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Investigating genetic relationships within Gryllotalpidae: a molecular hypothesis

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Abstract

We present a first preliminary molecular analysis of relationships among a sample of living members of the Gryllotalpidae, based on partial nucleotide sequence data of the 16S mitochondrial gene. Our analysis defines five groups that diverged from each other approximately 196 to 284 Mya in the Mesozoic era. This study supports the monophyly of the genus *Scapteriscus* and its placement in the subfamily Scapteriscinae, as well as the inclusion of the genus *Triamescaptor* in the subfamily Gryllotalpinae. The monophyly of the large genus *Gryllotalpa* is not supported, suggesting a revision of the genus is needed.

Key words

molecricket, mtDNA, molecular systematics, Gryllotalpidae

Introduction

While the insect order Orthoptera is one of the oldest extant insect lineages (~290 million years, Grimaldi & Engel 2005) and member taxa are often well known and common, putative relationships among the families based on morphological data have not gained consensus (see review in Gwynne 1995, Gorochov 1995, DeSutter-Grandcolas 2003). Molecular analyses are being added to the literature above the family level (*e.g.*, Jost & Shaw 2006, Panaram 2007, Fenn *et al.* 2008) to resolve this issue. Very few molecular sequences resolved to species level in the Gryllotalpidae have been deposited into GenBank (but see *Kim et al.* 2005, Fenn *et al.* 2008). A wealth of information on the Gryllotalpidae is now easily accessible through the Orthoptera Species File (Eades & Otte 2010), but within the family, only a preliminary analysis of the relationships among species based on morphological characters is available (Hill *et al.* 2002).

Representatives of the family Gryllotalpidae are distributed worldwide, other than in the polar regions (see distribution maps in Eades & Otte 2010). These molecrickets are distinguished by having mole-like fossorial forelimbs and the singing of sexual advertisement calls from specialized burrows that they construct in the soil (Bennet-Clark 1970, 1987; Daws *et al.* 1996). The family is represented by 99 described species in one extinct and six extant genera in two subfamilies, as well as four extinct genera that have not been resolved to subfamily level (Eades & Otte 2010, see also review of extinct species in Perrichot *et al.* 2002).

The genus *Gryllotalpa* is both the largest and the most widely distributed (63 extant species in North America, Europe, Asia, Africa and Australia), while representatives of the second largest genus (21 species), *Scapteriscus*, are found only in South America and as a single invasive species in North America. *Neocurtilla* (6 species, North and South America), *Indioscaptor* (4 species, Asia), *Gryllotalpella* (2 species, South America) and *Triamescaptor* (1 species, New Zealand) are much rarer (Eades & Otte 2010).

The two subfamilies are distinguished by the number of tibial dactyls, or claws, on the fossorial forelimb. Members of the Scapteriscinae have two dactyls and include the genera *Scapteriscus* and *Indioscaptor*, the latter separated from *Scapteriscus* in a recent revision (Nickle 2003). Members of the Gryllotalpinae (*Gryllotalpa*, *Neocurtilla*, and *Gryllotalpella*) have four dactyls. *Triamescaptor*, with three dactyls, has been grouped in the Scapteriscinae (Tindale 1928, Nickle 2003), but Eades and Otte (2010) include it with the Gryllotalpinae.

No hypotheses of relationships among the family or subfamilies based on molecular data have been proposed. Comparative studies of behaviors described in the extant Gryllotalpidae, such as communication via substrate-borne vibration (Hill & Shadley 1997, 2001), mating in leks (Hill 1999) or sprees (Walker 1983), or strategies employed in burrow construction (Hill *et al.* 2006), require a consensus phylogeny before any meaningful conclusions can be drawn (Brooks & McLennan 1991, Martins 1996). Here we present the first preliminary molecular analysis of relationships among a sample of living members of the Gryllotalpidae.

Methods

Taxon sampling.—A total of four live-caught (Oklahoma, USA, French Guiana, Portugal and New Zealand) and three laboratory-reared (Florida, USA) species, either fresh-frozen (-20°C) or preserved in ethanol (80-95%), were included in this study, along with eight 16S mitochondrial gene sequences deposited in GenBank (Fig. 1). These specimens represent at least 10 species from four of the six genera of the Gryllotalpidae, as well as a representative from the sister taxon Gryllidae, which was used to root the tree. Since three of the GenBank sequences were not identified to the species level, possibly as many as 14 species are represented in this analysis.

Extractions, amplifications, sequencing and alignment.—Qiagen DNeasy blood and tissue kits (Valencia, CA) were used to isolate DNA. The mitochondrial gene 16S was amplified via polymerase chain reaction (PCR) using the primer combinations: 16Sa (5'-CGC CTG TTT AAC AAA AAC AT-3'), 16Sb (5'-CCG GTC TGA ACT CAG ATC ACG T-3') (Kocher *et al.* 1989, Huang *et al.* 2000). PCR products were then run on a 1% agarose gel and successful products cleaned using EXOSAPIT (USB Corporation, Cleveland, OH). Sequencing reactions were performed using BigDye v3.1 (Life Technologies Corporation, Carlsbad, CA). Sequencing reactions were then cleaned using Sephadex G-50 (GE Healthcare Bio-Sciences Corporation,

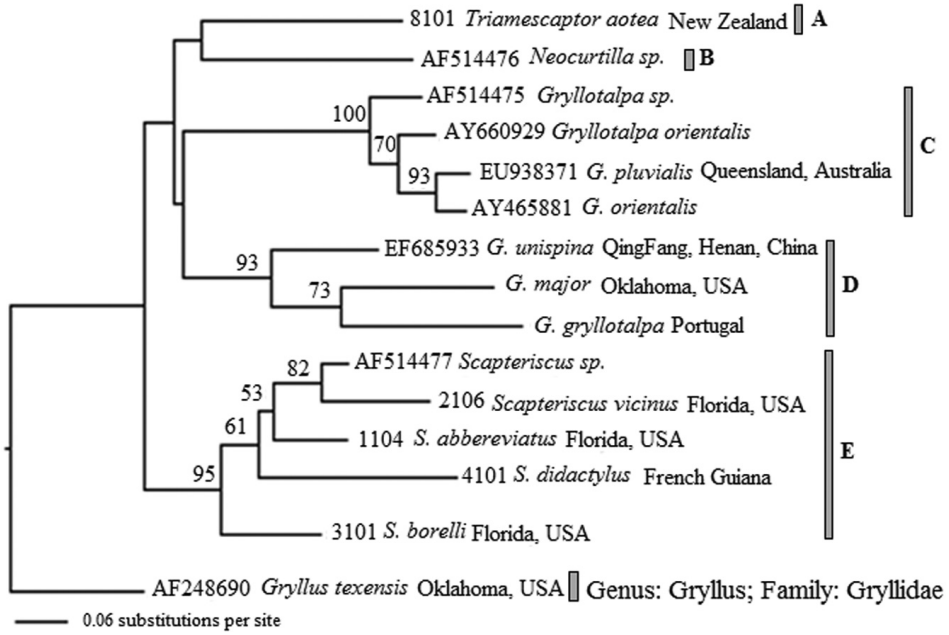

Fig. 1. ML tree depicting the evolutionary relationships among 13 species of molecricket belonging to the family Gryllotalpidae. The tree is rooted with *Gryllus texensis*. Bootstrap supports are mentioned at the base of the nodes. Bootstrap supports < 50 are not mentioned.

Piscataway, NJ) and reactions sequenced in both directions using an ABI 3130x automated genetic analyzer (Applied Biosystems, Foster City, CA). The 16S partial nucleotide sequences generated in the present study have been submitted to GenBank (accession numbers HQ259964-HQ259971).

Phylogenetic analyses.—Sequences were assembled and aligned using SEQUENCER and MEGA ver. 4 (Tamura *et al.* 2007), respectively. The appropriate model of nucleotide substitutions was selected by the Akaike Information Criterion (AIC) implemented in Modeltest ver. 3.7 (Posada & Crandall 1998). A maximum likelihood (ML) phylogeny with the appropriate model of nucleotide substitutions was reconstructed using PhyML ver. 2.4.4 (Guindon & Gascuel 2003). Using the same program, the bootstrap values were calculated from 100 replicates. The inferred tree was visualized using FigTree ver. 1.2.3 (Rambaut 2006). The pairwise percentage of evolutionary divergence among different groups was estimated using the Maximum Composite Likelihood method implemented in MEGA4 (Tamura *et al.* 2007). Using the same program, the standard errors were also estimated with 1000 bootstrap replicates.

Results

Phylogenetic analyses.—The GTR (General Time Reversable) model with the proportion of invariable site (I = 0.1824) and gamma distribution shape parameters (G = 1.1019) was the best-fit model selected by AIC. The nucleotide base frequencies for A, C, G, and T are 0.3441, 0.1173, 0.1913, and 0.3473, respectively—thus indicating the sequences are AT-rich (A% + T% = 69.14%). The substitution rate matrix is: [A-C] = 0.7855, [A-G] = 4.3102, [A-T] = 1.5611, [C-G] = 0.2693, [C-T] = 3.0822, [G-T] = 1.0000.

The ML tree shows the monophyly of the genus *Scapteriscus* (Fig. 1). However, the monophyly of the genus *Gryllotalpa* is not strongly supported. The evolutionary divergences between the five phylogenetically defined groups (A to E) are shown in Table 1. The evolutionary distances among the five groups are within the range of 30-36%. The genetic distances within C, D, and E are 8.131 ± 1.368 %, 22.770 ± 2. 427 and 9.658 ± 1.885, respectively.

By solving the equation $\lambda = d/2T$, where T is the time since divergence (in years) between the two species, d the genetic distance and λ the mutation rate, crude estimates can be made of divergence time. If the provisional mutation rate (λ) of 0.07% per one million years that has been reported for the 16S-mitochondrial gene of insects (Romano & Palumbi 1997, DeSalle *et al.* 1987) is applied, then based on the genetic distance estimates (Table 1), all five groups as shown in figure 1, diverged from each other approximately 216 ± 20 to 257 ± 26.6 Mya: this timing falls within early Jurassic to Permian periods. Similarly, based on this rate, the divergence among the species within groups C, D, and E are 58 ± 9.8 My, 162 ± 17.3 My, and 70 ± 13.5 My, respectively. These estimates suggest that within-group divergence occurred during the Cretaceous period, which is also the time period from which fossils of the four extinct genera that are not resolved to subfamily were found (Eades & Otte 2010).

Discussion

In this study using 16S mitochondrial gene partial nucleotide sequence data, we explored the evolutionary relationships among different species of molecrickets representing four genera of the family Gryllotalpidae. The monophyly of the genus *Scapteriscus* in our molecular tree is consistent with the placement of that genus in the subfamily Scapteriscinae, separate from the other genera in our study (Eades & Otte 2010). Our results support the placement by Eades and Otte (2010) of *Triamescaptor* within the Gryllotalpinae. Having so few specimens limits the conclusions that can be drawn about the larger clade of *Triamescaptor*, *Neocurtilla* and *Gryllotalpa*, especially in the absence of representation of the genus *Gryllotalpella*. Yet, the lack of monophyly in the genus *Gryllotalpa* suggests that a revision of that genus is in order. This lack of monophyly in *Gryllotalpa* is consistent with results of a preliminary study of relationships in the family based on morphology (Hill *et al.* 2003).

The present study shows the 16S region of molecrickets to be adenine (A) + thymine (T)-rich biased, which is characteristic of the Ensifera (Jost & Shaw 2006), as well as of other insects (DeSalle *et al.* 1987). Due to constraints imposed by a high AT content, it has been reported that insect 16S sequences are evolving much more slowly than those from other groups of organisms (Romano &

Table 1. Estimates of evolutionary divergence over sequence pairs between groups. Analyses were conducted using the Maximum Composite Likelihood method in MEGA4. Below and above diagonal (empty cells) is shown the evolutionary distance (in percentage) and standard errors, respectively.

Group	A	B	C	D	E
A		3.63	3.76	3.72	3.16
B	30.30		3.65	3.30	3.37
C	33.00	30.29		2.91	2.97
D	36.02	32.12	29.46		2.78
E	31.43	32.94	30.27	31.92	

Palumbi 1997, DeSalle *et al.* 1987).

If a provisional mutation rate (0.07% per My) that has been previously estimated for the mitochondrial 16S-sequence data of other insects (DeSalle *et al.* 1987, Romano & Palumbi 1997) is applied to our dataset, it appears that the five groups (Fig. 1) diverged from each other during the period spanning approximately from 196 to 284 Mya, the timing of which is comparable to previous reports (Sharov 1971, Walker & Masaki 1989, Panaram 2007).

The earliest members of the Gryllotalpidae are known from fossils of the Jurassic period (Sharov 1971, Walker & Masaki 1989), and the family is hypothesized to have diverged as a clade during the Triassic period, based on a molecular analysis of the Ensifera that was calibrated from the fossil record (Panaram 2007). However, some evidence points to possible molecricket burrows from the Permian (Perrichot *et al*. 2002). Based on these time-of-divergence estimates and the ubiquity of the Gryllotalpidae across all continents (except for the polar regions), we predict that vicariance biogeography explained by continental drift has a primary role in intercontinental diversification of this family. Further, it is interesting to note that species belonging to the subfamily Scapteriscinae that are endemic to South America are estimated to have a common ancestor at approximately 70 ± 13.5 Mya, a time when South America had already drifted away from Africa.

In conclusion, our study supports the monophyly of the genus *Scapteriscus*, which is endemic to South America, and also predicts an interesting biogeographic history of the family Gryllotalpidae that can be further explored with larger taxon sampling from all the continents and using multiple genetic markers.

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