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Molecular Phylogeny for the *Obolopteryx* Katydids of the Southwestern United States (Orthoptera: Tettigoniidae: Phaneropterinae)

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ABSTRACT

Obolopteryx Cohn *et al.* 2014, formerly *Dichopetala* Rehn and Hebard 1914, is a newly erected genus of North American phaneropterine katydids. Here, we provide the first molecular phylogeny for the genus *Obolopteryx* and compare our results to previous morphological studies. We used two mitochondrial genes (COI and Cytb) to resolve relationships in the genus. Molecular results failed to find congruence with any of the previous morphological hypotheses, suggesting that the genitalia traits used for morphological inferences are not necessarily phylogenetically informative. Further, we found preliminary evidence that the recently erected genus *Mactruchus* may be nested within the established *Obolopteryx*.

Key words: *Obolopteryx*, *Dichopetala*, mitochondrial DNA, phaneropterines, genitalia.

INTRODUCTION

Orthoptera is among the oldest orders of insects dating back more than 300 million years ago (MYA) by mitochondrial molecular clock estimates (Gaunt and Miles, 2002; Song *et al.,* 2015), and deep relationships within the taxon are mostly resolved (Flook and Rowell, 1998; Flook *et al.,* 1999; Song *et al.,* 2015). Among which, the Tettigoniidae are of great interest in understanding both acoustic communication and sexual selection (Bailey and Rentz, 1990; Gerhardt and Huber, 2002; Gwynne, 2001). However, a basic understanding of evolutionary relationships among genera is still lacking among members of the Tettigoniidae. For example, the mostly subtropical Phaneropterinae subfamily alone is estimated to include over 2300 species (Grzywacz *et al.,* 2014). The unresolved affinities among *Obolopteryx* is particularly problematic, as some populations and species (e.g., *O. oreoeca*) of the Chihuahuan Desert are imperiled, even a basic understanding of relationships within this group is valuable for conservation. Here, we present a preliminary molecular phylogeny for the relatively unstudied Southwestern United States members of the genus *Obolopteryx* (Cohn *et al.,* 2014).

The genus *Obolopteryx* (formerly *Dichopetala*), until 2015, was composed of eight morphologically similar species that overlap to varying degrees in geographic

distribution (Cohn *et al.,* 2014). They range from Arizona east to central Texas and south through northeastern Mexico, and recent data suggests there are additional species in Mexico (Orthoptera Species File). Little is known about their ecology or reproductive biology, with published reports limited to taxonomic studies describing male and female genitalia (Rehn and Hebard, 1909; Cohn *et al.,* 2014). Most phaneropterines display courtship behavior whereby females click in response to a specific "trigger pulse" of the male song, which in turn directs males to approach females. While such reversal of ancestral courtship behavior (females phonotaxing to singing males) periodically occurs among Old World phaneropterines, *Obolopteryx* are one of few North American genera that display this behavior. The tegmina in *Obolopteryx* females are so diminished that they do not contact each other, and are thus incapable of producing song. Worldwide, many phaneropterines show the diminished tegmina state (Bey-Bienko, 1954; Harz, 1969), although the condition is rare in the New World.

The phylogenetic relationships among the species in this genus are nontrivial due to increasingly recognized species diversity, overlapping distributions, and confounding morphology among *Obolopteryx* genitalia. Herein we provide the first molecular phylogeny for the United States *Obolopteryx*, and compare our results to inferences based on morphological traits.

METHODS

Samples included in our analysis were compiled from a combination of field collections, museum vouchers and previous published material (Table 1). Obtaining samples of *Obolopteryx* from the Southwestern United States is nontrivial, many of which are rare and no longer present at previously documented localities (BJK and colleagues, per. com.). Overall, within the *Obolopteryx* genus, we include all seven of the currently recognized species within the United States. At the time of this analysis, a single Mexican species was also described, but we were unable to obtain tissue from outside the United States. We collected individuals of *Obolopteryx oreoeca*, *O. brevihastata*, and *O. castanea* from multiple locations in Southwestern Texas and preserved them in 95% ethanol in the field. Specimens of *Obolopteryx gladiator*, *O. catinata*, *O. emarginata*, *O. seeversi, Mactruchus serrifer,* and *Arethaea gracilipes* (a thread legged katydid) were loaned from the University of Michigan, Museum of Zoology. We have included the species *Mactruchus serrifer* as part of our analysis, since it was formerly a member of *Dichopetala* (which included the species now placed in *Obolopteryx*). We included *Arethaea gracilipes* as an outgroup, as well as two additional outgroup sequences from GenBank: an old world cone-headed katydid, *Ruspolia dubia*, and a locust, *Locusta migratoria* (GenBank accession numbers EF583824 and JN858153, respectively). For all tissues, we isolated DNA from alcohol-preserved hind femurs using E.Z.N.A.® Insect D.N.A. Kits (Omega BioTek) according to the manufacturer's protocol.

We analyzed partial sequences for the cytochrome c oxidase subunit 1 (COI, 704 bp) and cytochrome b (Cytb; 751 bp) mitochondrial genes for nine phaneropterines

of the following species: *Arethaea gracilipes, Mactruchus serrifer, Obolopteryx brevihastata, O. castanea, O. catinata, O. emarginata, O. gladiator, O. oreoeca,* and *O. seeversi*. We amplified COI using published primers COI-F: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and COI–R: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Snyder *et al*., 2009). Primers for Cytb, (Cytb-F: 5'-CAA ATA TCY TTY TGA GGR GC-3' and Cytb-R: 5'-GTT TTC AAA ACR TAY GCT T-3'), were designed for this study from an alignment of related orthopteran taxa from GenBank. We amplified both genes under the following PCR conditions: an initial denaturing of 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30sec, 47°C for 1 min and 72°C for 1 min, with a final hold of 72°C for 7 min. PCR products were gel-purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). We sequenced in both directions using the PCR primers above on an Applied Biosystems ABI3730 genetic analyzer at the Core Facility of Oklahoma State University. We initially aligned sequences using CLC Main Workbench 6.8.2 (Qiagen), and then edited alignments manually in MEGA 5.2.2 (Tamura *et al.,* 2011). Initial phylogenetic analysis of each mtDNA gene yielded congruent results, so we concatenated both mitochondrial genes in our alignment for all analyses. We acknowledge the fundamental problem of potentially inflating bootstrap values when concatenated genes (Salichos and Rokas, 2013), but mitochondrial loci should be an exception to this problem since it is inherited as a single functional unit (Birky, 2001; Wolstenholme, 1985).

Table 1. List of material examined.

Species	N	Locality	Deposit
Arethaea gracilipes	1	Doña Ana Co., New Mexico	KCEM[2] #296
Mactruchus serrifer	1	Municipio de Huimilpan, Queretaro/ Mexico	UM[1] #10
Obolopteryx brevihastata	1	Brewster Co., Texas	KCEM[2] #301
Obolopteryx castanea	1	Webb Co., Texas	KCEM[2] #303
Obolopteryx catinata	1	Cameron Co., Texas	UM[1] #48
Obolopteryx emarginata	1	McMullen Co., Texas	UM[1] #39
Obolopteryx gladiator	1	Kenedy Co., Texas	UM[1] #43
Obolopteryx oreoeca	1	Jeff Davis Co., Texas	KCEM[2] #304
Obolopteryx seeversi	1	Bandera Co., Texas	UM[1] #2

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We generated phylogenies using maximum likelihood (ML) and Bayesian methods. The software PAUP 4.0 (Sinauer Associates, Inc. Publishers) and MrBayes 3.2 (Huelsenbeck and Ronquist, 2001) were used to estimate phylogenies for ML and Bayesian methods, respectively. Support for the ML topology was evaluated using 10,000 bootstrap replicates. For the Bayesian consensus tree we report posterior probabilities based on 37,500 posterior likelihoods from the trace file, ignoring a 25% burnin period.

For the ML tree, we used Modeltest 3.7 (Posada and Crandall, 1998) to select the GTR+I+G evolutionary model with the following parameters: *Lset Base* = (0.3245

0.2175 0.1346), *Nst* = 6, *Rmat* = (10.3206 49.7093 28.9093 6.2844 191.2924), *Rates* = gamma, *Shape* = 1.2301, *Pinvar* = 0.4995. We assigned the outgroup *Locusta migratoria*, and used the TBR branch swapping algorithm. For the ML tree, we enforced the constraint of monophyly of the *Obolopteryx* genus. For the Bayesian tree we used the following priors: *Nst*=6, *rate*=invgamma, and *Nchains*=6. We enforced a constraint on the monophyly of the Phaneropterinae subfamily, and assigned the outgroup *Locusta migratoria*. We set the MCMC for *nreps* = 5,000,000 and sampled every 100 runs. We generated a consensus tree using the *sumt* command with a 25% burn-in.

In a separate analysis, we used Beast 1.7 (Drummond *et al.,* 2012) to estimate divergence times within *Obolopteryx* using the same evolutionary model and similar parameters as used in our MrBayes analysis. We used the birth-death tree model of speciation and fixed a strict, uniform clock assuming a divergence rate of 2.3% per million years between taxa (Brower, 1994; Shapiro *et al.*, 2006).

RESULTS

We found that *Ruspolia dubia* and *Locusta migratoria* shared an unresolved polytomy with the Phaneropterinae subfamily of katydids (Fig. 1). However, the Phaneropterines outgroup *Arethaea gracilipes* was supported in both the maximum likelihood and Bayesian phylogenetic analyses as sister to the remaining dichopetalines. Two pairs of sister taxa shared moderate support by both methods. One pair clustered *O. castanea* as sister to *O. oreoeca*. The second grouped *O. brevihastata* as sister to the ancestor of *O. gladiator* and *O. seeversi*. Additionally, *O. gladiator* and *O. seeversi* had moderate support as sister taxa in the Bayesian analysis. Deeper within the *Obolopteryx* phylogeny there was uncertainty about the placement of *O. emarginata* in relation to the other groups. *Obolopteryx* was paraphyletic with respect to *M. serrifer*, which was nested within the genus. *Mactruchus serrifer's* sister relationship to *O. catinata* was highly supported by both Bayesian and ML methods.

Using the evolutionary rate of 2.3% divergence per million years (MY), the median ages of the *Obolopteryx* nodes ranged from 5.17± *CI* [4.19,6.27] MYA to 14.06± *CI* [12.14,16.17] MYA. Six of the species within *Obolopteryx* diverged from one another in the last 10.33± *CI* [8.92,11.90] MY. *Mactruchus serrifer* and *O. catinata* appear to have diverged approximately 12.05± *CI* [9.94,14.30] MYA.

DISCUSSION

This phylogeny marks the first molecular hypothesis for relationships within the genus *Obolopteryx*, and our results provide an initial framework for understanding evolution in this group. Mitochondrial genes are established as good initial approximations of phylogenetic relationships in the Orthoptera (Flook and Rowell,1997), although nuclear markers should be sought for further analysis (Toews and Brelsford, 2012). Still, many interspecific relationships are proposed by this analysis and provide direction for future work. However, of particular interest involves the unexpected position of *O. emarginata* and its relationships with the remaining well-resolved *Obolopteryx*.

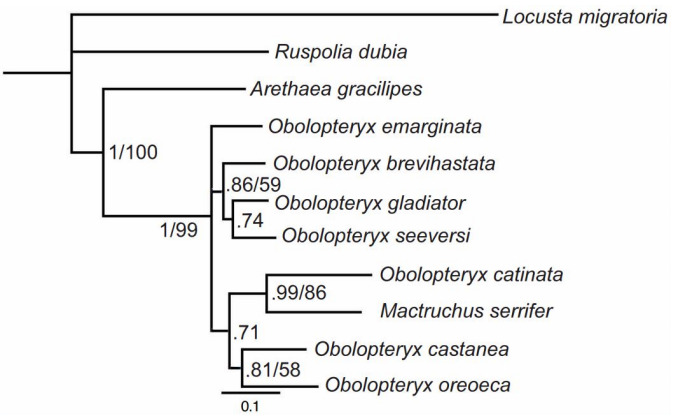

Fig. 1. *Obolopteryx* phylogeny constructed using Bayesian methods with posterior probabilities. Bootstrap values from ML analysis are secondarily included below. Branch support values ≥ 0.70 and ≥ 0.50 are shown for posterior probability and bootstrap analysis, respectively. Scale bar indicates substitutions per site.

Interestingly, this phylogeny supports almost none of the original relationships proposed by Rehn and Hebard (1914) based on morphological characteristics of male cerci. Rehn and Hebard's phylogeny hypothesized sister relationships for three taxon pairs included in our study, including *O. castanea* and *O. brevihastata*, *O. gladiator* and *O. emarginata* and *O. oreoeca* and *O. catinata,* although they refrain from further inferences among such pairs. Here, our molecular analysis provides some evidence to the contrary for all three pairings. While our results are consistent with cerci shape unifying all *Obolopteryx*, such characters are not informative in elucidating sister relationships. Cercal shape may exhibit considerable homoplasy. Thus, our results caution against using cercal morphology alone to reveal close affinities, much as Cohn *et al*. cautioned against depending on spinose ovipositors and the extent of tegmina reduction (Cohn *et al.,* 2014).

Cohn *et al.* (2014) also discussed the relationships within the *Obolopteryx*. They suggested that *O. gladiator, O. seeversi,* and *O. emarginata* share a common ancestor based on male cerci, subgenital plates and epiprocts. Our molecular results support the sister relationship of *O. gladiator* and *O. seeversi* (and recover *O. brevihastata* as sister to this pair).

Based on extant taxa in our analysis, it appears that *Obolopteryx* species radiated from one another in a relatively slow and stepwise fashion between 5 and 14 MYA during the second half of the Miocene, which was marked by a long and gradual cooling period preceding the most recent ice age. Notably, *M. serrifer* and *O. catinata* shared a common ancestor approximately 12 MYA during the warmest point of the Miocene. The Miocene epoch was marked by the expansion of grasslands, but it is unclear how modern distributions were influenced historically by this expansion, since much of the North American fauna was subsequently affected by the repeated glacial cycles during the Pleistocene (Hewitt, 1996). Interestingly, recent branches that precede our poorly supported, deep nodes are greater than one million years

apart, and thus we suspect that incomplete lineage sorting is unlikely contributing to the lack of resolution. Rather, resolution may be improved by including more slowly evolving nuclear markers.

Mactruchus serrifer has recently been moved to a separate genus based predominantly on the lack of forked cerci in males (Cohn *et al*., 2014). Further, the distinctiveness of this genus rests on the shape of the epiphallus rather than male cerci, a situation which Cohn *et al*. admit is systematically perplexing. Within the genus there appears to be no epiphallus commonalities between the three existing species. Our mitochondrial data presented here suggest that this new genus, *Mactruchus*, is nested within *Obolopteryx*, and further taxonomic study may support subsuming *Mactruchus serrifer* into *Obolopteryx*. The close relationship between *M. serrifer* and *O. catinata* was among the best supported in our phylogeny, and further reinforces that rapid evolution of male genitalia potentially confound species relationships among the dichopetalines.

When considering the spatial distributions of taxa included in our molecular analysis, two of the best-supported sister groups (*O. brevihastata* with (*O. gladiator/O. seeversi)* and *O. castanea* with *O.* oreoeca) suggest contrasting models of speciation. Species distributions seldom provide sufficient evidence to infer speciation (Coyne and Orr, 2004), and *O. castanea* and *O. oreoeca* occur in adjacent geographic proximity with only limited overlap, consistent with some forms of allopatric divergence. However, *O. gladiator, and O. seeversi* fall entirely within (but near the margins) of the distribution of *O. brevihastata*, and alternatively suggests a peripatric mode of speciation. This notion is consistent with our sampling over three years (2010-2012), and suggests that *Obolopteryx* species distributions are patchy in general, ephemeral from year to year, and potentially correlated with precipitation (BJK pers. comm.). *Obolopteryx* is typically abundant when adequate ground cover is available, but negligible when absent. Thus, it seems plausible that short periods of reproductive isolation are potentially generated by climatic influences in the already patchy deserts of the Southwestern U.S. and northeastern Mexico. Still, the above explanations are tentative, and do not alone provide a sufficient explanation for complete reproductive isolation between either of the above species pairs in *Obolopteryx*.

The contrasting molecular and morphological evidence suggests that in closely related species, male genitalia potentially evolve rapidly, and are decoupled from the putatively neutral molecular markers used here. In katydids, rapid evolution has been documented in the development of genitalic species differences as an effective means of reproductive isolation (Rentz, 1972), and male genitalia in general (Eberhard, 1985). While these traits are taxonomically informative for Obolopteryx, our data caution against using these traits alone for phylogenetic inference since hypotheses based primarily on male genitalia contrast markedly with molecular hypotheses.

Our results provide an initial molecular perspective on relationships within and among the *Obolopteryx* occurring within the Unites States. Logistical difficulties prevented us from sampling in Mexico, although recent work (Cohn *et al*., 2014) has since identified 20 additional species in multiple closely related New World genera, emphasizing the importance of a molecular phylogeny for this expanding group. Our

study provides a novel perspective on this taxonomic group and provides a framework to compare the newly described taxa. One major consideration of future systematic work is that male genitalia in this group conflict with this initial molecular analysis. However, such incongruence is not surprising due to the overlapping geographic distributions and potential hybridization of many closely related taxa, fostering the rapid evolution of these traits.

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