

Colonization of a newly cleaned cave by a camel cricket: Asian invasive or native?

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Crystal Cave in Kutztown, Pennsylvania, was discovered in 1871 while quarrying for limestone (Stone 1953). Crystal Cave is developed in a belt of Ordovician-age limestone and has an abundance of formations. The cave is about 110 m in extent with an upper level, and access is restricted by a blockhouse (Stone 1953). Crystal Cave is the oldest continually-operating commercial cave in the state, opening for a Grand Illumination in 1872 (Crystal Cave History 2010). It currently hosts about 75,000 visitors a year (K. Campbell, personal communication). Early visitors were guided using candles, oil, and kerosene lanterns, and for a grand lighting, kerosene was spilled onto flowstone and set ablaze to illuminate some of the larger rooms (Snyder 2000). By 1919, the cave was lit with battery-powered lights, and in 1929, 5000 feet of wiring with 225 light bulbs was installed. In 1974 new concealed wiring was installed with indirect sealed-beam spotlights (Snyder 2000). Crystal Cave has been heavily impacted by humans, and it showed. Soroka and Lavoie (2017) reported on work to clean up the cave to return it to more natural conditions by removal of soot and grime using power washing and scrubbing. Clean-up began in 2007 and was completed in 2010.

There are no historical records of cavernicolous animals from Crystal Cave. Rhaphidophorid crickets with reported distribution ranges in the area include the spotted camel cricket *Ceuthophilus maculatus* Harris, 1835 and the puteanus camel cricket *Euhadenoecus puteanus* Scudder 1877, which are both found in cave entrances, although they are primarily epigeal forest species (Hubbell 1936; Hubbell and Norton 1978). Biological inventories in Crystal Cave by visual census on and off trail showed no biota before cleaning began. Since 2007, occasional small animals have been found on the clean areas of the cave such as Collembolans on a water surface (Soroka and Lavoie 2017). In 2015, up to 50,000 camel crickets (**Figure 1**) were observed over the summer massing inside the entrance. Many species of crickets are found around entrances using the cave as a refuge and massing to exit the cave for nightly foraging (Campbell 1976; Lavoie et al. 2007). We used genetic barcoding to assist with identifying the Crystal Cave

cricket population.



Figure 1. (A) A juvenile cricket in Crystal Cave and (B) an adult female with younger crickets roosting near the entrance.

Methods

Crystal Cave cricket DNA samples were isolated from seven samples using the QIAGEN DNeasy Blood & Tissue Kit Quick-Start Protocol. All procedures were followed for Tissues. One hind femur was used as the tissue sample from each cricket. The cricket leg was cleaned with distilled water before it was cut into small pieces. Once the DNA was isolated, the DNA's concentration (OD_{260}) and purity ($OD_{260/280}$ and $OD_{260/230}$) were determined using the Nanodrop 2000 spectrophotometer (Thermo Scientific). Amplification of an 850-bp fragment of the cytochrome oxidase subunit I (COI) gene was done using the H7005 (Hafner et al. 1994) and the C1-J-1718 (Simon et al. 1994) primers. For PCR PuReTaq™ Ready-to-Go™ PCR beads (GE Healthcare) were used. To each PCR tube, 100-200 ng of DNA was added along with a final concentration of 1.6 μ M forward and reverse primers. The forward primer was 5'-CCGGATCCACNACRTARTANGTRTCRTG-3' (Hafner et al. 1994) and reverse primer 5'-GGAGGATTTGGAAATTGATTAGTTCC-3' (Simon et al., 1994). ddH₂O was added to bring it to a final volume of 25 μ l.

A C1000 thermocycler (Bio-Rad) was programmed as follows: 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 46°C for 30 seconds, and 72°C for 60 seconds, and a final 72°C for 5 minutes step. The PCR samples were separated and visualized (~800 base pairs) through DNA gel electrophoresis (1.25% agarose gel). The PCR products were excised from the agarose gels and purified using the New England BioLabs Monarch DNA Gel Extraction Kit. The DNA concentration and purity for each sample was determined using a Nanodrop spectrophotometer (Model 2000 Thermo Scientific). Each sample was prepared for sequencing by adding 10-20 ng DNA from each sample, 0.5 μ l of 2.0 μ M primer into a total volume of 3.5 μ l, by adding ddH₂O. The DNA samples were sequenced at the Clemson University Genomic Institute.

Sequences of each gene region were manually aligned with Geneious 5.6.3

(Biomatters Ltd., Auckland, New Zealand) following the similarity criterion (Simmons 2004). Sequence divergence was calculated with the Kimura-2-parameter (K2P) model (Kimura 1980). We used RAxML for maximum likelihood (ML) estimation (Stamatakis et al. 2008) through the online CIPRES portal (Miller et al. 2010), which uses the GTR+G model for heuristic searches and the GTR+CAT model when computing bootstrap replicates. We used PAUP* 4.0b10 (Swofford 2001) for maximum parsimony (MP) and performed analyses with heuristic parsimony searches, 1,000 stepwise random-addition-sequence replications, 10 trees held at each step, tree-bisection-reconnection (TBR) branch swapping, and saving all trees (MuLTrees). We assessed branch support with 1,000 nonparametric bootstrap replicates (Felsenstein 1985) following the same heuristic criteria. *Nausigaster meridonalis* and *Hybomitra rhombica* were used as outgroups.

Results and Discussion

Camel crickets (Orthoptera; Rhaphidophoridae) are widespread across North America with over 150 species descriptions published (Hubbell 1936; Arnett 2000). Many species are commonly found in houses, caves, and forested areas. Some are common in caves where they range from true troglobionts restricted to caves, to troglonexes found in both cave and surface environments. Like cellars and basements, caves are cool, dark, and damp. In Italy, the camel cricket *Dolichopoda schiavazzii* Capra, 1934 is found both in caves and subterranean environments of human origin (Allegrucci et al. 1997). In nutrient-limited environments, cave and camel crickets may be keystone species in importing fixed nutrients from the surface to support the base of the ecosystem (Poulson et al. 1995; Lavoie et al. 2007).

The basic local alignment search tool (BLAST) was used to determine percent sequence similarity among the Crystal Cave sequences and other raphidophorids that have been accessioned into the nucleotide database in GenBank. Accession numbers for the seven Crystal Cave cricket sequences in NCBI GenBank are MF44176–MF441182.

Mean pairwise sequence divergence of the seven Crystal Cave sequences was 0.8%. The COI results (**Figure 2**) were unexpected. BLAST revealed no direct match to the Crystal Cave cricket. The closest match on GenBank was *Diestrammena asynamora* Brunner von Wattenwyl, 1888 from China, with 87% sequence similarity, and an 85% match to *Diestramima* sp. Storozenkho, 1990 from Southeastern Asia.

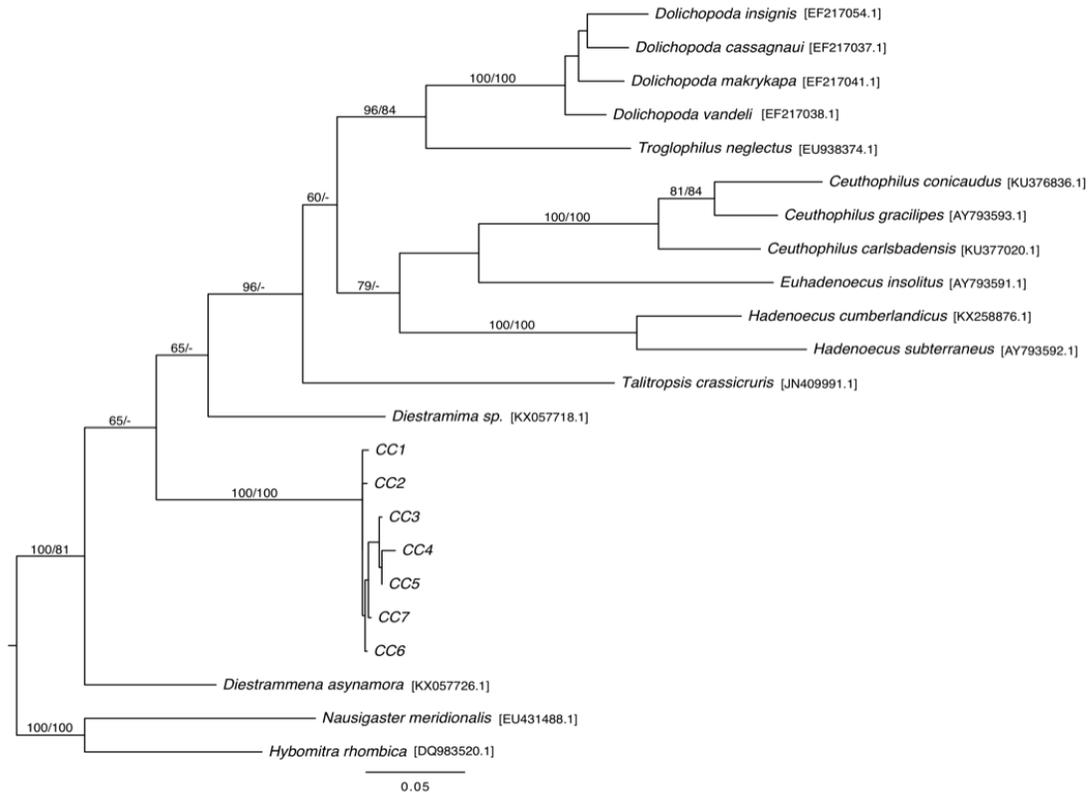


Figure 2. Maximum likelihood phylogram of cytochrome oxidase subunit I (COI) of all Crystal Cave (CC) accessions (MF44176-MF441182), plus selected Raphidophoridae sequences from Genbank. Support values are maximum likelihood bootstrap/maximum parsimony bootstrap. Genbank sequence identification codes are in brackets.

The Crystal Cave cricket clade is strongly supported and does not nest within any previously sequenced taxon. The position of the Crystal Cave clade as sister to a clade including all sampled raphidophorids, except *D. asynamora*, is moderately supported and suggests the Crystal Cave taxon is an early divergent lineage. Morphologically, the striking camouflage pattern of the Crystal Cave cricket closely resembles that of *Diestrammena* based on published images (Arnett 2000). The pattern on the native cricket *C. maculatus* is much more subdued, consisting of small spots on the head and thorax. A diagnostic feature of *Diestrammena* is the presence of a fastigium on the forehead with the appearance of down-pointing “horns” (Ferguson 2010). The Crystal Cave crickets lack a fastigium (**Figure 3**). Given our phylogenetic and morphological results, and the genetic distance between the other crickets in the database, the Crystal Cave crickets may be a new genus, which is usually set at differences greater than 5%, or sequences may not yet be accessioned into GenBank.



Figure 3. Close-up of the head of a Crystal Cave cricket. showing the absence of the fastigium that are characteristic of *Diestramenna*, and the lack of the typical spots seen with *C. maculatus*. (To see a *Diestramenna* fastigium, see Ferguson 2010.)

A continental-scale citizen science project was conducted by Epps et al. (2014) to determine the distribution and frequency of native and non-native camel crickets in basements and cellars of houses throughout North America, especially in southeastern states. In what they described as a “cryptic invasion,” Epps et al. showed that the exotic invasive species, *Diestramenna asynamora* Adelung, 1902, the greenhouse camel cricket, along with a second species, *D. japonica* Batchley, 1843, are more common in houses in the eastern United States than native camel crickets. In homes with identifiable camel crickets, 88% had *Diestramenna*, with 94% *D. asynamora* and 6% *D. japonica*. *D. japonica* was found only from the northeastern United States in Massachusetts, Pennsylvania, and New Jersey (Epps et al. 2014). *Diestramenna* crickets are native to caves in Asia and Russia. Gorochov et al. (2006) described six new species and one new subspecies of *Diestramenna* from caves in China. The crickets are described as troglomorphic.

Diestramenna camel crickets have been reported in North America since the 1890s (Scudder 1898). Morse (1904) speculated they arrived from the commercial importation of foreign plants, and are now found in all 50 states (Texas Institute of Invasive Species 2014). There are no published records of invasive, non-native camel crickets colonizing North American caves. The invasion of homes in North America by Asian camel crickets was only recently recognized (Epps et al. 2014), so perhaps they are more common in caves than we know. Colonization of Crystal Cave was into a newly-restored environment with no life before the cleanup. Can the Asian camel crickets out-compete native camel crickets or co-exist with them? Native camel crickets (*Ceuthophilis stygius*) and native cave crickets (*Hadenoeus subterraneus*) are commonly found together in entrance areas in caves in Mammoth Cave National Park (Lavoie et al. 2007). Given the cryptic invasion of basements by *Diestramenna* camel crickets replacing native crickets, we believe the same thing will happen in cave entrances with native camel crickets being

replaced. It will also be interesting to see if guano from invasive camel crickets can support development of an ecosystem of cavernicolous organisms in Crystal Cave.

Conclusion

The Crystal Cave cricket is likely a raphidophorid, but may be a new or unsequenced genus of camel cricket. The closest-related genera in GenBank are *Diestramenna* and *Diestramima* spp., both of Asian origin, with an 85–87% similarity match. If it is an Asian invasive, then it represents the first reported colonization of a cave by an invasive Asian camel cricket, identical to what is happening in basements in the United States (Epps et al. 2014). Our next approach is to do field collections in other Pennsylvania caves and basements to determine what species are present. We expect to find large populations of the Crystal Cave cricket since the 50,000 crickets that arrived in 2015 had to come from nearby sources.

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