem. River systems and their hydrogeological history have influenced isolation and speciation in fish (Hocutt et al. 1986, Mayden 1988), frogs (Lemmon et al. 2007), and salamanders (Kozak et al. 2006, Kuchta et al. 2016) of the Atlantic Slope and the Appalachian Mountains. Additionally, the longleaf pine ecosystem has experienced considerable fragmentation due to anthropogenic habitat loss and degradation (Peet and Allard 1993). Given the flightless behavior of the katydids we studied and the fragmentation of the Atlantic Slope by rivers and anthropogenic change, gene flow among populations is probably reduced. Reduced gene flow contributes to a complex spatial structure among populations and provides opportunities for local genetic changes through drift or selection that might lead to divergence and speciation (Fig. 15). Interestingly, *A. arenicola*, a member of the flight-capable *uhleri* complex, co-occurs with *A. bartrami* in the sandhill populations we sampled in north Florida and North Carolina. While the populations we studied were found to differ genetically across that ~700 km distance, *A. arenicola* does not (unpublished data).

Genetic variation: Gene trees vs species trees.—Discordance between gene trees and species trees can be caused by various evolutionary processes, including incomplete lineage sorting, gene duplication, hybridization, and gene flow (Mallo and Posada 2016). We used multiple genes and a multispecies coalescent analysis to account for incomplete lineage sorting, and here we discuss the gene trees from our analysis to consider the potential problems with each in determining the species/population trees.

Nuclear mitochondrial pseudogenes (numts) may be co-amplified with COI. These pseudogenes are difficult to detect and may influence barcoding results. Mitochondrial pseudogenes occur in a wide diversity of Orthoptera. Hawlitschek et al. (2017) showed that only 76% of the orthopteran species they studied were reliably identified by barcoding genes and mostly agreed with traditional taxonomy. However, some DNA barcoding sequences had large genetic distances within a species and,
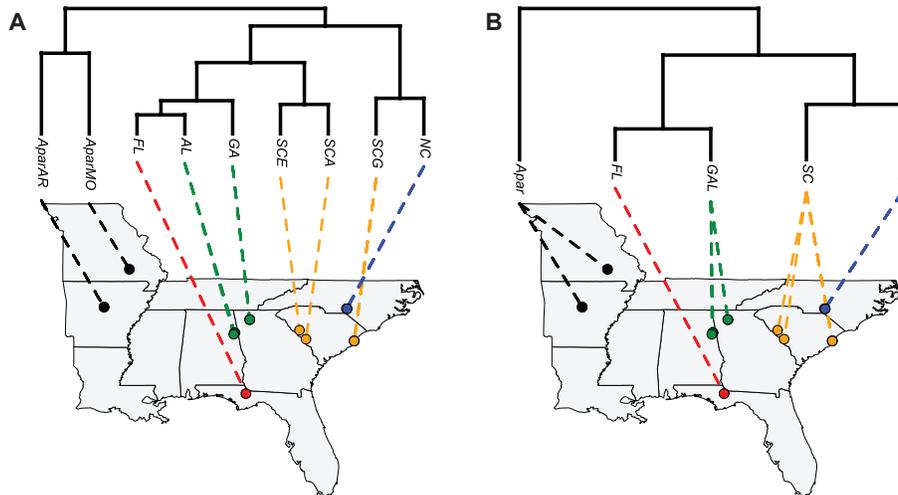

Fig. 15. Phylogeography of '*A. bartrami*'. Phylogenies were determined by a Bayesian multi-locus multi-species coalescent model using a Markov Chain Monte Carlo process. **A.** Phylogeographic distribution of sampled populations of supposed *A. bartrami* (AL, FL, GA, NC, and SC) and *A. parvipennis*; **B.** Phylogeographic distribution of song morphs that differed in syllable rates as a function of temperature. Plots were constructed using plot.to.map function of the R package phytools (Revell 2012).

in some cases, had identical DNA haplotypes between morphologically/ecologically divergent species, which were most likely caused by incomplete lineage sorting or hybridization (Hawlitschek et al. 2017). Using only DNA barcoding genes, genealogical paraphyly is common in many groups of closely related animals (see Trewick 2008). Research using only COI to determine relationships among closely related phaneropterines has not always confirmed relationships and species predicted from morphology, acoustic signals or ecology (e.g. Kensinger et al. 2017, Kocinski 2020). Our gene tree for COI using the Bayesian coalescent approach showed high support for most populations and, in general, agreed with data from our behavioral (acoustic signals) and morphological analyses. One COI sequence from an individual from Edgefield Co., SC was very different from the other *A. nr bartrami* (SCE, Fig. 13A), even though the BLAST search of this sequence closely matched *Amblycorypha floridana* and *Amblycorypha oblongifolia* sequences (HQ983649.1 and HQ983655.1, respectively).

In addition to COI, we sequenced the 5.8S ribosomal gene and internal transcribed spacers ITS1 and ITS2. ITS1 and ITS2 sequences have been useful in finding species-level differences in some insect groups, including katydids. Ullrich et al. (2010) investigated ITS1 and ITS2 from barbistine katydids and tested whether the secondary structure, which is determined by the interdependency of the interacting nucleotides of these ribosomal genes, might be useful in phylogenetic analyses. They found that ITS2 had two secondary structures similar to those known from other eukaryotes. ITS1 was much more variable, and it was, therefore, more difficult to predict its secondary structure. We did not investigate the secondary structure of the ITS DNA sequences. The 5.8S and ITS sequences appeared to improve our phylogenetic analysis and grouped AL and GA populations as well as NC with some SC populations.

Tubulin-alpha I genes have evolved through gene duplication in insects, and paralogues of tubulin may have different sequences (Nielsen et al. 2010) that could have influenced our gene tree. The information from our TUB sequences did not sort populations and song morphs in ways that we expected. Interestingly, eliminating TUB sequences from the analysis did not change the topology of the resulting species trees. Indeed, support values for grouping song morphs were greater when TUB sequences were included in the coalescent analysis. The WNG gene tree, although having many branches with low support values, consistently grouped populations of *A. parvipennis* and grouped individuals from the song morphs (GA with AL populations, NC populations, and SC populations).

Including nuclear, ribosomal, and mitochondrial genes in our analysis probably improved our overall understanding of the relationships among the populations we studied. Our results were similar to those of Kim et al. (2016), who found lower divergence in nuclear genes (<1% divergence) compared with mitochondrial genes (3–7% divergence) in *Tettigonia* from South Korea. Adding nuclear genes to their analysis contributed to an improved phylogenetic signal, which aided in identifying cryptic species.

Songs and diversity.—In phaneropterines, speciation may begin as the result of sexually selected changes occurring in the song structure of local populations that cause divergence (Heller et al. 2015). Because duets play important roles in phaneropterine courtship and allow for eavesdropping by individuals outside the duet, song complexity has probably evolved as countermeasures to eavesdropping (Villarreal and Gilbert 2014, Heller et al. 2015, Heller et al. 2017, Heller and Hemp 2020). Heller et al. (2017) found cryptic ethospecies in *Ducetia japonica* (long-winged and widespread) that showed little or no difference in genitalic diversity but had very different songs, and the file teeth associated with the stridulatory apparatus differed in shape, number, and size.

Small changes in timing and temporal song structure are enough to cause behavioral isolation in phaneropterines. Female *Mecopoda elongata* from populations that 'chirp' distinguish between trilling and double chirp songs (Dutta et al. 2017). The double chirpers of *M. elongata* have higher song rates. Similarly, we found that NC males produce doublet syllables with higher rates compared with their closely related geographic neighbors in SC.

Flightlessness, which probably decreases gene flow, may increase the rates of divergence among populations. Hemp et al. (2009) found that flightless *Monticolaria* katydids in Africa were isolated on mountain ranges, resulting in speciation. We believe that river systems have probably isolated populations of flightless members of the *rotundifolia* complex inhabiting the coastal plain of the southeastern United States and that this isolation allowed for divergences in their song, morphology, and genetics.

Taxonomy.—Based on our work and the differences in song, morphology, and genetics that we found among the populations we studied, we describe three new species of round-winged katydids: *A. carolina* sp. nov., *A. peedee* sp. nov., and A. *tallapoosa* sp. nov.

Tettigoniidae Krauss, 1902
Phaneropterinae Burmeister, 1838
Amblycoryphini Brunner von Wattenwyl, 1878

Amblycorypha **Stål, 1873**

Type species.—*Amblycorypha oblongifolia* (De Geer, 1773).

Amblycorypha carolina **Spooner & Forrest, sp. nov.**
https://zoobank.org/A279436F-23BC-492F-B8C6-9EA7A6E7E430
Figs 2, 7–16, Table 2

Material examined.— **Holotype:** USA • ♂; South Carolina, Georgetown, Hobcaw Barony, Kings Rd; 33.3480°N, 79.227°W; 5 Jun. 2009; T.G. Forrest and L.D. Block leg.; Anb-M04-2009; DNA (MS-034) NCBI accession SAMN31929333; REC (2009 Tape02 PGM 1023); UNCA to be transferred to FSCA.

Allotype: USA • ♀; South Carolina, Georgetown, Hobcaw Barony, Kings Rd; 33.3480°N, 79.2271°W; 5 Jun. 2009; T.G. Forrest and L.D. Block leg.; Anb-F03-2009; DNA (MS-004) NCBI accession SAMN31929331; REC (2009 Tape02 PGM 1024 duet with M06), REC (2009 Tape02 PGM 1025 duet with M02); UNCA to be transferred to FSCA.

Paratypes: (16♂, 16♀) USA • 1♀, 1♂; South Carolina, Aiken Co; 14 May 2008; J.D. Spooner leg.; UNCA to be transferred to FSCA • 1♀; South Carolina, Aiken Co; 24 May 2008; J.D. Spooner leg.; UNCA to be transferred to FSCA • 1♀, 1♂; South Carolina, Aiken Co; 30 May 2008; J.D. Spooner leg.; UNCA to be transferred to FSCA • 2♀, 3♂; South Carolina, Aiken Co; 4 Jun 2008; J.D. Spooner leg.; UNCA to be transferred to FSCA • 1♂; South Carolina, Aiken Co; 27 May 2019; T.G. Forrest leg.; UNCA to be transferred to FSCA • 2♀, 1♂; South Carolina, Edgefield Co; 21 May 2008; J.D. Spooner leg.; UNCA to be transferred to FSCA • 3♂; South Carolina, Edgefield Co; 24 May 2010; J.D. Spooner leg.; UNCA to be transferred to FSCA • 6♀, 1♂; South Carolina, Edgefield Co; 5 Jun.

Fig. 16. Holotype of *Amblycorypha carolina*, Carolina round-winged katydid. **A.** Dorsal view; **B.** Lateral view. Scale bars: 5 mm.

2007; J.D. Spooner leg.; UNCA to be transferred to FSCA • 3♀, 5♂; South Carolina, Georgetown Co; 5 Jun. 2009; T.G. Forrest and L.D. Block leg.; UNCA to be transferred to FSCA.

Other specimens.—4♂, 4♀ from Walker et al. 2003: USA • 2♂; South Carolina, Aiken Co; 17 Jun. 68; J.D. Spooner leg.; FSCA • 1♀; South Carolina, Aiken Co; 10 Jul. 87; J.D. Spooner leg.; FSCA • 1♂; South Carolina, Aiken Co; 7 Jun. 88; J.D. Spooner leg.; FSCA • 1♂; South Carolina, Aiken Co; 21 Jun. 88; J.D. Spooner leg.; FSCA • 1♀; South Carolina, Aiken Co; 14 Jun. 93; J.D. Spooner leg.; FSCA • 1♀; South Carolina, Aiken Co; 21 Jun. 93; J.D. Spooner leg.; FSCA • 1♀; South Carolina, Edgefield Co; 7 Jun. 88; J.D. Spooner leg.; FSCA.

Size measurements (mm).—Holotype: PrnL: 6.1, PrnW: 4.4, TegL: 28.0, TegW: 8.9, HwEx: 2.2, FemL: 28.2, and TibL: 29.7 mm (Fig. 16). Allotype: PrnL: 6.7, PrnW: 4.4, TegL: 26.4, TegW: 7.5, HwEx: NA, FemL: 28.9, TibL: 29.0, OviL: 8.8.

Etymology.—This species is named for its geographic location within South Carolina, north of the Savannah River and south of the Pee Dee River.

Common name.—Carolina round-winged katydid.

Differential diagnosis.—Members of this species are best distinguished from other members of the *rotundifolia* species group and from *A. bartrami*, in particular by calling song. Syllable rates as a function of temperature are ~5.8s⁻¹ at 25°C (Fig. 8) and differ from all other eastern members of the *rotundifolia* complex. Although the syllable rates of *A. carolina* are similar to *A. parvipennis* from the western USA, *A. carolina* have significantly longer tegminal lengths and hindwing exposure (Table 2). Calling songs of *A. carolina* have a sustained series of about 25 syllables compared to more than 100 syllables in the sustained portion of songs from Florida *A. bartrami* (Fig. 7). Series that preceded the main, sustained series also tend to have fewer syllables than Florida *A. bartrami* (2–4 vs 5–6, respectively Fig. 7).

Description.—Individuals are typically green and have all attributes of members of the *rotundifolia* complex of *Amblycorypha* (Walker et al. 2003). Female size measurements ($\bar{x}\pm$SE in mm, N) are on average PrnL: 6.60±0.07, 17; PrnW: 4.12±0.08, 17; TegL: 26.6±0.47, 13; TegW: 8.12±0.25, 15; FemL: 27.6±0.40, 15; TibL: 28.7±0.40, 14; OviL: 9.93±0.25, 17. Males size measurements are on average PrnL: 6.03±0.09, 17; PrnW: 4.00±0.09, 17; TegL: 26.2±0.44, 17; TegW: 8.21±0.17, 17; FemL: 27.1±0.42, 15; TibL: 28.2±0.34, 15 (Table 2). Male calling songs are composed of several series of single syllables with initial series having 2 to 4 syllables leading up to a sustained final series with about 25 syllables (Figs 2, 7). During the sustained portion of the final series the syllable rates are about ~5.8s⁻¹ at 25°C. Syllable rates change with temperature following the linear relationship: rate=0.293(temp)-1.467 (Fig. 8).

Amblycorypha peedee Forrest, sp. nov.
https://zoobank.org/40A9EF19-9D67-481C-8EB3-6BBA1F0EFA99
Figs 5, 7–15, 17, Table 2

Material examined.—**Holotype:** USA • ♂; North Carolina, Richmond Co., Sandhills Gamelands; 35.0528°N, 79.6035°W; 1 Jul. 2006; T.G. Forrest leg.; Ambar?-M03-2006; DNA (MS-044) NCBI accession SAMN31929325; REC (2006 Tape01 PGM 04); UNCA to be transferred to FSCA.

Allotype: USA • ♀; North Carolina, Richmond Co., Sandhills Gamelands; 35.06139°N, 79.63982°W; 16 Jul. 2004; T.G. Forrest leg.; Ambar?-F01-2004; **DNA** (NA); **REC** (NA); UNCA to be transferred to FSCA.

Paratypes: (8♂, 0♀) USA • 1♂; North Carolina, Richmond Co.; 16 Jul. 2004; T.G. Forrest leg.; UNCA to be transferred to FSCA • 6♂; North Carolina: Richmond Co.; 1 Jul. 2006; T.G. Forrest leg.; UNCA to be transferred to FSCA • 1♂; North Carolina, Richmond Co.; 19 Jul. 2007; T.G. Forrest leg.; UNCA to be transferred to FSCA.

Other specimens:—2♂, 0♀: North Carolina specimens from Walker et al. 2003 deposited in Florida State Collection of Arthropods. USA • 1♂; North Carolina, Moore Co.; T.J. Walker leg.; (doublet song); FSCA • 1♂; North Carolina, Hoke Co; 26 Jul. 1964; T.J. Walker leg.; FSCA.

Size measurements (mm).—Holotype: PrnL: 5.9, PrnW: 4.45, TegL: 27.8, TegW: 10.0, HwEx: 3.9, FemL: 27.2, TibL: 28.8mm (Fig. 17). Allotype: PrnL: 7.0, PrnW: 46, TegL: 28.0, TegW: 9.6, HwEx: 2.2, FemL: 29.9, TibL: 31.0, OviL: 11.0mm.

Etymology.—This species is named for its geographic location, with populations north of the Pee Dee River, which isolates it from populations of *A. carolina*.

Common name.—Pee Dee round-winged katydid.

Differential diagnosis.—*Amblycorypha peedee* is best distinguished from other species in the *rotundifolia* complex by calling song. Syllables are almost always produced in doublets with syllable rates within doublets of about 11.6s⁻¹ at 25°C (Fig. 8) and rates of 4.0s⁻¹ at 25°C between doublets. The sustained portion of calling songs of *A. peedee* has about 17 syllables with 2–3 syllables in each of the series preceding the sustained portion (Fig. 7).

Fig. 17. Holotype of *Amblycorypha peedee*, PeeDee round-winged katydid. **A.** Dorsal view; **B.** Lateral view. Scale bars: 5 mm.

Fig. 18. Holotype of *Amblycorypha tallapoosa*, Tallapoosa round-winged katydid. **A.** Dorsal view; **B.** Lateral view. Scale bars: 5 mm.

Description.—Individuals are typically green with characteristics of the *rotundifolia* complex of *Amblycorypha* (Walker et al. 2003). The male's calling song consists of a single syllable type that are produced in several (4–12) bouts of a 2–3 syllables preceding a sustained series of about 17 syllables (Figs 5, 7) that occur in pairs (doublets) (Fig. 5D). During the sustained portion of the song, at 25°C the syllable rate between doublets is about $4.0s^{-1}$ and is about $11.6s^{-1}$ within the doublet. The function of syllable rate within a doublet in response to changes in temperature is rate=0.583(temp)-2.92 (Fig. 8). Female size measurements for the single individual that was collected are PrnL: 7.05; PrnW: 4.58; TegL: 28.0; TegW: 9.63; FemL: 29.9; TibL: 31.0; OviL: 11.0. Males' measurements ($\overline{x}$±SE in mm, N) are on average PrnL: 5.84±0.04, 9; PrnW: 4.32±0.06, 9; TegL: 28.2±0.21, 9; TegW: 9.32±0.18, 9; FemL: 27.3±0.38, 6; TibL: 28.7±0.44, 6 (Table 2).

Amblycorypha tallapoosa Forrest, sp. nov.
https://zoobank.org/9F0FEE14-A7D9-45B6-8B72-CD2C52562766
Figs 3, 4, 7–15, 18, Table 2

Material examined.—**Holotype:** USA • ♂; Alabama, Cleburne Co., Heflin, Talladega Nat Forest, CR 548; 33.78012°N, 85.52666°W; 2 Jun. 2007; T.G. Forrest leg.; Ambar-M05j-2007; DNA (MS-030) NCBI accession SAMN31929312; REC (2007 Tape03 PGM 10); UNCA to be transferred to FSCA.
    Allotype: USA • ♀; Alabama, Cleburne, Pinhoti Trl, Coleman Lake; 33.78624°N, 85.56705°W; 2 Jun. 2007; T.G. Forrest leg.; Ambar-F02j-2007; DNA (MS-144) NCBI accession SAMN31929311; REC (2007 Tape03 PGM 05 duet with AmuGA-M01j-2007), REC (2007 Tape03 PGM 07 duet with Ambar-M04j-2007), REC (2007 Tape03 PGM 10 duet with Ambar-M05j-2007); UNCA to be transferred to FSCA.
    Paratypes: (6♂, 4♀) USA • 1♂; Alabama, Cleburne Co.; 2 Jun. 2007; T.G. Forrest leg.; UNCA to be transferred to FSCA • 1♂ Alabama, Cleburne Co.; 3 Jun. 2007; T.G. Forrest leg.; UNCA to be transferred to FSCA • 2♂; Georgia, Gordon Co.; 9 Jul. 2005; J.A. Hamel and T. Richardson leg.; UNCA to be transferred to FSCA

• 2♀, 1♂; Georgia, Gordon Co.; 5 Jul. 2006; T.G. Forrest leg.; UNCA to be transferred to FSCA • 2♀, 1♂; Georgia, Gordon Co.; 1 Jun. 2007; T.G. Forrest leg.; UNCA to be transferred to FSCA.

Other specimens:—One specimen from Walker et al. 2003 •1♂; Alabama, Cleburne Co.; 29 Aug.1964, T.J. Walker leg.; FSCA.

Size measurements (mm).—Holotype: PrnL: 4.9, PrnW: 3.4, TegL: 24.3, TegW: 7.7, HwEx: 3.3, FemL: 23.7, TibL: 25.5mm (Fig. 18). Allotype: PrnL: 5.3, PrnW: 3.7, TegL: 24.1, TegW: 7.3, HwEx: 2.2, FemL: 26.5, TibL: 26.2, OviL: 9.2mm.

Common name.—Tallapoosa round-winged katydid

Etymology.—This species is named for its geographic location, with populations north of the Tallapoosa River and within the boundaries formed by its confluence with the Coosa River.

Differential diagnosis.—Although most of the size measurements of this species are smaller than the other eastern species we studied in this project (Table 2), calling songs are the best way to determine members of *A. tallapoosa*. The syllable rates as a function of temperature for *A. tallapoosa* males ($\sim$13s^{-1} at 25°C) are the fastest among the species we studied (Fig. 8). Additionally, the main syllable series of the songs of *A. tallapoosa* have fewer syllables (7.5±1) than the other species we studied (Fig. 7).

Description.—Individuals have all the characteristics typical of the *rotundifolia* complex (Walker et al. 2003). Size is generally small for the *rotundifolia* group. On average males' sizes are ($\overline{x}$±SE in mm, N) PrnL: 5.08±0.14, 7; PrnW: 3.64±0.08, 7; TegL: 25.4±0.70, 7; TegW: 7.83±0.21, 7; FemL: 23.3±0.11, 7; TibL: 25.1±0.20, 7 and females are on average PrnL: 5.99±0.23, 5; PrnW: 3.86±0.13, 5; TegL: 26.0±0.67, 5; TegW: 8.07±0.34, 5; FemL: 25.9±0.49, 5; TibL: 26.6±0.59, 5; OviL: 10.7±0.38, 5 (Table 2). The main portion of the calling songs of males are series of about 8 syllables produced at rates of about $13s^{-1}$ at 25°C (Figs 3, 4, 8). Preceding the sustained portion of the song,

males produce 1–6 shorter bouts of 3–8 syllables (Fig. 7). During the steady portion of the song, the relationship of syllable rate with changes in temperature (°C) is rate=0.750(temp)-5.86 (Fig. 8).

Future work.—More data from other geographic locations would help resolve several interesting questions. For example, why do populations of *A. carolina* differ so much genetically among the three sites we sampled in South Carolina? Also, it would be important to sample katydids on each side of the major rivers to determine the degree of isolation and reduction of gene flow. This would be particularly interesting around 1) the Pee Dee River where the doublet songs of *A. peedee* are found north of the river (Hoke Co., Richmond Co., Moore Co., NC) but not south of the river (Stanley Co., NC), 2) on either side of the Savannah River where song rates of *A. carolina* are much slower to the north (Edgefield Co., Aiken Co., and Georgetown Co., SC) than they presumably are to the south in GA, and 3) in AL where the calling songs of *A. tallapoosa* have fast rates in the region between the Coosa and Tallapoosa Rivers (Cleburne Co.) but have rates similar to FL *A. bartrami* farther south and west (Perry Co., AL see pink in Fig. 8).

Acknowledgments

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Supplementary material 1

Author: Timothy G. Forrest
Data type: xls
Explanation note: Spreadsheet with morphological measurements of specimens that are included in the paper.
Link: https://doi.org/10.3897/jor.32.96295.suppl1

Supplementary material 2

Author: Timothy G. Forrest
Data type: xls
Explanation note: Spreadsheet with the acoustical measurements of song rates as a function of temperature for recordings in the paper.
Link: https://doi.org/10.3897/jor.32.96295.suppl2

Supplementary material 3

Author: Timothy G. Forrest
Data type: xls
Explanation note: Spreadsheet containing counts of syllables for recordings in the paper.
Link: https://doi.org/10.3897/jor.32.96295.suppl3

Supplementary material 4

Author: Timothy G. Forrest
Data type: xls
Explanation note: Spreadsheet showing the distance/dissimilarity matrices for spectral analyses in the paper.
Link: https://doi.org/10.3897/jor.32.96295.suppl4