Journal of Biogeography (J. Biogeogr.) (2016)

ORIGINAL ARTICLE

Comparative phylogeography of two codistributed subgenera of cave crickets (Orthoptera: Rhaphidophoridae: *Ceuthophilus* spp.)

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ABSTRACT

Aim We compare the phylogeographical structure among caves for co-occurring cave dwelling crickets (*Ceuthophilus*) in two subgenera *Ceuthophilus* (*Ceuthophilus*) (hereafter, called *Ceuthophilus*) and *Ceuthophilus* (*Geotettix*) (hereafter, called *Geotettix*). In our study area (central Texas), cave-inhabiting members of the subgenus *Ceuthophilus* are trogloxenes, roosting in the caves but foraging above ground and occasionally moving between caves, whereas members of the subgenus *Geotettix* are near-obligate cave dwellers, which forage inside the caves, and only rarely are found above ground. Differences in potential dispersal ability and ecology provide a framework for understanding their effects on the phylogeographical structure and isolation of populations of cave dwelling organisms.

Location Edwards Plateau, Texas, USA.

Methods We sequenced 1263 bp of two mitochondrial genes for a total of 309 individual rhaphidophorid cave crickets primarily in two subgenera of *Ceuthophilus* (Rhaphidophoridae). We reconstructed phylogenetic trees for each subgenus using Bayesian inference and then assessed whether their recent evolutionary history exhibited patterns of geographical structure.

Results Both *Ceuthophilus* and *Geotettix* exhibited strong geographical structure. Rather than exhibiting the expected lower levels of divergence and genetic structure, the trogloxenes of the subgenus *Ceuthophilus* show deeper divergences than the more cave-limited *Geotettix* taxa. *Ceuthophilus* has a higher proportion of unique haplotypes than does *Geotettix*. Mismatch distributions of *Ceuthophilus* and *Geotettix* differ, with *Ceuthophilus* exhibiting a multimodal mismatch distribution and *Geotettix* exhibiting a unimodal mismatch distribution.

Main conclusions Both cave cricket subgenera display strong geographical structuring. However, their phylogenetic trees differed in their geographical orientation, which could be explained by timing of colonization, association with caves and underground connections, and above-ground landscape or ecological barriers. For *Ceuthophilus*, the relatively high proportion of unique haplotypes and the multimodal mismatch distribution are consistent with relatively larger population size as compared to *Geotettix*.

Keywords
caves, *Ceuthophilus*, Edwards Plateau, genetic structure, mitochondrial DNA, phylogeography, troglobite, trogloxene

Journal of Biogeography

INTRODUCTION

Cave dwelling species provide exciting opportunities for phylogeographical studies because these species are often isolated, and mechanisms for divergence may be similar to those of oceanic island dwelling species (Barr, 1968; Culver, 1982; Culver & Pipan, 2009). Like island species, cave-adapted species often have small geographical ranges and high levels of endemism (Porter, 2007). Thus, caves can provide a simplified system in which to study evolutionary processes and historical factors affecting biogeographical patterns of species diversity and diversification (Juan *et al.*, 2010).

Many cave dwelling species are morphologically and physiologically adapted to life below ground (Packard, 1888; Barr, 1967, 1968; Culver *et al.*, 1995; Racoviță, 2006). However, there is variation in both the geological connections between cave systems (Moulds *et al.*, 2007) and in the degree of cave dependence of particular species (Caccone, 1985). These two factors can affect dispersal ability, levels of biogeographical isolation and phylogeographical patterns. For example, obligate cave dwellers, termed *troglobites*, are completely tied to caves for their survival and are therefore less capable of dispersal between disconnected cave systems. In contrast, *trogloxenes*, while dependent upon the caves for portions of their life histories, must spend some time outside of cave environments and thus might disperse between isolated caves more easily (Caccone, 1985). Therefore, we might expect cave organisms to exhibit varying genetic and species diversification patterns in relation to both dispersal ability in above-ground environments and connectivity of cave systems.

Several studies have examined the population genetic and phylogeographical structure of cave organisms, including both invertebrates and vertebrates. The results of these studies are somewhat variable depending on the connectivity of the geological features (Caccone & Sbordoni, 1987), the dispersal ability of the organisms (Rivera *et al.*, 2002), and relative timing of isolation in caves for the lineage being studied (Sbordoni *et al.*, 1981). For example, Caccone & Sbordoni (1987) generally found low rates of gene flow between populations of cave dependent *Hadenoecus* crickets, but they also noted that populations in regions with continuous and highly fissured limestone features were less genetically differentiated in comparison to higher levels of differentiation found between populations in regions where the limestone distribution was more fragmented. In general, molecular genetic studies of cave-adapted organisms have uncovered high levels of genetic differentiation (Hedin, 1997; Strecker *et al.*, 2003; Snowman *et al.*, 2010), cryptic diversification (Lefébure *et al.*, 2006; Niemiller *et al.*, 2012), and genetic variation structured by geographical and geological features (Allegrucci *et al.*, 1997; Allegrucci *et al.*, 2005). However, there are also cases where there is evidence of some recent dispersal between caves (Rivera *et al.*, 2002; Ketmaier *et al.*, 2013; Lefébure *et al.*, 2006) tied to ecological characteristics of the species being studied or geological connections between caves (Moulds *et al.*, 2007). Few of these studies have compared patterns of genetic diversity (e.g. Caccone, 1985) and phylogeography directly between co-occurring *troglobites* and the more facultative *troglophiles* and *trogloxenes*. Thus, the role of differences in dispersal ability and level of cave adaptation in generating differences in population structure and phylogeographical patterns is not well understood. Studies that compare co-occurring closely related species that differ in their potential for dispersal are needed to understand the relative contributions of dispersal versus geographical structures on the diversification of cave organisms.

Cave crickets in the genus *Ceuthophilus* are ideal for studying dispersal differences in cave associated organisms because species from two subgenera (*Ceuthophilus* and *Geotettix*) co-occur in caves of Texas, northern Mexico and New Mexico. We focused on sampling and studying species in these two subgenera from caves across the Edward's Plateau of Texas. Most of these caves are formed in karstified marine carbonates of the lower Cretaceous Edwards Limestone Group (Cooke, 2005). These two subgenera have contrasting ecologies, in particular with respect to above-ground dispersal abilities. In our study area, cavernicolous members of the subgenus *Ceuthophilus* are trogloxenes, utilizing caves to roost and lay eggs, but also utilizing the surface environment to forage (Taylor *et al.*, 2005) and disperse between caves that are in close proximity (< 1 km; Taylor *et al.*, 2004). In contrast, members of the subgenus *Geotettix*, in the study area, are near-obligate cave dwellers and forage within caves, only rarely emerging from caves, and have not been documented dispersing between of caves.

We focused on sampling and studying cave crickets in these two subgenera from caves across the Edwards Plateau of Texas. The Edwards Plateau is one of the largest karst areas in the United States and by far the most significant in the state. It has a north–south width of *c.* 250 km and extends west 500 km from the central part of the state. Karstified limestone crops out throughout most of the Edwards Plateau, and has minimal or no soil cover. The predominant limestone unit is the Lower Cretaceous Edwards Group and its equivalent units. Significant Palaeozoic carbonate rocks occur in the Llano Uplift region, which is geologically distinct from the plateau but geographically is often considered part of it.

For the purposes of the study, the Edwards Plateau karst is divided into karst regions (Fig. 1) defined by type of cave and karst development characteristic of each region, as well as by boundaries that also function as major barriers or restrictions to subsurface dispersion of cave-limited species (Smith & Veni, 1994; Veni, 2009). Hydrogeological barriers to subterranean dispersal between and within regions, and hence features that promote speciation within those areas, primarily include (1) the absence of cavernous rock horizontally or vertically due to erosional removal of the rock, faulting that laterally juxtaposes karstified with non-karstified rock, the presence of overlying and/or underlying non-karsti-

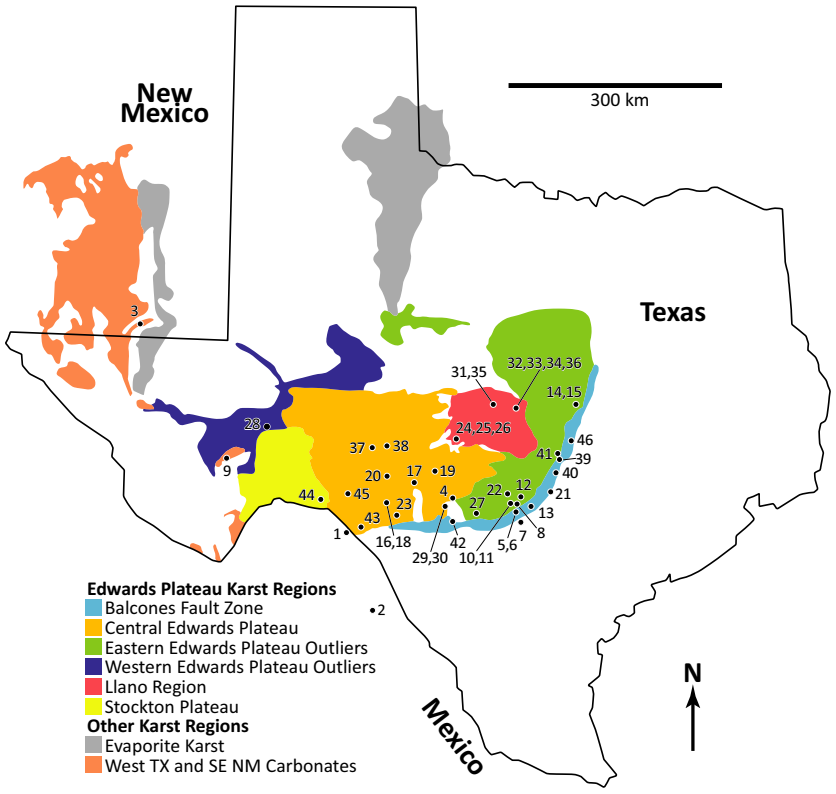

Figure 1 Distribution of sample sites and karst regions (adapted from Smith & Veni, 1994) (excluding Missouri and Kentucky outgroups). Mexico, Coahuila: 1. Cueva de Casa Blanca; 2. Cueva de la Azufrosa. USA, New Mexico, Eddy County: 3. Carlsbad Cavern. USA, Texas, Bandera County: 4. near Haby Salamander Cave. Bexar County: 5. MARS Shaft; 6. Poor Boy Baculum Cave; 7. Robber Baron Cave; 8. Tall Tales Cave. Brewster County: 9. 400 Foot Cave. Comal County: 10. Camp Bullis Bat Cave; 11. Camp Bullis Cave No. 1; 12. Preserve Cave; 13. Temple of Doom. Coryell County: 14. Mixmaster Cave; 15. Rocket River Cave. Edwards County: 16. Deep Cave; 17. Devils Sinkhole; 18. Punkin Cave; 19. Schroeder Bat Cave; 20. Writing on the Rocks Cave. Hays County: 21. Ezell's Cave. Kendall County: 22. Dead Man's Cave. Kinney County: 23. Kickapoo Cavern. Mason County: 24. Behren's Grotto; 25. Porcupine Pit; 26. Swift Cave. Medina County: 27. Ground Hog Cave. Pecos County: 28. Amazing Maze Cave. Real County: 29. All the Wonders and Joys Cave, 30. Little Dry Frio. San Saba County: 31. unnamed cave; 32. Cicurina Cave; 33. Lemons Ranch Cave; 34. Puberty Pit; 35. Rattlesnake Drop; 36. Turtle Shell Cave. Sutton County: 37. Caverns of Sonora; 38. IH-10 Cave. Travis County: 39. Lamm Cave; 40. Lost Oasis Cave; 41. Testudo Tube. Uvalde County: 42. Finley Bat Cave. Val Verde County: 43. unnamed cave; 44. Big Tree Cave; 45. Fern Cave. Williamson County: 46. Temples of Thor.

fied units; and (2) perennial streams where high water tables prevent terrestrial troglobites from reaching subterranean habitat on the opposite bank even if karstified rock occurs on both sides and below the stream.

Restrictions to dispersal are where these features are not fully present and allow relatively small areas where dispersal may take place. Such barriers and restrictions are especially well developed along the southern and eastern margin of the plateau along the Balcones Fault Zone.

In this study, we directly compare the phylogeographical and genetic structure of co-occurring populations of species in the subgenera *Ceuthophilus* and *Geotettix* in caves throughout central Texas. We used mitochondrial DNA sequences from two genes, cytochrome oxidase I (COI) and nicotinamide adenine dinucleotide dehydrogenase subunit 5 (ND5) to reconstruct phylogeographical history of codistributed cave associated species from these two subgenera, because mtDNA evolves rapidly over relatively short timescales and has been particularly useful for comparing levels of population genetic structure in other cavernicoles (e.g. Caccone & Sbordoni, 2001; Martinsen *et al.*, 2009; Bryson *et al.*, 2014). We compare the level of genetic structure between these two cricket subgenera to assess the scale at which differences in dispersal ability might translate into differences in genetic structure. We hypothesize that differences in dispersal ability result in more highly structured populations of *Geotettix* as compared to *Ceuthophilus*. Furthermore, the taxonomy of species in these two subgenera has been problematic and we assess our results in relation to morphology and taxonomy of these groups and thus identify genetically divergent lineages that are likely cryptic species.

MATERIALS AND METHODS

Specimens and collections

In total we sequenced mtDNA from 309 raphidophorid cave crickets (see Appendix S1 in Supporting Information)

including 179 individuals from the subgenus *Ceuthophilus* comprised of *C.* (*Ceuthophilus*) *secretus* Scudder, 1894, *C.* (*Ceuthophilus*) *conicaudus* Hubbell, 1936; and the undescribed taxon *C.* (*Ceuthophilus*) 'species B.' and 122 individuals from the subgenus *Geotettix* comprised of *C.* (*Geotettix*) *cunicularis* Hubbell, 1936 and *C.* (*Geotettix*) *polingi* Hubbell, 1936. We collected specimens primarily from counties within the Balcones Escarpment and Edwards Plateau of Texas (Fig. 1). This sampling design was aimed at testing whether these subgenera, which potentially differ in dispersal ability, also differ in phylogeographical structure. We sampled multiple *Ceuthophilus* individuals for the ingroups of each subgenus at each cave (where possible) from 43 caves distributed across 20 Texas counties, one cave in New Mexico and two caves in Mexico. To obtain a representative sample across the study area, we sampled a number of caves in each relevant Texas county in rough proportion to the number of caves that are known from that county. Because these counties are roughly equal in size, we used counties as approximately equal sampling units. We also obtained other rhaphidophorid cave cricket specimens primarily for use as outgroups from caves in Kentucky [*Hadenoecus subterraneus* (Scudder, 1861); $n = 1$], Missouri [*Ceuthophilus* (*Ceuthophilus*) *gracilipes* (Haldeman, 1850); $n = 1$, *Ceuthophilus* (*Ceuthophilus*) *williamsoni* Hubbell, 1934; $n = 1$], and New Mexico [*Ceuthophilus* (*Ceuthophilus*) *longipes* Caudell, 1924; $n = 2$, *Ceuthophilus* (*Geotettix*) *carlsbadensis* Caudell, 1924].

Specimens were captured by hand and placed in 95% ethanol to preserve DNA and morphology. An effort was made to collect several adult individuals of both sexes and both subgenera at each cave site. In the lab, a single leg was removed from each specimen for use in DNA extraction and the remaining portion of each specimen was deposited in the insect collection of the Illinois Natural History Survey as a voucher available for future morphological analyses.

DNA extraction, PCR and sequencing

We extracted total genomic DNA from a single leg of each specimen using a Qiagen Dneasy extraction kit (Qiagen, Valencia, CA, USA) following the kit protocol for animal tissues. For each specimen we sequenced 1263 bp of mitochondrial DNA (mtDNA) including 850 bp of COI and 413 bp of ND5. We amplified an 850 bp fragment of COI using the primers C1-J-1718 (Simon *et al.*, 1994) and H7005 (Hafner *et al.*, 1994) and sequenced this fragment using the C1-7-1718, H7005, and two of three internal primers designed specifically for this study: CeuthCOIL (5′-GATCCTGCTGG TGGAGGAGATCC-3′), and either CeuthCOIH (5′-GAATTG GATCTCCTCCACCAGCAGG-3′) or CeuthCOIHcunn (5′-G AATTGGATCTCCTCCTCCTGCYGG-3′). We amplified and sequenced a 413 bp fragment of ND5 using the primers F7081 and R7495 (Yoshizawa, 2004). For COI polymerase chain reaction (PCR) amplifications, we used the following thermal cycling profile: 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 46 °C for 30 s, 72 °C for 1 min, and 72 °C for 7 min. For ND5 PCR amplifications, we used the following thermal cycling profile: 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 42 °C for 30 s, 65 °C for 30 s and 65 °C for 7 min. We verified all PCR products on a 1% agarose gel and purified them using a QIAquick PCR Purification kit (Qiagen). Cycle sequencing reactions were performed at the University of Illinois DNA sequencing facility using an ABI Big Dye kit (Applied Biosystems, Foster City, CA, USA), the above-listed primers, and *c.* 75 ng of purified PCR product. We ran purified sequencing reaction products on an ABI 3730 capillary electrophoresis system (Applied Biosystems) and used SEQUENCHER (ver. 4.5; GeneCodes Co., Ann Arbor, MI, USA) to reconcile double-stranded sequences and to align sequences for analysis. All DNA sequences generated for this study have been deposited in GenBank (accession numbers KU376526–KU376834 and KU376835–KU377143).

Phylogenetic and phylogeographical analyses

We calculated uncorrected pairwise sequence divergence (*p*-distance) using PAUP* 4.0 BETA10 (Swofford, 2003) between taxa within each of these two subgeneric clades to compare the relative levels of divergence within each of the subgenera. For each subgenus, we plotted a frequency histogram of these pairwise genetic differences (mismatch distribution) to assess levels of divergence and structuring within each ingroup. To compare the level of within versus between cave genetic diversity, we calculated the proportion of unique haplotypes in each cave for each subgenus. This was done by counting the number of haplotypes recovered from each cave and dividing by the number of individuals sampled from that cave. Note that this measure is different from the standard population genetic parameter haplotype diversity, which requires larger sample sizes for its calculation than we obtained from each cave sampled for this study. *Ceuthophilus* in particular exhibited deep genetic divergences and multiple species in some caves (see below). Therefore, we calculated this proportion of unique haplotypes measure separately for each major clade of *Ceuthophilus* (see Fig. 2). For *Geotettix*, which showed shallower haplotype clades, we calculated this proportion both with and without accounting for major clades (Fig. 2). Note that calculating the proportion of unique haplotypes without splitting the *Geotettix* tree into major clades is conservative with respect to the hypothesis that the more cave-limited *Geotettix* has a smaller proportion (diversity) of haplotypes, because this calculation doesn't take into account cases in which haplotypes from divergent clades were found in the same cave (only two cases). We then compared the ratios of the number of haplotypes in each cave divided by the number of individuals sampled in each cave between *Ceuthophilus* and *Geotettix* using the non-parametric Wilcoxon rank sum test.

We estimated separate phylogenetic trees for the *Ceuthophilus* subgenera (*Ceuthophilus* and *Geotettix*) using Bayesian Inference (BI) under a maximum likelihood model as implemented in MRBAYES 3.1.1 (Ronquist & Huelsenbeck, 2003).

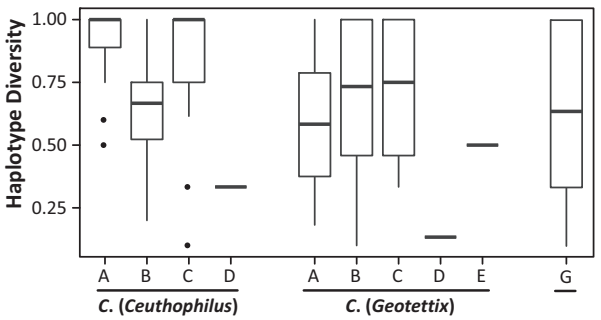

Figure 2 Proportion of unique haplotypes measured separately for each major clade of *Ceuthophilus* (*Ceuthophilus*), *Ceuthophilus* (*Geotettix*) and for all *Ceuthophilus* (*Geotettix*) combined (indicated as G). Letters indicate clades/lineages from the phylogenetic tree (Figs 4 & 5).

For both subgeneric data sets we conducted three BI analyses, each with a different partitioning scheme, including (all data combined), (2) two partitions (COI and ND5) and (3) three partitions (three mtDNA codon positions). We used MrModeltest 2.3 (Nylander, 2004) to determine which model of molecular evolution was most appropriate for each partition. We chose among the three partitioning schemes using Bayes factors (Brandley *et al.*, 2005) calculated using the harmonic mean from the sump command within MrBayes 3.1.1 (Ronquist & Huelsenbeck, 2003) and considered a difference of 2 ln Bayes factor > 10 as the minimum value to discriminate between partitioning schemes. For both subgenera, the Bayes factor analysis determined that the three-partition (mtDNA codon positions) scheme is the most appropriate and this is the one that we present here. For both subgenera, the three-partition scheme had likelihood models set for mtDNA 1st codon positions as GTR+I+G, mtDNA second codon positions as HKY+I, and mtDNA third positions as GTR+G with the state frequencies set as direchlet. All model parameters except the topology and branch lengths were set as unlinked between partitions and were estimated from the data as part of the analysis. For each data set, we ran two parallel runs of five million generations with four Markov chains to ensure that our analyses were not stuck at local optima (Huelsenbeck & Bollback, 2001). We sampled Markov chains every 500 generations yielding 10,000 parameter point estimates and used these point estimates minus the burn-in (500 generations) to create a 50% majority-rule consensus tree and to calculate Bayesian posterior probabilities as an assessment of nodal support. We used the outgroup *H. subterraneus* to root the topology.

To conduct a test of geographical phylogenetic signal we coded each terminal taxon for the karst region and county in which it was collected. We used maximum parsimony (MP) as implemented in MacClade 4.05 (Maddison & Maddison, 2002) to map the karst regions (n = 7), defined on the basis of geomorphology, geomorphical history, stratigraphy, geological structure, cave density and type of cave formation (Smith & Veni, 1994), and counties (n = 24) onto each subgeneric tree and then for each analysis we collapsed monophyletic groups from the same geographical region (karst region or county) to a single terminal taxon to prevent multiple sampling of closely related crickets from the same region, which would bias the test towards rejecting the null hypothesis. For each pruned subgeneric tree we also counted the number of character state changes for karst region and county and used MacClade to perform a Maddison & Slatkin (1991) randomization test to assess the significance of phylogenetic signal for karst region and county on each of the pruned subgeneric phylogenies. For each test, we randomized the region (karst region or county) 1000 times on each of the pruned subgeneric phylogenies. Then we compared the actual number of changes in region/county on the phylogeny to the random distribution of changes in region/county on each phylogeny to calculate a *P*-value.

To reconstruct the phylogeographical patterns and assess whether there was an influence of geography on the phylogenetic results we used the application GenGIS 2.0.2 (Parks *et al.*, 2009) to construct geophylogenies, which associate the leaf nodes of the *Ceuthophilus* and *Geotettix* trees with their geographical location of collection. GenGIS also provides a count of the number of edge crossings between leaf nodes and their geographical locations as a measure of the amount of geographical structure exhibited by a phylogenetic tree. Thus, the fewer edge crossings between leaf nodes and their geographical location, the more geographically structured a phylogeny. We used GenGIS to perform a linear axis analysis on each of the subgeneric geophylogenies to find the optimal orientation(s) that minimize the number of edge crossings and to generally determine how orientation of the geophylogeny affects the number of edge crossings between associated leaf nodes and their geographical locations. We conducted this linear axis analysis for all possible linear orientation gradients for each tree and produced a graph showing the number of crossings for all possible linear gradients and then ran 10,000 permutations at P = 0.05 level to identify all of the linear orientations with significantly low numbers of crossing events. We conducted these analyses on trees pared down to include only individuals from caves where both subgenera were sampled, allowing us to make comparisons of phylogeographical signal between both subgeneric trees.

RESULTS

P-distances and phylogenetic analyses

Comparisons of uncorrected *p*-distances within the ingroup ranged from 0% to 9.3% (average 2.9) for *Ceuthophilus* and 0% to 2.3% (average 1.1) for *Geotettix* (Fig. 3a,b respectively). Divergences between the ingroup and outgroup taxa averaged 10.7% (range 8.2–20.5) for *Ceuthophilus* and 11.0% (range 5.8–19.7) for *Geotettix*. The mismatch distribution of uncorrected *p*-distances for *Ceuthophilus* showed evidence of

multiple peaks (raggedness), whereas the *Geotettix* mismatch distribution was unimodal and relatively close to the *y*-axis. Phylogenetic trees for both subgenera were highly structured geographically (Figs 4 & 5). In many cases individuals within a given haplotype clade from the same cave had identical or nearly identical haplotypes. Comparisons between subgenera of the proportion of unique haplotypes in each cave revealed that on average *Geotettix* had a lower diversity of haplotypes in each cave compared to the number of individuals sampled than did *Ceuthophilus* ($W = 1054.5$, $P = 0.0019$ in test accounting for *Geotettix* clades, $W = 1015$, $P = 0.0023$, not accounting for major clades within *Geotettix*).

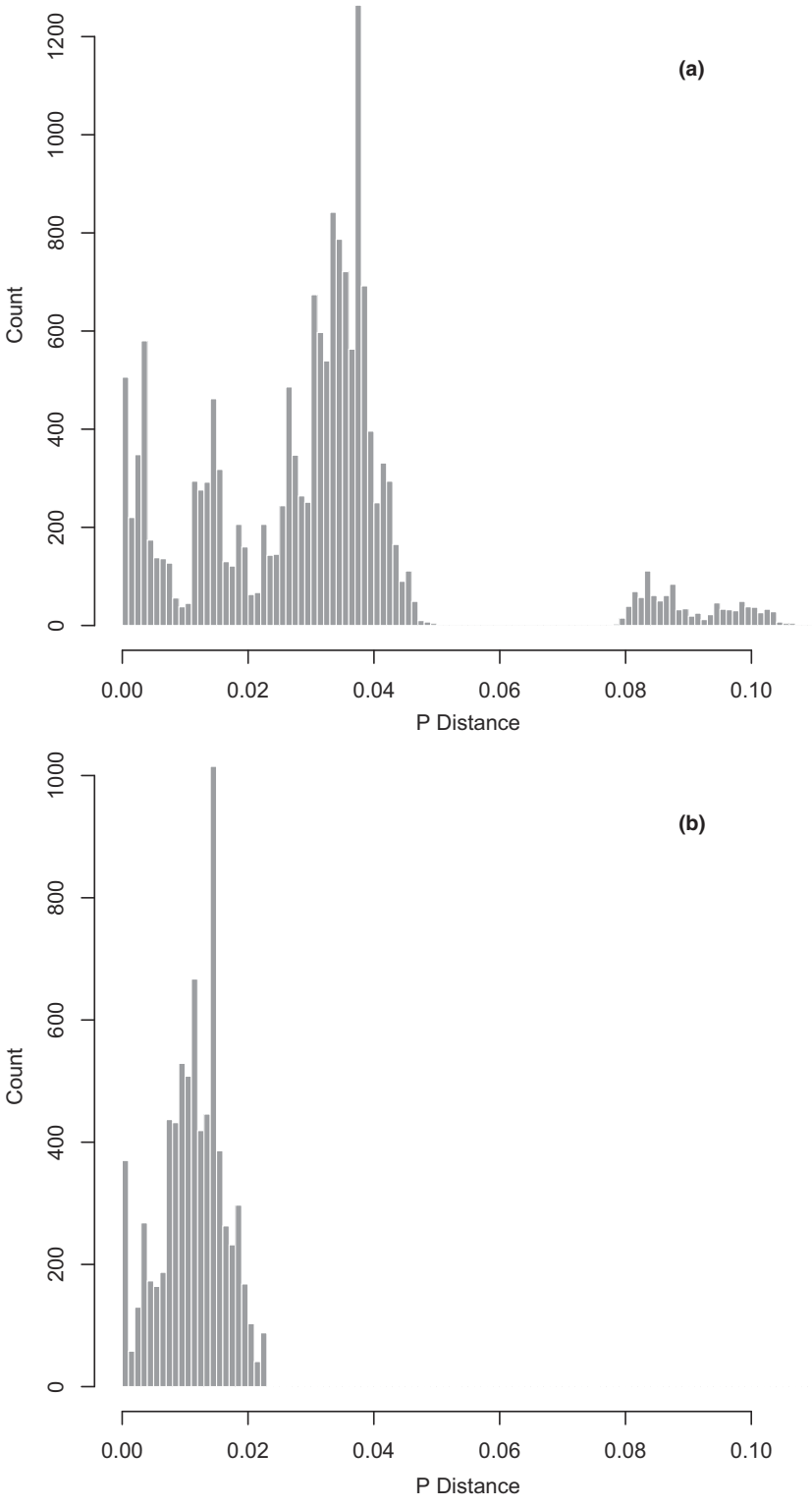

Figure 3 Frequency histograms (mismatch distributions) of uncorrected *p*-distances. Outgroup taxa are excluded.
(a) *Ceuthophilus* (*Ceuthophilus*), based on 15,931 pairwise comparisons.
(b) *Ceuthophilus* (*Geotettix*), based on 7381 pairwise distances.

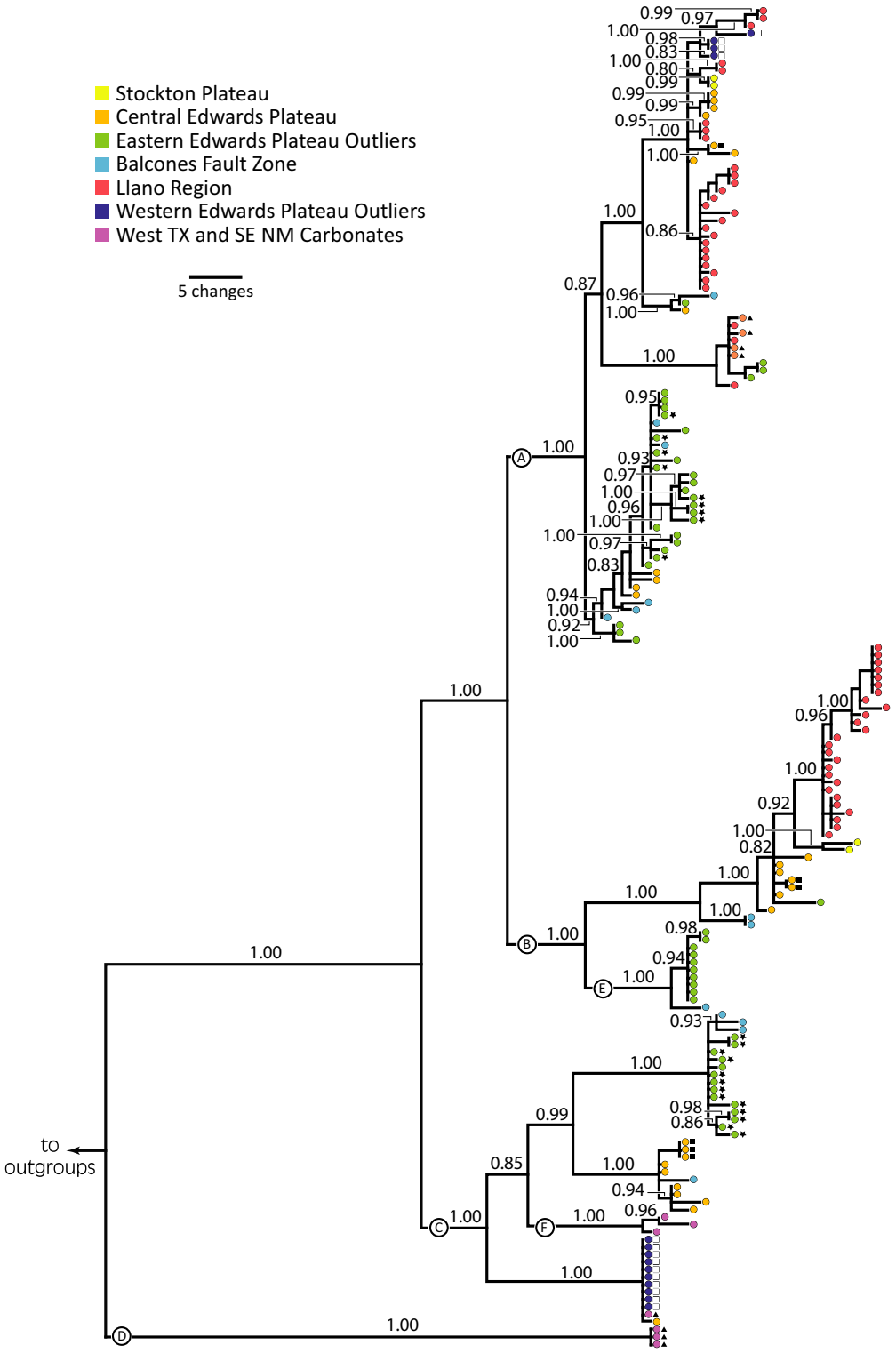

Figure 4 *Ceuthophilus* (*Ceuthophilus*) Bayesian consensus tree with minimum changes reconstructed as branch lengths. Numbers above branches are Bayesian posterior probabilities ≥ 0.80. Colours of circles, representing terminal taxa correspond to karst regions. Letters mark clades that are discussed in the text. Specific caves discussed in text are as follows: solid square, Writing on the Rocks Cave (Edwards County, Texas); open square, Amazing Maze Cave (Pecos County, Texas); triangle, 400 Foot Cave (Brewster County, Texas); star, Tall Tales Cave (Bexar County, Texas). Appendix S2 includes a Bayesian consensus tree with labels indicating the taxon number and cave name (see Appendix S1). All specimens are identified, based on morphology, to *Ceuthophilus* (*Ceuthophilus*) sp., unless indicated otherwise in Appendices S1 and S2.

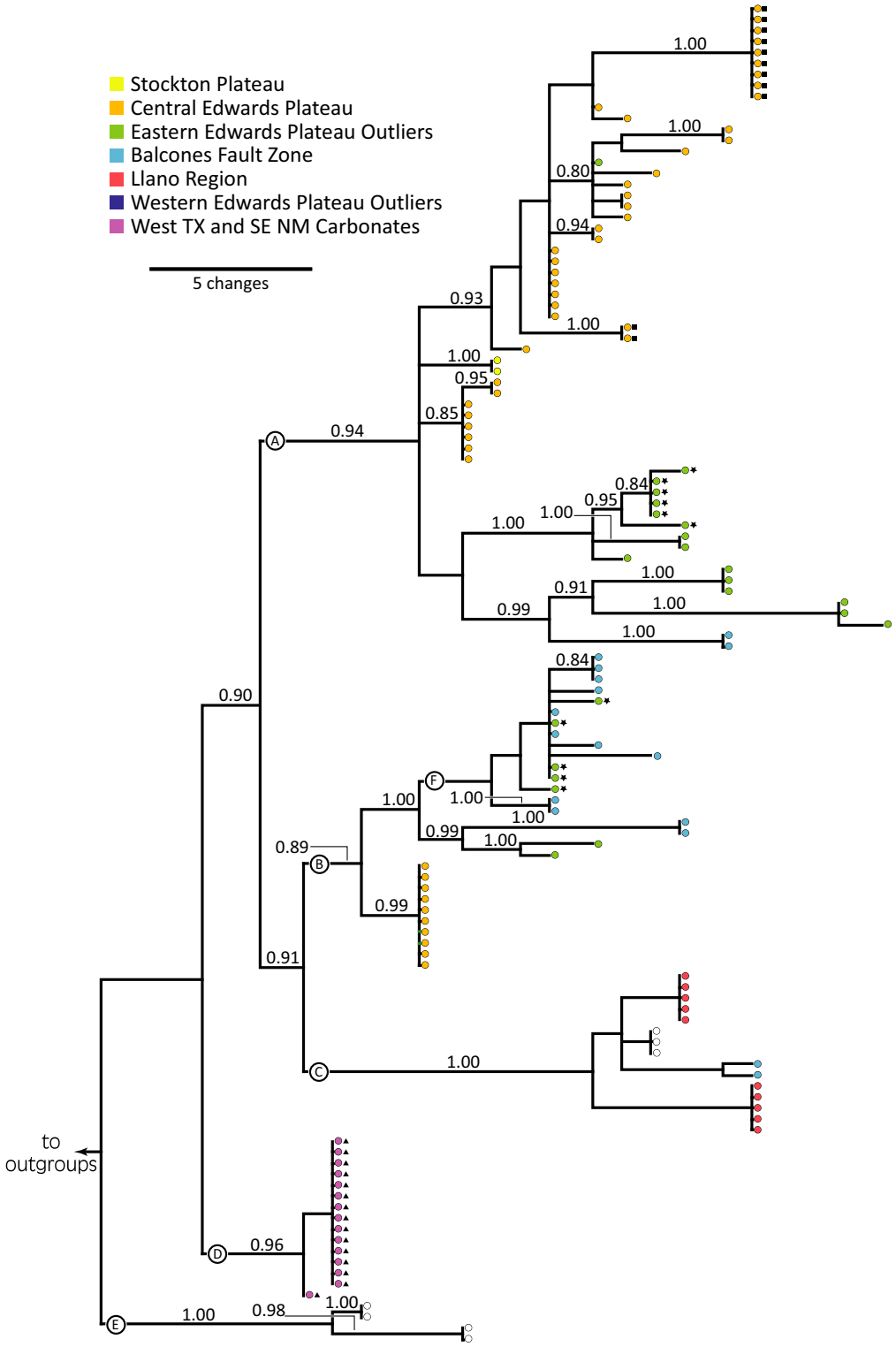

Figure 5 *Ceuthophilus* (*Geotettix*) Bayesian consensus tree with minimum changes reconstructed as branch lengths. Numbers above branches are Bayesian posterior probabilities ≥ 0.80. Colours of circles, representing terminal taxa correspond to karst regions. Letters mark clades that are discussed in the text. Specific caves discussed in text are as follows: solid square, Writing on the Rocks Cave (Edwards County, Texas); triangle, 400 Foot Cave (Brewster County, Texas); star, Tall Tales Cave (Bexar County, Texas). Appendix S3 includes a Bayesian consensus tree with labels indicating the taxon number (see Appendix S1) and cave name. All specimens are identified, based on morphology, to *Ceuthophilus* (*Geotettix*) sp., unless indicated otherwise. All collections are from Texas, unless indicated otherwise in Appendices S1 and S3.

Subgenus *Ceuthophilus*

The *Ceuthophilus* phylogenetic tree can be divided into four major haplotype clades that are strongly supported by 1.00 Bayesian posterior probabilities (Fig. 4, clades A–D). One of these clades (Clade D) includes a few individuals from 400 Foot Cave, and divergence between the haplotypes of these individuals and all other *Ceuthophilus* haplotypes (excluding those in the outgroup) ranged between 7.9–9.3% (average 8.4%) suggesting a relatively long period of isolation. Crickets from two of the other three genetically divergent *Ceuthophilus* haplotype clades (Clades A and C) were also found in 400 Foot Cave. 400 Foot Cave is one of only two caves (the other being Writing on the Rocks Cave) that had more than two of the *Ceuthophilus* haplotype clades. Even within clades A, B, and C, there was substantial genetic structure, with a number of well-diagnosed and relatively deeply diverged clades.

Based on morphology, we definitively identified individuals from two well-supported subclades to previously described species level morphotaxa. Clade E, supported by 1.00 Bayesian posterior probability consists of *C.* (*Ceuthophilus*) *secretus* whereas Clade F includes *C.* (*Ceuthophilus*) *conicaudus* (Fig. 4). The average divergence between these named individuals in clade E and clade F was 3.8% (range 3.7–3.9). Overall, between-clade divergences in the *Ceuthophilus* tree for clades A–D averaged 3.8% (range 2.1–9.3), whereas within-clade divergences in the *Ceuthophilus* tree for clades A–D averaged 1.2% (range 0–2.9).

Subgenus *Geotettix*

The *Geotettix* phylogenetic tree can be divided into five major haplotype clades (Fig. 5, clades A–E), with some (clades C–E) supported by > 0.95 Bayesian posterior probability and others more marginally supported (clade A = 0.94, clade B = 0.89). In only one case did we find individuals from two different haplotype clades (Fig. 5, clades A and B) in the same cave (Tall Tales Cave). Within *Geotettix* clades A–C, there was substantial genetic structure with multiple well-supported subclades and many caves having unique haplotypes not found in any other cave. However, unlike for *Ceuthophilus* for which we found uncorrected *p*-distances up to *c.* 11% (Fig. 3a), there were no pronounced deep divergences within the *Geotettix* tree and uncorrected *p*-distances were only up to *c.* 2% (Fig. 3b).

Based on morphology we definitively identified one well-supported clade and one more weakly supported subclade as belonging to previously described species level morphotaxa. Clade C, supported by 1.00 Bayesian posterior probability, consists of individuals belonging to *C.* (*Geotettix*) *polingi*, and individuals from subclade F, which is not strongly supported by Bayesian posterior probability (< 0.95), belong to *C.* (*Geotettix*) *cunicularis*. The average divergence between these named individuals in clade C and subclade F was 1.4% (range 1.2–1.8). Overall between-clade divergences in the *Geotettix* tree for clades A–E averaged 1.4% (range 0.8–2.3) whereas within-clade divergences in the *Geotettix* tree for clades A–E averaged 0.6% (range 0–1.4).

Tests of phylogeographical structure

All four Maddison & Slatkin (1991) randomizations indicated that geographical region had significant phylogenetic signal for both *Ceuthophilus* and *Geotettix*. MP reconstructions on the pruned *Geotettix* tree identified 15 changes in karst region and 24 changes in county over the ingroup and for both karst region and county the Maddison & Slatkin (1991) test of phylogenetic signal was significant ($P < 0.001$). For *Ceuthophilus*, the MP reconstructions on the pruned tree identified 31 changes in karst region and 55 changes in county over the ingroup and for both karst region and county the Maddison & Slatkin (1991) test of phylogenetic signal was significant ($P < 0.001$).

Analyses of trees pared down to caves that included sampled individuals from both *Ceuthophilus* and *Geotettix* in GenGIS provided an assessment of both the optimal orientation of the trees with respect to geographical locations and the number of edge crossing between leaf nodes and their geographical locations (a measure of the amount of geographical structure in the phylogeny) (Fig. 6). The linear axis analysis in GenGIS indicated that for *Ceuthophilus* the optimal orientation of the tree with least number of edge crossings (842) was 162.69°, whereas for *Geotettix* the optimal orientation of the tree with least number of edge crossings (806) was 195.03° (Fig. 6). *Ceuthophilus* linear orientations with significantly ($P = 0.05$) low numbers of edge crossings ranged from 138.84 to 219.73 degrees, a north–south orientation, whereas for *Geotettix* linear orientations with significantly ($P = 0.05$) low numbers of edge crossings range from 190.18 to 279.04°, a southwest-northeast orientation (Fig. 6, and see Appendices S4 and S5).

DISCUSSION

Comparison of phylogeographical structure in the subgenera *Ceuthophilus* and *Geotettix*

The cave cricket subgenera *Ceuthophilus* and *Geotettix* are excellent models for studying phylogeographical histories replicated across the same geography to ask whether particular landscape features might have concordant effects on these two codistributed cave cricket lineages. Furthermore, the species complexes in these two subgenera have contrasting ecology, in particular with respect to their potential for aboveground dispersal. The effects of ecological differences between these two taxa can be assessed with respect to population divergence and phylogeographical history. Species in the subgenus *Ceuthophilus* in the region are trogloxenes, sometimes leaving the cave to forage during the night and even occasionally spending the day hiding underneath rocks,

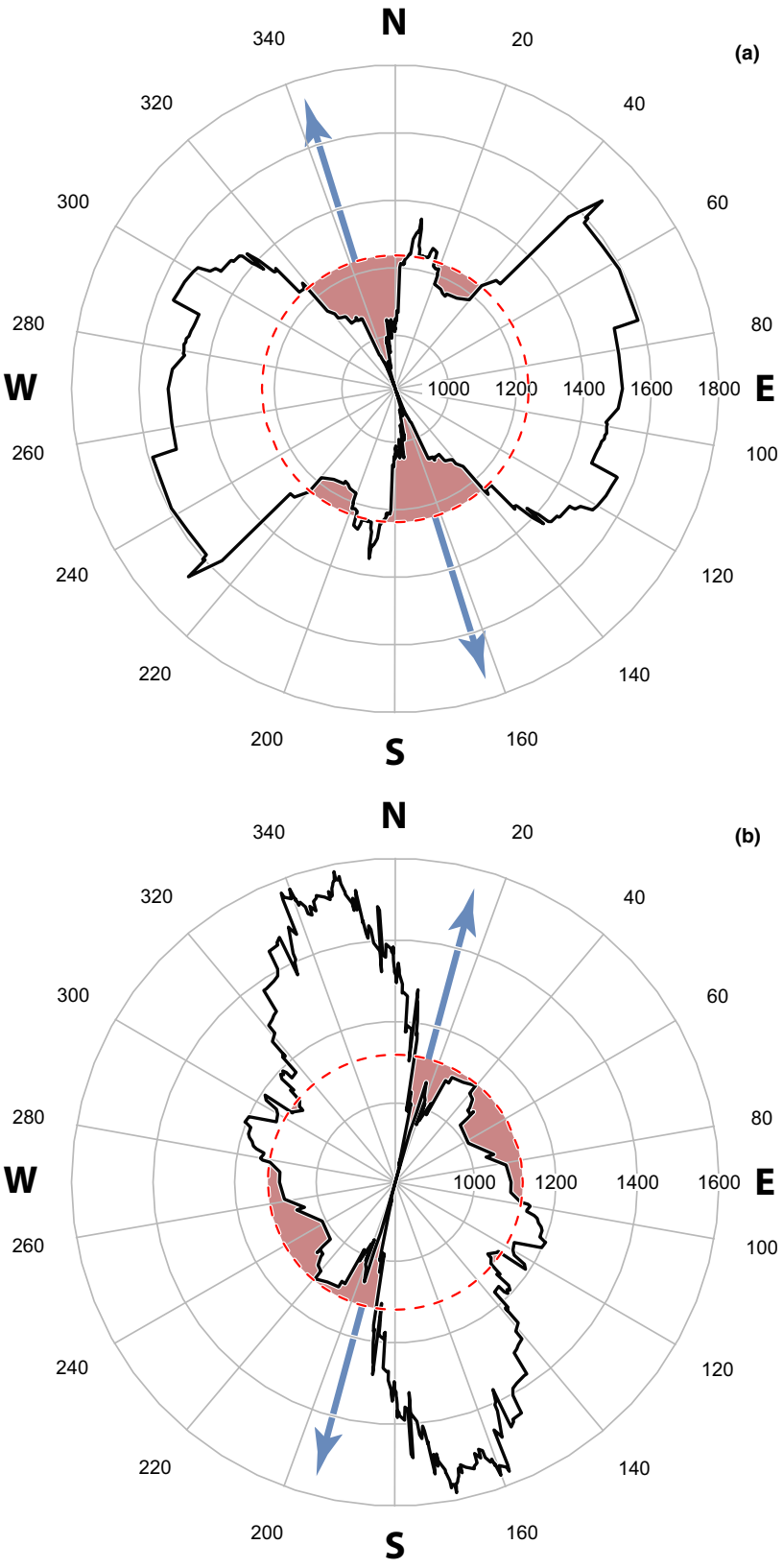

Figure 6 Optimal Linear Axis Analysis of GenGIS trees for data restricted to caves where both subgenera were sampled. Dashed line indicates cutoff for significance at 0.05, shaded area indicates axes with significant geographical structure. Arrow indicates axis with least number of crossings (most geographical structure). Outgroup taxa are excluded from the analysis. (a) *Ceuthophilus* (*Ceuthophilus*), (b) *Ceuthophilus* (*Geotettix*).

whereas species in the subgenus *Geotettix* in this region are more cave-limited, almost never seen outside of a cave, and not recorded anywhere on the surface beyond a cave entrance (Taylor *et al.*, 2005). Thus, we originally predicted that *Ceuthophilus* would show lower levels of geographical structure than *Geotettix*.

Comparisons of the pattern of phylogeographical structure in these two geographically codistributed cave dwelling cricket subgenera (*Ceuthophilus* and *Geotettix*), across the Edwards Plateau of Texas, revealed both similarities and differences. Three salient findings emerged from our analysis. First, both *Ceuthophilus* and *Geotettix* exhibited strong geographical structure using both a Maddison & Slatkin (1991) test of phylogenetic signal for karst region and county and an analysis of geographical structure and linear orientation conducted using GenGIS. However, the linear orientation for *Ceuthophilus* and *Geotettix* trees differed, with *Ceuthophilus* exhibiting a north-south orientation and *Geotettix* exhibiting a southwest-northeast orientation. This difference in orientation could be explained by these two subgenera responding to different landscape features. For example, *Geotettix* phylogeographical structure may be more influenced by cave proximity and subsurface connectivity because this subgenus is a near-obligate cavernicole. Whereas for the subgenus *Ceuthophilus*, phylogeographical structure may be more influenced by above-ground landscape or ecological barriers, such as rivers, because this subgenus is better able to move among caves, at least in a localized proximity, by travelling above ground.

Second, rather than exhibiting the expected relatively lower levels of divergence and genetic structure, the *Ceuthophilus* phylogenetic tree shows deeper divergences than *Geotettix*. This might be due to the radiation of *Ceuthophilus* in Texas caves being a relatively older event. One possibility is that the proclivity for *Ceuthophilus* to leave caves during the night may have provided opportunities, over long periods of time, for small numbers of individuals from primarily isolated lineages that have diverged to periodically recolonize caves. Thus, *Ceuthophilus* would have radiated more than once across the Edwards Plateau, explaining why multiple clades of *Ceuthophilus* are found in the same cave (e.g. 400 Foot, Writing on the Rocks, Amazing Maze, and Tall Tales caves; Reddell, 1994). The dichotomy in genetic divergence and structure for these two subgenera is also borne out by the mismatch distributions, which are multimodal for *Ceuthophilus*, indicating relatively deeply divergent clades in the tree and a long period of stable population size (Slatkin & Hudson, 1991), and unimodal and close to the *y*-axis for *Geotettix*, indicating little divergence between clades. The lesser divergence between clades in *Geotettix* is also consistent with a recent demographic expansion from a population bottleneck (Slatkin & Hudson, 1991) or range expansion with migration between regions (Ray *et al.*, 2003).

Third, the proportion of unique haplotypes is higher for *Ceuthophilus* than for *Geotettix*, which suggests that the cave-inhabiting species of the subgenus *Ceuthophilus* in central Texas have a relatively larger effective population size than do those in *Geotettix*. This hypothesis is confirmed by cave census data (e.g. Taylor *et al.*, 2003, 2007) and is consistent with the ragged mismatch distribution of *Ceuthophilus* and the unimodal mismatch distribution of *Geotettix*.

Comparison with previously published studies of cavernicoles

Terrestrial cave animals tend to show patterns of genetic divergence that are consistent with evolution in a somewhat isolated setting, sometimes as specific as a cave or group of caves (Snowman *et al.*, 2010; Dixon and Zigler, 2011). Cavernicoles span a range of ecological adaptations to cave living—troglobites, troglophiles, and trogloxenes—and a concomitant difference in degree of genetic isolation or speciation due to a combination of factors including vicariance and dispersal (Porter, 2007), which may include isolation of cavernous rock units and reduced dispersal capacity across harsh surface habitats (Barr, 1967; Caccone, 1985). Within the Rhaphidophoridae, both Martinsen *et al.* (2009) and Allegrucci *et al.* (2011) found that both vicariance and dispersal were important factors in southern Europe, and similar patterns of variation in geographic genetic structuring of rhaphidophorids previously has also been documented in the United States (Caccone & Sbordoni, 1987). Our findings suggest that for the two codistributed raphidophorid subgenera in the caves of central Texas, colonization occurred either at different times or in different ways, a finding consistent with Allegrucci *et al.*'s (2009, 2011) analysis of *Dolichopoda* species in Greece and Cook *et al.*'s (2010) analysis of New Zealand Rhaphidophoridae.

Both *Ceuthophilus* and *Geotettix* exhibit strong geographic structure in their phylogenies. Trogloxenic *Ceuthophilus* species, in central Texas, exhibit higher levels of genetic divergence than the co-occurring, near-obligate cave dwellers in *Geotettix*. Like other molecular studies of cave-adapted organisms, our work on *Ceuthophilus* and *Geotettix* has uncovered both high levels of genetic differentiation (Hedin, 1997; Strecker *et al.* 2003; Snowman & Zigler, 2010) and cryptic diversity (Lefébure *et al.*, 2006; Juan *et al.*, 2010; Hamilton *et al.*, 2011; Niemiller *et al.*, 2012).

Published molecular phylogeographic studies of cave arthropods specifically from the Edwards Plateau have found similar patterns to those exhibited by *Ceuthophilus*. For example, Paquin & Hedin (2004), who studied the federally endangered cave spider genus *Cicurina*, showed that species in this genus exhibited considerable genetic divergence and structuring across caves. Our data also showed relatively high genetic divergence and geographic structure between DNA sequences within the *Ceuthophilus* and *Geotettix* subgenera.

Taxonomic implications

From a taxonomic perspective, our data suggest that it is likely that both subgenera of cave crickets include multiple unnamed species level taxa and that taxonomic work on this genus is desperately needed. In fact, one undescribed species, C. (*Ceuthophilus*) 'species B', has been recognized by central Texas cave researchers for some time (Reddell, 1994; Taylor *et al.*, 2005), but has never been formally described. Our

genetic data are consistent with the recognition of this species because it forms a distinct group within *Ceuthophilus* in our analyses, underscoring the need for its description. Furthermore, the co-occurrence of multiple divergent *Ceuthophilus* haplotype clades in the same cave (e.g. 400 Foot, Amazing Maze, and Tall Tales caves) also suggests that these co-occurring subclades may behave like divergent species level taxa, suggesting that more than one undescribed species level taxon may be present in each of these caves. The taxonomy of the Nearctic genus *Ceuthophilus*, comprised of 87 described species, suffers from a long period of inactivity, with most species described more than 75 years ago, no new species described in nearly 50 years, and no revisionary work undertaken since Hubbell's (1936) monographic revision of the genus. Consequently, our understanding of species boundaries within the genus, and even the capacity to identify collected material to the species level, is hampered by a limited understanding of the morphology of the genus. The combination of morphological taxonomic uncertainty, our molecular phylogeographical data identifying cryptic diversity and structure, and the importance of *Ceuthophilus* species in cave ecosystems points towards an urgent need to better describe the diversity of the genus *Ceuthophilus*. Presently, optimal preserve design for federally listed terrestrial cave invertebrates in central Texas (USFWS, 2012) takes into account the foraging range of *C.* (*Ceuthophilus*) *secretus*, based on the foraging range established by Taylor *et al.* (2005). However, our findings in the present study suggest that several phenotypes occur across central Texas, most notably 'Species B' in the subgenus *Ceuthophilus*. If optimal management of the endangered terrestrial cave invertebrate communities is to include cricket foraging ranges, further work is needed to establish foraging ranges of each of the major lineages and to describe the co-occurring species, as this could change optimal preserve size and help better prioritize units of conservation.

ACKNOWLEDGEMENTS

We thank the innumerable landowners and land managers, both private and government (including Texas Parks and Wildlife Department, The Nature Conservancy, Texas Cave Management Association, U.S. Fish & Wildlife, Fort Hood, Camp Bullis, Fort Leonard Wood, Caverns of Sonora, etc.) and the cavers and others who created maps of caves, facilitated our visits, or helped us through phone calls and conversations to gain access to caves. The following individuals made special efforts to help us acquire the samples needed for this study: Peter Sprouse, Kristopher Megahan, N. Parker, Rob Meyers, Andrew Gluesenkamp, Vivian Loftin, Cindy A. Lee, Justin Shaw, Alan King, Jim Kennedy, Mark Sanders, Kathleen O'Connor, John Chenowith, Alan King, Wayne Peplinski, Don Evan Selby, Allan Cobb, Christopher A. Phillips, Charles Pekins, Bill Sawyer, Michael Cunningham, Michael E. Slay and Kurt L. Helf. Many of the above individuals participated in trips and spent long hours driving between sites, crawling through caves and fending off rattlesnakes. We thank Don Selby, who spent time trying to find *C.* (*Ceuthophilus*) *secretus* in the crawl spaces of homes in Dallas, Texas (the type locality). This project was funded by the Texas Parks and Wildlife Department.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Specimen numbers and associated metadata.
Appendix S2 *Ceuthophilus* (*Ceuthophilus*) Bayesian consensus tree with cave names and specimen numbers.
Appendix S3 *Ceuthophilus* (*Geotettix*) Bayesian consensus tree with cave names and specimen numbers.
Appendix S4. Geophylogeny of *Ceuthophilus* (*Ceuthophilus*).
Appendix S5. Geophylogeny of *Ceuthophilus* (*Geotettix*).

BIOSKETCH

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Author contributions: S.J.T., K.P.J., J.D.W. and J.K.K. conceived the project; J.K.K. and S.J.T. collected field samples; J.D.M. and J.D.W. conducted laboratory analysis of samples; J.D.W., K.P.J. and S.J.T. analysed the data; D.M.T. evaluated morphology and taxonomic status of specimens; G.V., J.K.K. and J.R.R. contributed to understanding geological and biospeleological context for interpretation of results; J.D.W., K.P.J. and S.J.T. wrote the paper. All authors contributed comments to earlier drafts of the manuscript, which was approved by all authors.

Editor: Michael Patten