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The power and perils of 'molecular taxonomy': a case study of eyeless and endangered *Cicurina* (Araneae: Dictynidae) from Texas caves

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Abstract

Rapid development in karst-rich regions of the US state of Texas has prompted the listing of four *Cicurina* species (Araneae, Dictynidae) as US Federally Endangered. A major constraint in the management of these taxa is the extreme rarity of adult specimens, which are required for accurate species identification. We report a first attempt at using mitochondrial DNA (mtDNA) sequences to accurately identify immature *Cicurina* specimens. This identification is founded on a phylogenetic framework that is anchored by identified adult and/or topotypic specimens. Analysis of ~1 kb of cytochrome oxidase subunit I (CO1) mtDNA data for over 100 samples results in a phylogenetic tree that includes a large number of distinctive, easily recognizable, tip clades. These tip clades almost always correspond to a priori species hypotheses, and show nonoverlapping patterns of sequence divergence, making it possible to place species names on a number of immature specimens. Three cases of inconsistency between recovered tip clades and a priori species hypotheses suggest possible introgression between cave-dwelling *Cicurina*, or alternatively, species synonymy. Although species determination is not possible in these instances, the inconsistencies point to areas of taxonomic ambiguity that require further study. Our molecular phylogenetic sample is largest for the Federally Endangered *C. madla*. These data suggest that *C. madla* occurs in more than twice the number of caves as previously reported, and indicate the possible synonymy of *C. madla* with *C. vespera*, which is also Federally Endangered. Network analyses reveal considerable genetic divergence and structuring across caves in this species. Although the use of DNA sequences to identify previously 'unidentifiable' specimens illustrates the potential power of molecular data in taxonomy, many other aspects of the same dataset speak to the necessity of a balanced taxonomic approach.

Keywords: caves, conservation, introgression, phylogeny, species determination, TCS, troglomorphic species

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Introduction

Animal genomes are huge, presenting a potentially endless supply of evolutionary and systematic information. This character data can obviously be used to infer species relationships, but in addition, might be used more fundamentally in species diagnosis, description and identification. Such 'molecular taxonomy' has a long history in morphologically 'simple' groups such as bacteria, viruses and fungi. Recently, several authors have argued that a similar model might profitably be used in the animal world (e.g. Baker *et al*. 2003; Blaxter & Floyd 2003; Hebert *et al*. 2003; Proudlove & Wood 2003; Tautz *et al*. 2003). Cited strengths include the potential universality and objective nature of DNA data as taxonomic information, the usefulness of molecular data in animal groups characterized by morphological crypsis and the use of DNA characters to identify otherwise 'unidentifiable' biological material (e.g. body parts or immature specimens).

We summarize our efforts to identify immature, US Federally Endangered cave spiders (genus *Cicurina*) via phylogenetic analyses of mitochondrial DNA (mtDNA) sequence data. This general goal is similar to, for example,

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'DNA surveillance' research on cetaceans, where DNA sequences from unknowns are matched (using phylogenetic analysis) to clades of reference sequences, allowing subsequent identification of the unknowns (see Baker *et al*. 2003). Although our research shows the power of this 'molecular taxonomic' approach, at the same time, the data illustrate the need to maintain a taxonomic framework built upon multiple types of biological information. First, the identification of unknown biological material necessarily requires an a priori taxonomic foundation which is typically built using a combination of evidence (morphology, behaviour, molecules, etc.). Second, gathering multiple types of biological information allows taxonomy to grow. In cave spiders, we show that the a priori taxonomic foundation is probably imperfect in some cases, but it is the combination of information that allows us to see these imperfections. Finally, we reveal evidence for gene tree/species tree incongruence in our data. If unaccounted for, such incongruence will positively mislead a purely molecular taxonomic framework. In combination, our data support those who have argued against one-dimensional taxonomy (see Bond & Sierwald 2003; Dunn 2003; Lipscomb *et al*. 2003; Mallet & Willmott 2003; Seberg *et al*. 2003; Bond 2004; Lee 2004; Will & Rubinoff 2004).

Cave spiders

The spider genus *Cicurina* Menge includes 131 species separated into four subgenera (Chamberlin & Ivie 1940; Cokendolpher 2004a). The core of this diversity is found in North America (> 100 species), with fewer species in eastern Asia and a single species from Europe. These spiders are generally found under rocks and in rotten logs, mostly in forested regions, although a few species are found in more xeric habitats (e.g. Roth & Brame 1972). Many *Cicurina* species are found only in caves, and display obvious morphological features (troglomorphisms) associated with this lifestyle (e.g. eyelessness). The US state of Texas is rich in such eyeless, cave-limited *Cicurina*. Nearly 60 troglomorphic species have been formally described, all placed in the subgenus *Cicurella* Chamberlin & Ivie (Gertsch 1992; Cokendolpher & Reddell 2001; Cokendolpher 2004a,b).

Four cave-limited *Cicurina* species from Bexar County, Texas are listed as US Federally Endangered species (Longacre 2000). Conservation biologists are concerned about these taxa for several reasons. The spiders are restricted to easily perturbed cave habitats, in an area of exceedingly rapid commercial and residential development (summarized in Rappaport Clark 1998; Longacre 2000; Cokendolpher 2004a). This combination threatens many cave habitats and their unique faunas. Most cave-limited *Cicurina* have small geographical distributions — a majority are known only from a handful of caves, and over 20 species are thought to be single-cave endemics (Gertsch 1992; Cokendolpher & Reddell 2001; Cokendolpher 2004a,b). Finally, these spiders are relatively rare in cave habitats. While quantitative abundance data are lacking for eyeless *Cicurina*, collections conducted by teams of several cavers typically result in the collection of few individuals in caves of modest dimension (Gertsch 1992; Cokendolpher 2004b). Several described species are known only from a single adult specimen (Gertsch 1992).

A prerequisite for the management and protection of threatened or endangered species (*Cicurina* included) is detailed distributional knowledge, which itself is dependent upon a solid taxonomic foundation. In the case of *Cicurina*, an ideal management framework would include information on the full geographical distribution of all relevant taxa for all caves in a region. This knowledge would allow one to single out species with truly remarkable or susceptible distributions (e.g. species known only from a single, threatened site), and would also allow managers to identify sites with remarkable attributes (e.g. caves with multiple endemic species from several disparate taxa). Historically, species-level identification in *Cicurina* has relied almost entirely on genital morphology of adult specimens. This reliance is standard in the field of arachnology (see Eberhard 1986), but represents a serious constraint in the Texas cave *Cicurina* system, primarily because adult spiders are very rare in caves. This rarity means that the majority of collected specimens are immature, particularly if one strives to collect relatively few specimens per cave (i.e. minimize over-collecting). Immature eyeless *Cicurina* are impossible to identify to species using morphology alone. The result is that there are over 100 Texas caves known to house eyeless, 'unidentifiable' *Cicurina* (Gertsch 1992; Cokendolpher & Reddell 2001; Cokendolpher 2004a), leaving considerable gaps in our knowledge of these taxa.

The over-reaching goal of the research presented here is to increase the 'taxonomic value' of immature eyeless *Cicurina* by developing a means to confidently identify such specimens. Because immature morphology is uninformative, we have focused on the use of readily assayed DNA characters as a primary diagnostics tool. In our case, we use genealogical concordance as a primary criterion to identify immature specimens (Avise & Ball 1990). Under this framework, we expect adult specimens of the same taxonomic species to fall together into discrete, exclusive genetic clades. If this expectation holds, then we are able to tentatively identify immature specimens that also fall into such clades. When these previously unidentifiable specimens are from new cave locations, we increase our knowledge of species distributions. As shown below, DNA sequences from immature specimens sometimes form divergent genetic clades separate from those clades anchored by identified specimens. In such cases, we hypothesize that these immature specimens represent undescribed and/or unsampled species, and make predictions that require

confirmation via the collection of DNA data from adults. Because of the large number of Texas cave *Cicurina*, the taxon sampling reported here is necessarily preliminary. Despite this limitation, our data have important implications for the taxonomy, biogeography and conservation of many eyeless *Cicurina* taxa. Moreover, our results imply value for the thousands of immature *Cicurina* specimens that now reside, with limited value, on museum shelves.

Materials and methods

Taxon sampling and identification

Species authorship information for all *Cicurina* species discussed below is provided in the Appendix. Specimens of multiple eyeless *Cicurina* species, plus the eyed species *Cicurina pampa* and *C. varians*, were collected in caves located along the Balcones Escarpment of central Texas (Appendix; Fig. 1). Eyeless *Cicurina* were collected by professional cavers and biologists (see Acknowledgements), as access to caves and collection of protected species requires special permits. Other surface *Cicurina* species were collected at various places in North America (Appendix). Following suggestions of Cokendolpher (2004a), we used *Yorima* sequences to root our *Cicurina* trees. We felt that the use of a single outgroup taxon was justified, as our objective here is not a detailed exploration of internal relationships within *Cicurina*.

Nineteen *Cicurina* species are represented by one or more adult specimens in our analysis (Appendix). Adults were identified to species using morphological criteria by one of the authors (PP) or by J. Cokendolpher, an authority on Texas cave *Cicurina* (Appendix). In a few cases, we placed *tentative* a priori species names on immature eyeless specimens collected at known type localities (following Gertsch 1992). Sympatry of eyeless Texan *Cicurina* is very rare, having been 'authenticated' in only a single case (Cokendolpher & Reddell 2001), and suggested in two others (see Cokendolpher 2004a). In most instances, only one troglomorphic species occupies any particular cave. We felt that this one-to-one relationship justified our a priori assignment of species names to some immature eyeless specimens, which we made for the following taxa: *C. ezelli*, *C. reddelli* and *C. vespera*. For a single surface-dwelling

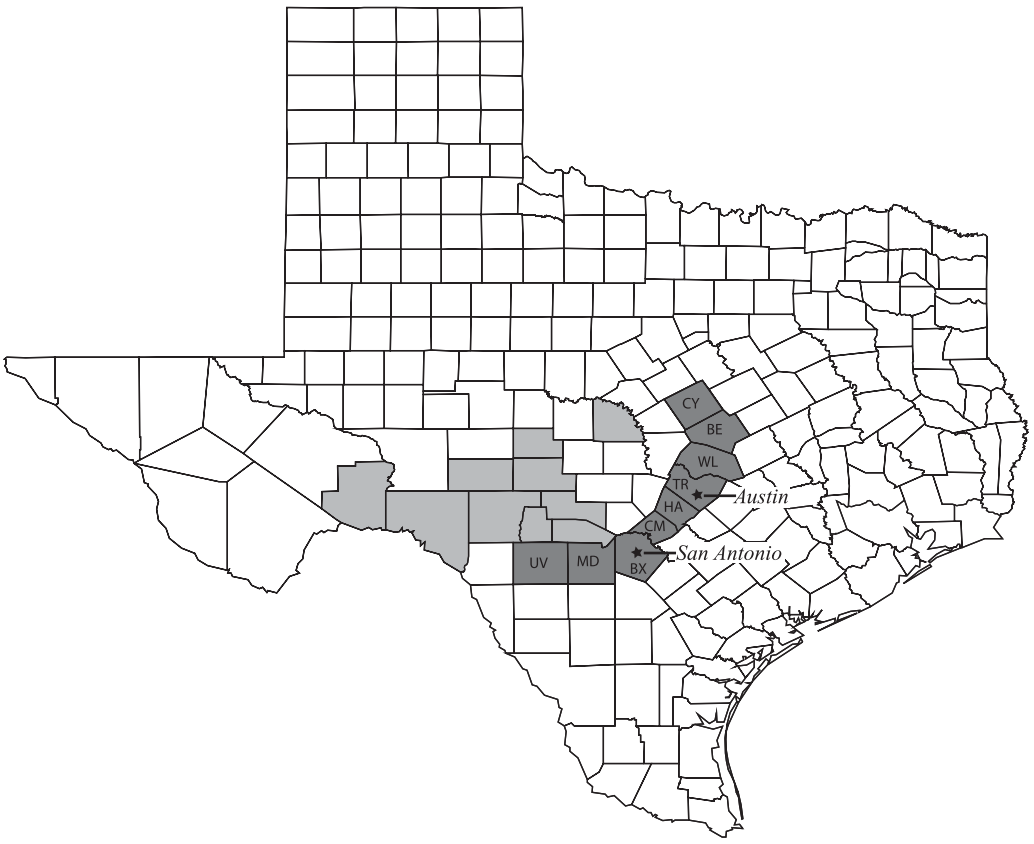


Fig. 1 Map of Texas. Shaded counties include caves that house eyeless *Cicurina* (following Gertsch 1992; Cokendolpher & Reddell 2001; Cokendolpher 2004a,b). Our sample of eyeless taxa comes from counties with both shading and abbreviations, as follows: CY = Coryell, BE = Bell, WL = Williamson, TR = Travis, HA = Hayes, CM = Comal, BX = Bexar, MD = Medina and UV = Uvalde.

species (*C. pacifica*), we collected a series of adult and immature specimens from a site, but used only immature specimens in the DNA analysis. We assume a lack of *Cicurina* sympatry in this instance. All other specimens included in phylogenetic analyses were immatures, and were not identified a priori. In these cases we used our phylogenetic results to suggest a posteriori identifications.

DNA data collection

Specimen deposition information is provided in the Appendix. Two legs (more for spiderlings) were removed from specimens for DNA extractions. Genomic DNAs of *C. bullis*, *C. hoodensis* and *C. caliga* were extracted using the CTAB protocol of Shahjahan *et al*. (1995). Other genomics were extracted using the DNeasy tissue kit (Qiagen) following manufacturer's suggestions. Partial fragments of the mitochondrial CO1 gene (~1 kb) were amplified via the polymerase chain reaction (PCR) using the primer C1-J-1751SPID, paired with either C1-N-2776 or C1-N-2568 (Hedin & Maddison 2001). This gene region has shown prior utility in spider molecular phylogenetic studies (e.g. Hedin 2001; Hedin & Wood 2002; Vink & Paterson 2003), is relatively easy to amplify and sequence and is the same gene used in the molecular 'barcoding' studies of Hebert *et al*. (2003). Importantly, researchers have also successfully amplified this gene region from museum-preserved spider specimens (see Colgan *et al*. 2002), a consideration that may apply to future research efforts in *Cicurina*. PCR amplifications were conducted on a MJ Research PTC100 thermocycler with the following protocol: 92 °C initial denaturation for 30 s, 30 cycles of 92 °C for 30 s, 44 °C for 45 s plus 0.2 °C per cycle, 72 °C for 1 min 30 s, final extension at 72 °C for 5 min. PCR products were purified on polyacrylamide gels (Sambrook *et al*. 1989), and sequenced using Big Dye Terminator 3.0 chemistry on an ABI 377 automated sequencer. Templates were sequenced in both directions using PCR primers. Sequences were manually aligned and edited using a combination of SEQAPP v. 1.9a169 (Gilbert 1994) and MACCLADE 4.0 (Maddison & Maddison 2001).

Phylogenetic analysis

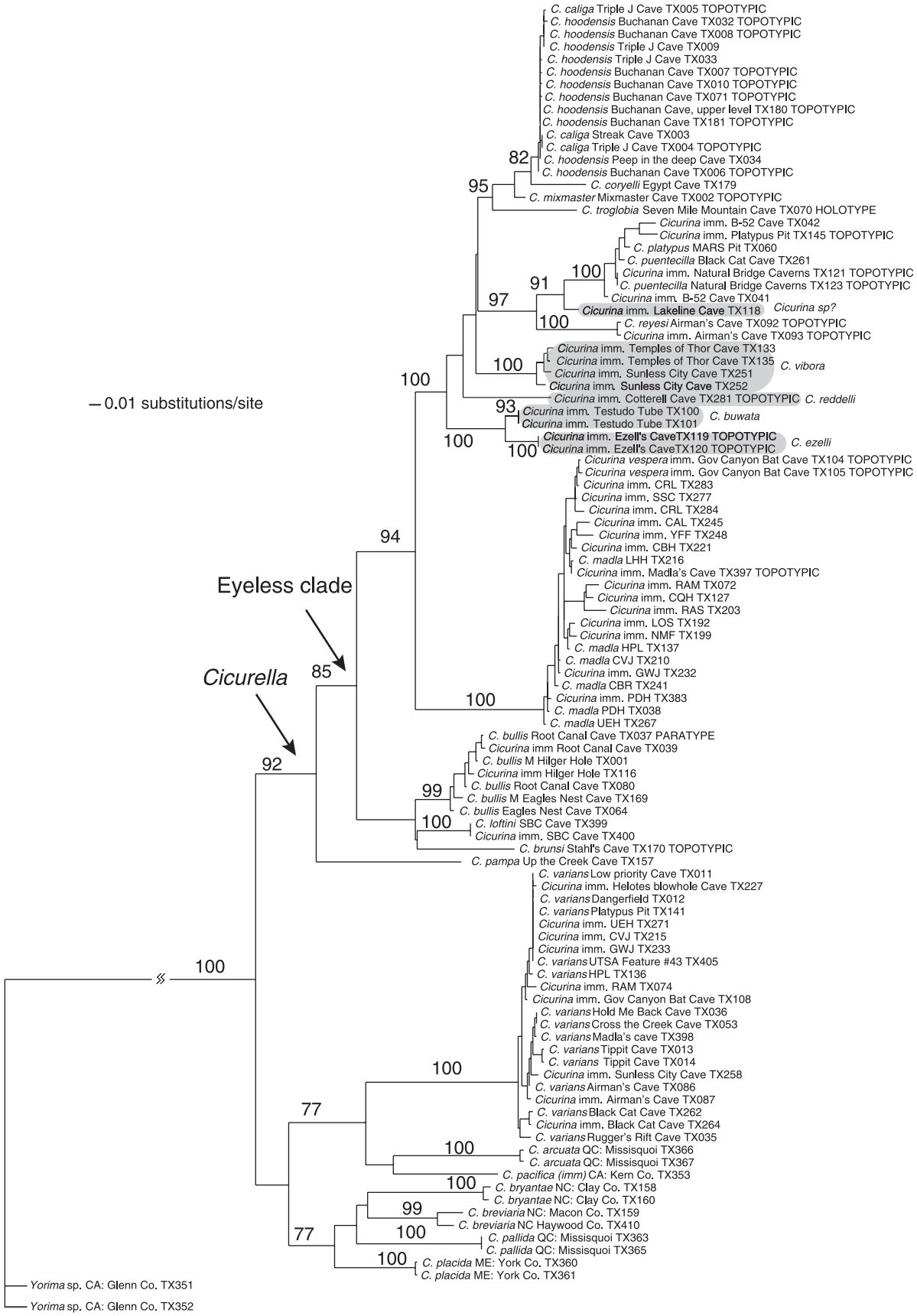
Three types of phylogenetic analysis were conducted, including neighbour joining (NJ), Bayesian and TCS network analyses. NJ trees were constructed using maximum likelihood distances (using PAUP* v. 4.0b10, Swofford 2002), assuming a model and model parameters estimated by MODELTEST v. 3.04 (Posada & Crandall 1998). One thousand NJ bootstrap replicates were conducted to assess nodal support. Bayesian analyses were used to estimate tree topologies with MRBAYES v. 3b4 (Huelsenbeck & Ronquist 2001), using nst = 6, rates = invgamma model parameters, consistent with MODELTEST results. Analyses were conducted with four heated chains, and run for 2 million generations, sampling every 100th tree. To assess convergence (Huelsenbeck *et al*. 2002), we replicated Bayesian analyses two additional times. Trees were imported into PAUP* and a majority rule consensus tree was constructed, discarding trees generated over the first 200 000 generations as burn-in. The Templeton *et al*. (1992) network estimation procedure, as implemented in the TCS v. 1.13 software of Clement *et al*. (2000), was used to resolve intraspecific haplotype relationships within the species *C. madla*. TCS was developed for population genetic data, where phylogenetic divergences are often low, ancestral haplotypes are typically extant, and multifurcations are common (see Templeton *et al*. 1992; Posada & Crandall 2001).

Results

We generated 95 CO1 sequences comprising 984 bp, and nine shorter sequences of 804 bp. Haplotype and Accession no. information is provided in the Appendix. No indels or stop codons were found in the data, suggesting that all sequences are functional, coding sequences. The GTR + I + Γ model was determined as the best-fit model, and used to reconstruct a NJ tree (Fig. 2). This tree is topologically similar to those recovered from Bayesian analyses (Fig. 3), with identical resolution of tip clades (see below). There were minor differences in the interrelationships of tip clades, and differences in how haplotypes within such clades were resolved, but neither of these differences influences the general interpretation of our results. Replicate Bayesian analyses were visually compared and revealed the same topological structure, suggesting that each analysis had converged to the same general solution.

Although we discuss our phylogenetic results in the context of the NJ tree (Fig. 2), our discussion applies equally well to Bayesian trees (Fig. 3). There is a primary division deep in the tree between species belonging to the subgenus *Cicurella*, which in our analysis includes all eyeless Texas taxa plus the surface-dwelling *Cicurina pampa* from Texas, and surface species (including *C. varians* from Texas). Within *Cicurella* itself, there is a second deep split separating

Fig. 2 Results of NJ distance analysis, with boostrap values (1000 pseudoreplicates). Tip clades that lack adult specimens are indicated in shaded boxes — species determinations for these clades are tentative. Full locality data and Accession no. information provided in the Appendix. To protect the identity of unpublished cave locations for the federally listed *Cicurina madla*, we have used three-letter codes, corresponding to those also found in the Appendix. Cave names and locations associated with these codes are available upon request from the authors.


C. caliga Triple J Cave TX005 TOPOTYPIC
C. hoodensis Buchanan Cave TX032 TOPOTYPIC
C. hoodensis Buchanan Cave TX008 TOPOTYPIC
C. hoodensis Triple J Cave TX009
C. hoodensis Triple J Cave TX033
C. hoodensis Buchanan Cave TX007 TOPOTYPIC
C. hoodensis Buchanan Cave TX010 TOPOTYPIC
C. hoodensis Buchanan Cave TX071 TOPOTYPIC
C. hoodensis Buchanan Cave, upper level TX180 TOPOTYPIC
C. hoodensis Buchanan Cave TX181 TOPOTYPIC
C. caliga Streak Cave TX003
C. caliga Triple J Cave TX004 TOPOTYPIC
C. hoodensis Peep in the deep Cave TX034
C. hoodensis Buchanan Cave TX006 TOPOTYPIC
C. coryelli Egypt Cave TX179
C. mixmaster Mixmaster Cave TX002 TOPOTYPIC
C. troglobia Seven Mile Mountain Cave TX070 HOLOTYPE
Cicurina imm. B-52 Cave TX042
Cicurina imm. Platypus Pit TX145 TOPOTYPIC
C. platypus MARS Pit TX060
C. puentecilla Black Cat Cave TX261
Cicurina imm. Natural Bridge Caverns TX121 TOPOTYPIC
C. puentecilla Natural Bridge Caverns TX123 TOPOTYPIC
Cicurina imm. B-52 Cave TX041
Cicurina imm. Lakeline Cave TX118
Cicurina sp?
C. reyesi Airman's Cave TX092 TOPOTYPIC
Cicurina imm. Airman's Cave TX093 TOPOTYPIC
Cicurina imm. Temples of Thor Cave TX133
Cicurina imm. Temples of Thor Cave TX135
Cicurina imm. Sunless City Cave TX251
Cicurina imm. Sunless City Cave TX252
C. vibora
Cicurina imm. Cotterell Cave TX281 TOPOTYPIC
C. reddelli
Cicurina imm. Testudo Tube TX100
Cicurina imm. Testudo Tube TX101
C. buwata
Cicurina imm. Ezell's CaveTX119 TOPOTYPIC
Cicurina imm. Ezell's CaveTX120 TOPOTYPIC
C. ezelli
Cicurina vespera imm. Gov Canyon Bat Cave TX104 TOPOTYPIC
Cicurina vespera imm. Gov Canyon Bat Cave TX105 TOPOTYPIC
Cicurina imm. CRL TX283
Cicurina imm. SSC TX277
Cicurina imm. CRL TX284
Cicurina imm. CAL TX245
Cicurina imm. YFF TX248
Cicurina imm. CBH TX221
C. madla LHH TX216
Cicurina imm. Madla's Cave TX397 TOPOTYPIC
Cicurina imm. RAM TX072
Cicurina imm. CQH TX127
Cicurina imm. RAS TX203
Cicurina imm. LOS TX192
Cicurina imm. NMF TX199
C. madla HPL TX137
C. madla CVJ TX210
Cicurina imm. GWJ TX232
C. madla CBR TX241
Cicurina imm. PDH TX383
C. madla PDH TX038
C. madla UEH TX267
C. bullis Root Canal Cave TX037 PARATYPE
Cicurina imm Root Canal Cave TX039
C. bullis M Hilger Hole TX001
Cicurina imm Hilger Hole TX116
C. bullis Root Canal Cave TX080
C. bullis M Eagles Nest Cave TX169
C. bullis Eagles Nest Cave TX064
C. loftini SBC Cave TX399
Cicurina imm. SBC Cave TX400
C. brunsi Stahl's Cave TX170 TOPOTYPIC
C. pampa Up the Creek Cave TX157
C. varians Low priority Cave TX011
Cicurina imm. Helotes blowhole Cave TX227
C. varians Dangerfield TX012
C. varians Platypus Pit TX141
Cicurina imm. UEH TX271
Cicurina imm. CVJ TX215
Cicurina imm. GWJ TX233
C. varians UTSA Feature #43 TX405
C. varians HPL TX136
Cicurina imm. RAM TX074
Cicurina imm. Gov Canyon Bat Cave TX108
C. varians Hold Me Back Cave TX036
C. varians Cross the Creek Cave TX053
C. varians Madla's cave TX398
C. varians Tippit Cave TX013
C. varians Tippit Cave TX014
Cicurina imm. Sunless City Cave TX258
C. varians Airman's Cave TX086
Cicurina imm. Airman's Cave TX087
C. varians Black Cat Cave TX262
Cicurina imm. Black Cat Cave TX264
C. varians Rugger's Rift Cave TX035
C. arcuata QC: Missisquoi TX366
C. arcuata QC: Missisquoi TX367
C. pacifica (imm) CA: Kern Co. TX353
C. bryantae NC: Clay Co. TX158
C. bryantae NC: Clay Co. TX160
C. breviaria NC: Macon Co. TX159
C. breviaria NC Haywood Co. TX410
C. pallida QC: Missisquoi TX363
C. pallida QC: Missisquoi TX365
C. placida ME: York Co. TX360
C. placida ME: York Co. TX361
Yorima sp. CA: Glenn Co. TX351
Yorima sp. CA: Glenn Co. TX352
0.01 substitutions/site
Eyeless clade
Cicurella

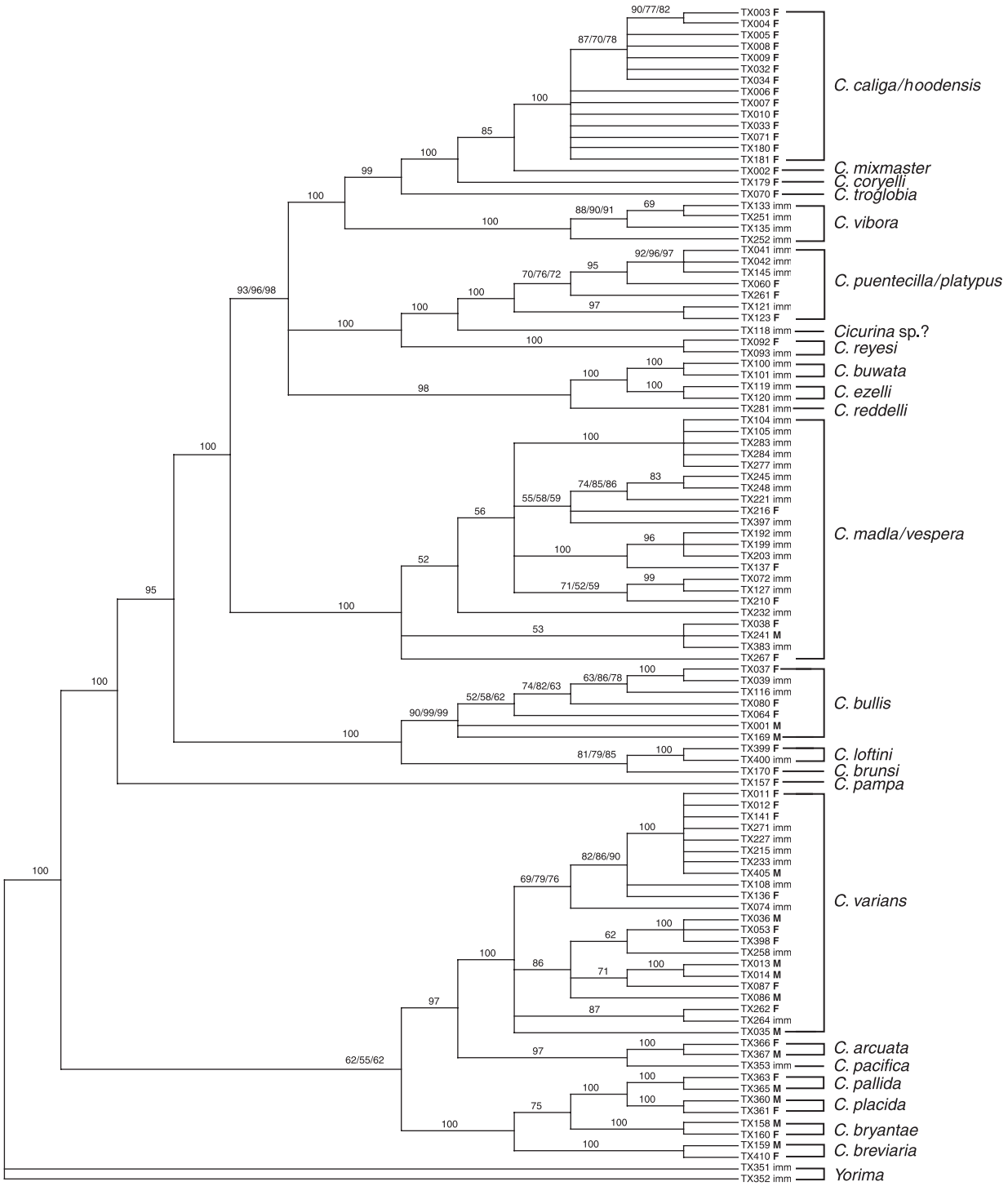


Fig. 3 Bayesian consensus tree, based on results of one of three replicate analyses. Posterior probability values are shown for each of the three replicate analyses, unless values were found to be identical across analyses.

C. pampa from sampled eyeless *Cicurella*. This topology suggests that eyeless *Cicurina* from Texas form a clade, although many key surface-dwelling taxa are missing from our analysis (see Gertsch 1992).

Terminal clades on the CO1 gene tree, hereafter called 'tip clades', conform reasonably well to hypothesized a priori species boundaries. Tip clades are not only well supported as monophyletic, but also show the characteristic

Table 1 Summary of genetic distances within and between tip clades

	Tip clade (Species)	*N*	Within	Between
SURFACE	*Cicurina arcuata*	2	0.1	10.06
	C. bryantae	2	0.91	8.84
	C. breviaria	2	3.09	8.84
	C. pacifica	1	—	10.06
	C. pallida	2	0	9.2
	C. pampa	1	—	12.03
	C. placida	2	0	9.15
	C. varians	22	2.54	10.98
CAVE	*C. brunsi*	1	—	8.33
	C. bullis	7	1.63	6.32
	C. caliga/hoodensis	14	0.41	1.73
	C. coryelli	1	—	3.55
	C. elliotti	2	0	3.15
	C. ezelli	2	0	3.15
	C. loftini	2	0	6.32
	C. madla/vespera	22	3.96	10.06
	C. mixmaster	1	—	1.73
	C. puentecilla/platypus	7	2.35	4.67
	C. reddelli	1	—	8.39
	C. reyesi	2	0	8.47
	C. troglobia	1	—	7.01
	C. vibora	4	1.42	7.03
	Cicurina sp. 1	1	—	4.67

Within-clade distances reported as the maximum pairwise sequence divergence found within a given tip clade, estimated as *p* distances (the observed proportion of sites that differ between sequences) × 100. Between-clade divergences are minimum *p* distances (× 100) from one tip clade to the nearest (in genetic distance) tip clade. *N* represents the number of individuals sampled for each tip clade.

of low sequence divergence within, but high sequence divergence between, tip clades (Table 1). With few exceptions, these divergence values are completely nonoverlapping. Different surface-dwelling species that were identified, a priori, using adult specimens fall into separate, well-defined, tip clades (Fig. 2). Similarly, different eyeless species that were determined from adult specimens *typically* fall into separate tip clades (Fig. 2). Two exceptions involve the species pairs *C. caliga*/*C. hoodensis* and *C. puentecilla*/*C. platypus*. In these cases, it is the *species pair* that forms a tip clade, but the individual species themselves are phylogenetically indistinguishable within such clades.

Most eyeless specimens that were determined a priori to species using the topotypic criteria also fall into distinguishable tip clades (*C. reddelli* from Cotterell Cave, *C. ezelli* from Ezell's Cave). We predict that CO1 sequences from adult specimens of these species will fall into the genetic clades recovered here. The assignment of immature specimens from Cotterell Cave to the species *C. reddelli* is complicated by the potential sympatry of eyeless taxa in this cave, as Gertsch (1992) also lists *C. buwata* (under *C. elliotti*) as occurring in Cotterell Cave. However, sequences from immature specimens collected in Testudo Tubes, Williamson County (a known site for *C. buwata*, Cokendolpher 2004a) fall into a distinct, and nonsister, genetic clade from sequences of immature specimens collected in Cotterell Cave. We hypothesize that material collected in Cotterell Cave represents *C. reddelli*, and that material collected in Testudo Tubes represents *C. buwata*. This hypothesis is consistent with the suggestion of Cokendolpher & Reddell (2001), who regard the Cotterell Cave record of *C. buwata* as erroneous. Sequences collected from eyeless specimens from Government Canyon Bat Cave, the type locality of *C. vespera*, do not form an isolated tip clade, but instead cluster within the *C. madla* tip clade. This situation is discussed further below.

Phylogenetic analyses suggest the placement of many immatures that lack a priori species determinations. Sometimes haplotypes from unidentified immatures are embedded within tip clades that include adult specimens, as was found for the eyeless taxa *C. madla*, *C. bullis*, *C. puentecilla*, *C. loftini* and *C. reyesi*, and the troglophilic *C. varians*. This phylogenetic evidence suggests new occurrence records for four of these taxa. The greatest increase in distribution involves the Federally Endangered species *C. madla* (Fig. 4).

Three tip clades were recovered that include neither adult nor topotypic 'anchoring' material (Fig. 2). As discussed above, we hypothesize that the tip clade from Testudo Tubes represents *C. buwata*. A tip clade that includes sequences from Temples of Thor Cave and Sunless City Cave is hypothesized to represent *C. vibora*, as Temples of Thor Cave is a published record for this species. Finally, the sequence from an immature specimen from Lakeline Cave (TX118) is divergent. We hypothesize that this specimen represents either an undescribed species, or a new record from a described species not otherwise included in our sample.

We have data from 12 cave locations where an eyeless taxon is found in sympatry with the eyed, troglophilic species *C. varians*. Phylogenetic examination of these cases shows that haplotypes are always perfectly sorted to species, suggesting a lack of hybridization and molecular introgression between these taxa (Figs 2 and 3). This lack of introgression probably reflects the deep phylogenetic divergence separating these taxa (classified into different subgenera). As discussed below, our data are less decisive about possible genetic introgression between three pairs of eyeless species (*C. caliga*/*C. hoodensis*, *C. vespera*/*C. madla* and *C. puentecilla*/*C. platypus*).

A TCS network was reconstructed for haplotypes falling within the well-sampled *C. madla* tip clade (Fig. 5). This network reveals considerable genetic divergence across geographically proximate populations, and genetic clustering associated with geography. The conservation implications of this preliminary population sample are further discussed below.

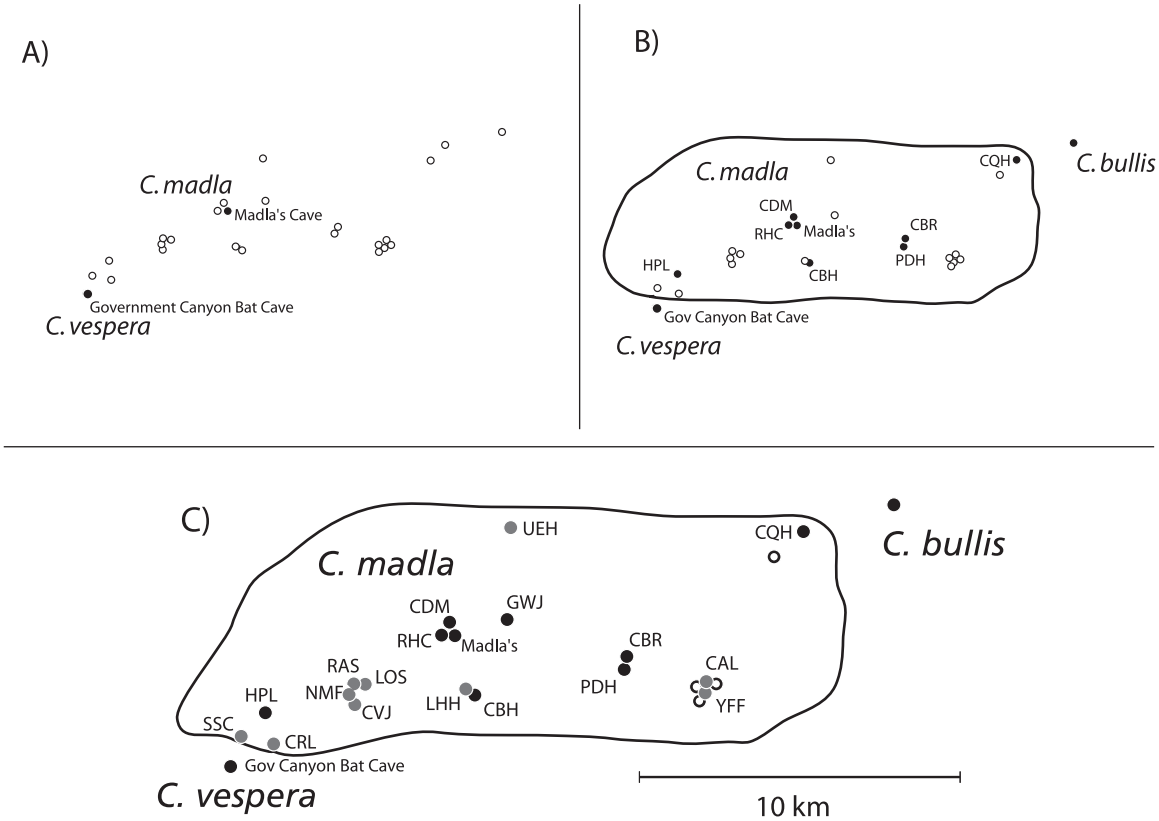


Fig. 4 The distribution of eyeless *Cicurina* in northern Bexar County, Texas (A) following Gertsch (1992), (B) following Cokendolpher (2004a) and (C) as suggested by this study. Unpublished cave locations for *C. madla* are indicated by three-letter codes. Filled dark circles correspond to caves with adult *Cicurina*; unfilled circles are cave locations, many with immature eyeless *Cicurina*; grey filled circles (C) are caves with immatures that have been identified as *C. madla* using phylogenetic criteria.

Discussion

Eyeless *Cicurina* of Texas present a challenge to taxonomists and conservation biologists alike. Most species are known from very few sites and few adults, a situation that constrains both traditional taxonomic progress and conservation efforts. To overcome these constraints, we have attempted to use DNA sequences to extract accurate species and biogeographical information from previously unidentifiable immature specimens. Although we have just started to build our phylogenetic framework, and expect the predictive value of this framework to increase as our taxon sample grows, we feel that preliminary taxonomic and biogeographical findings are promising. These findings have tangible conservation implications. That said, our results also illustrate shortcomings of a one-dimensional molecular approach, clearly demonstrating the need for multidimensional taxonomy.

Patterns of genealogical congruence in Cicurina

Cross-validation is a principal justification for needing to collect multiple lines of taxonomic evidence. The literature is full of examples of morphological species that exhibit striking levels of internal, phylogenetically structured, molecular variation (e.g. Bond *et al*. 2001; Hedin & Wood 2002). Conversely, entire clades of morphological species can sometimes show limited genetic divergence (e.g. Maddison & Hedin 2003). Importantly, interpreting these differences in patterns of variation requires multiple lines of evidence. Just as we need independent data to interpret and investigate hypothesized morphological species boundaries, we also need such data to interpret and investigate hypothesized molecular species boundaries (Lipscomb *et al*. 2003; Mallet & Willmott 2003; Bond 2004; Lee 2004).

The necessity for cross-validation is clearly seen in the *Cicurina* data. Although genealogical congruence is prevalent in our data (i.e. most molecular tip clades correspond to *a priori* morphological species), the nature of this congruence is not equivalent across the phylogeny. Some nominal species of *Cicurina* show conspicuous internal morphological and molecular divergence (e.g. *Cicurina varians*, Fig. 6), whereas other relatively tight molecular clades include several different morphological species (e.g. the *C. mixmaster* clade). Only by collecting multiple types of data are we able to recognize and interpret these contrasts.

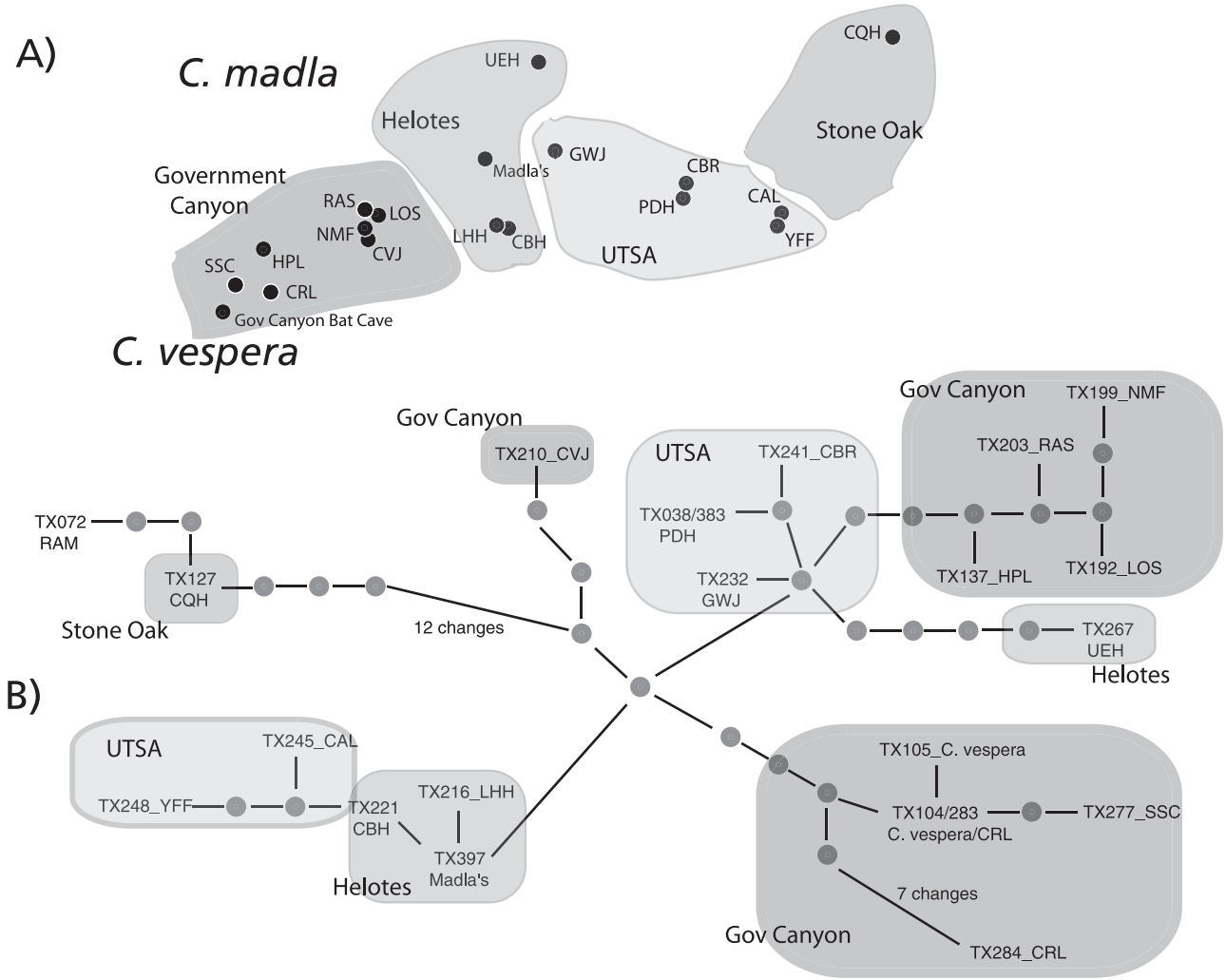


Fig. 5 (A) Distribution of *Cicurina madla* populations across the karst faunal units of Veni (1994). These include the Stone Oak and Helotes regions, the latter of which has been further subdivided into subregions (Government Canyon, Helotes and UTSA). (B) TCS network of *C. madla* haplotypes, with geographical origin of haplotypes indicated. Unsampled and/or extinct haplotypes indicated by filled circles. Two long branches have been truncated, showing only the actual number of reconstructed changes.

Genealogical incongruence in Cicurina

Genealogical congruence is not the only pattern observed in the *Cicurina* data. The molecular data indicate three cases in which recovered tip clades are inconsistent with a priori predictions based on morphology. These cases involve the species pairs *C. caliga*/*C. hoodensis*, *C. madla*/*C. vespera* and *C. puentecilla*/*C. platypus*. Species in these pairs do not form distinguishable molecular clades (Figs 2 and 3). Instead, both members of each pair fall within the same tip clade, without evidence for internal phylogenetic structuring (i.e. they *do not* form clades nested within clades). As argued below for two of the three cases, these inconsistencies may reflect inadequate morphological taxonomy (i.e. members of the species pair are actually synonymous), the retention of ancestral molecular polymorphism since speciation or ongoing gene flow across species boundaries (see Funk & Omland 2003).

The eyeless troglomorphs *C. caliga* and *C. hoodensis* each occur in a handful (three and five caves, respectively) of geographically proximate caves in Bell County (Figs 1 and 7). These taxa are believed to co-occur in two separate caves, including the large Buchanan and Triple J caves (Fig. 22 of Cokendolpher & Reddell 2001; our Fig. 7). The size and complexity of these caves is thought to have facilitated species sympatry, the first 'authenticated' case of such sympatry between eyeless *Cicurina* in Texas. These taxa appear to differ in degree of troglomorphism (leg length to carapace ratios, see Cokendolpher & Reddell 2001), and show minor genitalic differences.

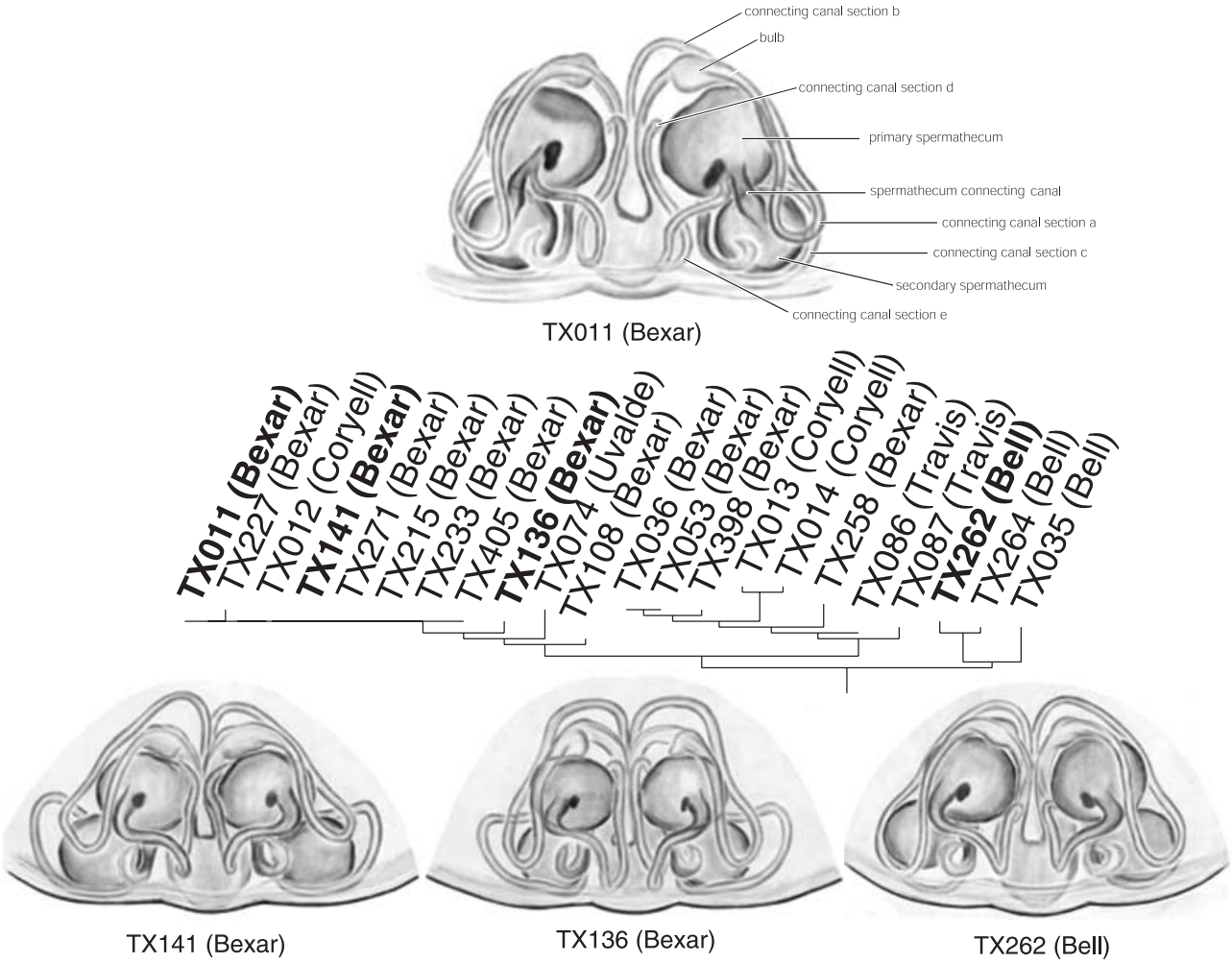


Fig. 6 Examples of female genitalic variation observed within the *Cicurina varians* tip clade. *C. varians* is a troglophilic species found in both cave and favourable surface habitats in parts of Texas, New Mexico and Colorado (summarized in Cokendolpher 2004a), and is one of the most common and wide-ranging spiders found in Texas caves (Reddell 1965). Female specimens (TX011, TX136, TX141 and TX262) were illustrated from dorsal views. Morphological terminology follows Chamberlin & Ivie (1940). Phylogeny inset from Fig. 2.

Our molecular results fail to indicate a phylogenetic separation of these species (Figs 2 and 3), as specimens identified as *C. caliga* carry sequences that are very close to those of *C. hoodensis* (we note that all specimens of both species were identified by J. Cokendolpher). Indeed, specimens from both species collected in Triple J Cave share the same CO1 sequence. Even sequences from caves where the species are allopatric (*C. caliga* in Streak Cave; *C. hoodensis* in Peep in the Deep Cave) are mixed within this tight genetic clade (Figs 2 and 3). Two alternatives seem clear. Either these closely related species share sequence variation because of molecular introgression (incomplete lineage sorting is also possible), or the taxa are, in fact, synonymous. Although we lean towards the synonymy hypothesis, the available data do not allow us to distinguish these alternatives. Further studies of possible niche divergence within caves, increased genetic sample sizes and nuclear DNA data are needed to resolve this incongruence issue.

A second problematic case involves *C. madla* and *C. vespera*, both listed as US Federally Endangered species. Government Canyon Bat Cave is the type and only known locality for *C. vespera* (Gertsch 1992), a species known only from a single adult female. Government Canyon Bat Cave is geographically adjacent to the main distribution of *C. madla*, and there are no known subterranean geological barriers separating caves inhabited by these taxa (see Fig. 5A). Contrary to a priori expectations, sequences from two immatures from Government Canyon Bat Cave are well embedded within the *C. madla* tip clade (Fig. 2). This situation differs from the *C. hoodensis*/*C. caliga* situation, as *C. vespera* and *C. madla* are not obviously morphologically allied taxa (Cokendolpher 2004a), and the species were not expected to be in sympatry.

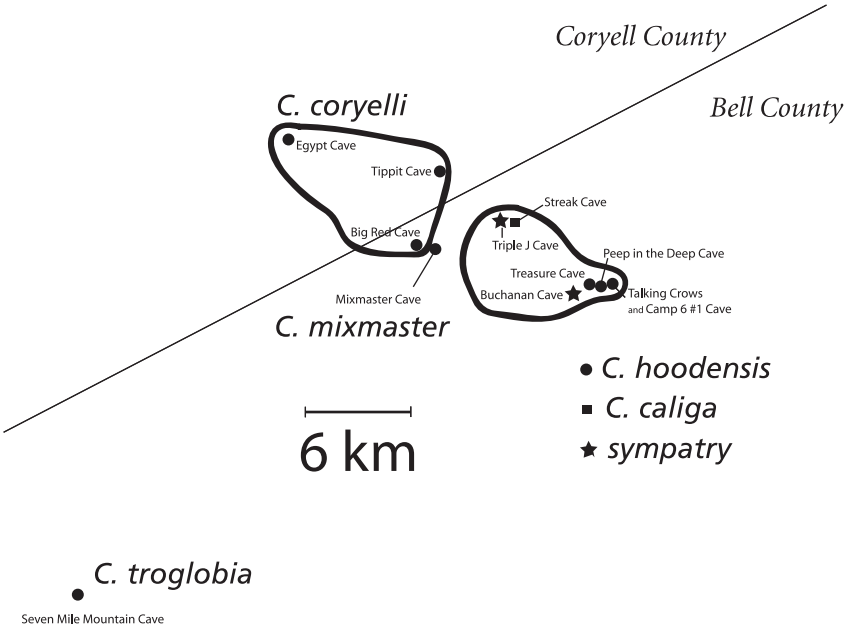


Fig. 7 Map of Coryell and Bell Counties showing the distribution of *Cicurina coryelli*, *C. troglobia*, *C. mixmaster*, *C. caliga* and *C. hoodensis*, as based on published records (Cokendolpher & Reddell 2001; Cokendolpher 2004b). The distribution of *C. caliga* and *C. hoodensis* is bounded by a single line, reflecting the possible synonymy of these species (see text).

We see three alternatives. First, *C. madla* actually occurs in sympatry with *C. vespera* in Government Canyon Bat Cave, and our immatures represent *C. madla*. Second, *C. madla* occurs in sympatry with *C. vespera* in Government Canyon Bat Cave, our samples represent *C. vespera*, but molecular introgression has occurred. Third, *C. vespera* is, in fact, synonymous with *C. madla*. This last hypothesis would be surprising, given the apparent morphological differences between these species. We view all three alternative hypotheses as potentially viable, but not resolvable with current data. There is a critical need for the collection and analysis of DNA sequences from more adult females from Government Canyon Bat Cave. If these females reveal morphological variation consistent with that seen in *C. madla*, and continue to cluster within the *C. madla* tip clade, we would favour a synonymy hypothesis.

Although the examples discussed above are not conclusive, they illustrate a fundamental weakness of one-dimensional molecular taxonomy—genealogical incongruence is impossible to detect without an external reference. That is, if our taxonomy is based entirely on a gene tree, we can never observe incongruence, because the species tree *is* the gene tree. The ability to detect incongruence is vital in taxonomy for two reasons. First, incongruence directs our taxonomic attention to species in need of additional research, as argued for the taxa highlighted above. Second, incongruence raises a 'red flag', reminding us that it is inappropriate to interpret gene trees at face value. There are now dozens of empirical examples illustrating the processes of incomplete lineage sorting, balancing selection, and molecular introgression (e.g. Ballard 2000; Sota *et al*. 2001; Beltran *et al*. 2002; Shaw 2002). These processes result in situations where taxonomically valid species are mixed or indistinguishable on a gene tree, presenting an obvious problem for molecular taxonomy.

Conservation implications

Bexar County, Texas is not only a hotbed for troglomorphic arthropod species, including several eyeless *Cicurina* species (see Cokendolpher 2004a), but also a region of recent and rapid economic and housing development (Rappaport Clark 1998; Longacre 2000). The city of San Antonio has developed at a remarkable rate during the past 20–30 years, infringing upon extensive karst habitats in the northern sector of the county. This growth, and the coincident threats and habitat destruction associated with such growth, have prompted the listing of nine terrestrial arthropods from Bexar County as US Federally Endangered (Longacre 2000). Four of these species are eyeless *Cicurina*, including *C. madla*, *C. vespera*, *C. venii* and *C. baronia*.

Effective conservation of endangered (and other) *Cicurina* requires accurate and approximately complete taxonomic information, which we have attempted to supplement via the molecular identification of previously unidentifiable immatures. Our results have several tangible conservation implications. First, we have gathered data suggesting the possible synonymy of *C. vespera* and *C. madla*. This synonymy issue has clear implications for the conservation of these listed species, and we have suggested possible ways to settle this issue. Second, we feel that additional attention should be directed at eyeless *Cicurina* species that appear to be single cave endemics. Our data support the existence of such endemics (e.g. *C. troglobia*, *C. brunsi*), which would

seem to deserve higher conservation status than currently afforded. Finally, our relatively large geographical sample for *C. madla* allows novel insight into the distribution and genetic structuring of this species.

Gertsch (1992) described *C. madla* from a single individual female collected from Madla's Cave (Fig. 4A). Following the listing of this species, regional collecting efforts intensified, resulting in the discovery of additional adults from new sites (Fig. 4B; White *et al*. 2001; Cokendolpher 2004a). Our DNA evidence suggests that the distribution of *C. madla* may actually encompass up to 20 caves (Fig. 4C), although most of the new records are based on immature specimens only. Although we view these data with due caution, we feel strongly that the genetically inferred distribution of *C. madla* should be used as the most up-to-date working hypothesis when making near-term management decisions involving this species. The data are imperfect, but are much more than we have had in the past. The alternative is to wait for verification via the collection or rearing of adult specimens. Both of these options require a waiting time that may be too costly in a climate of very rapid change and development.

Our data also allow preliminary insight into the geographical structuring of genetic variation in *C. madla*. *C. madla* is distributed over a karst landscape of northern Bexar County that has been subdivided into four 'karst faunal units' (Fig. 5A). These units are hypothesized areas of endemicity for terrestrial cave faunas (see Veni 1994; White *et al*. 2001; Cokendolpher 2004a), reflecting (to a greater or lesser degree) geological isolation across units. For terrestrial cave dwellers, the downcutting of major streams is thought to represent the most significant barrier to movement between units (Veni 1994). However, the ultimate impact of these barriers depends very much upon the biology and regional history of the taxonomic group of interest. We might expect some arthropod taxa to show endemic species in all four areas, whereas other taxa may only show regional phylogeographical divergence, or perhaps no divergence (see White *et al*. 2001). Our study of *C. madla* is the first modern genetic study conducted for a terrestrial troglobite in the region, and as such, provides initial insight into this issue.

Consideration of female genital morphology in *C. madla* suggests possible divergence across Helotes Creek, separating Government Canyon and Helotes populations from those found further east (see Cokendolpher 2004a; Fig. 47; our Fig. 5A). This evidence thus suggests at least two population units in *C. madla* that might be considered in management and recovery plans. In contrast, TCS analysis of sampled haplotypes reveals unique genetic variation in all four areas (Fig. 5B). Most tight genetic clusters in the network are from the same karst faunal unit, but the units themselves are not necessarily monophyletic on the network. Moreover, the data do not reveal a simple east/west trend as predicted by morphology. Although larger genetic samples are needed to further explore these population genetic patterns, the available data suggest two avenues for further study. First, our data suggest unanticipated genetic connections across karst faunal regions, a finding foreshadowed by predictions of White *et al*. (2001). Understanding these connections will require the collection of much more genetic data, and a more detailed analysis of geological barriers in the region. Second, it is apparent that all four faunal units carry at least some novel genetic variation. To maintain extant genetic variation in *C. madla*, we recommend conservation activities in all four regions.

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Pierre Paquin is currently a postdoctoral researcher working with Marshal Hedin at San Diego State University. His research interests include spider taxonomy and related biodiversity/conservation topics. Marshal Hedin is a member of the Evolutionary Biology Program Area in the Department of Biology at SDSU. He has interests in spider diversity and evolution, cave biology, and the speciation process. Our study of *Cicurina* conservation phylogenetics represents a convergence of interest in spiders, caves, and habitat conservation.

Appendix

Sample Information

Species name	Voucher number	Sex	Locality	Voucher location	ID	Genbank Accession no.
Eyeless species						
Cicurina brunsi Cokendolpher	TX170	F	TX: Bexar County, Stahl's Cave	1	JCC	AY633098
Cicurina bullis Cokendolpher	TX001	M	TX: Bexar County, Hilger Hole (Camp Bullis)	1	*post*	AY633005
	TX116	imm	TX: Bexar County, Hilger Hole	2	*post*	AY633010
	TX037 paratype	F	TX: Bexar County, Root Canal Cave (Camp Bullis)	1	JCC	AY633006
	TX039	imm	TX: Bexar County, Root Canal Cave	2	*post*	Ay633007
	TX080	F	TX: Bexar County, Root Canal Cave	1	JCC	AY633009
	TX064	F	TX: Bexar County, Eagles Nest Cave (Camp Bullis)	1	JCC	AY633008
	TX169	M	TX: Bexar County, Eagles Nest Cave	1	*post*	AY633011
Cicurina buwata Chamberlin & Ivie	TX100, 101	imm	TX: Williamson County, Testudo Tube	2	*post*	AY633084, AY633085
Cicurina caliga Cokendolpher & Reddell	TX003	F	TX: Bell County, Streak Cave (Fort Hood)	1	JCC	AY633016
	TX004, 005	F	TX: Bell County, Triple J Cave (Fort Hood)	1	JCC	AY633017, AY633018
Cicurina coryelli Gertsch	TX179	F	TX: Bell County, Egypt Cave (Fort Hood)	1	JCC	AY633074
Cicurina ezelli Gertsch	TX119, 120	imm	TX: Hays County, Ezell's Cave	2	*post*	AY633087, AY633088
Cicurina hoodensis Cokendolpher & Reddell	TX006, 007, 008, 010, 032, 071, 180, 181	F	TX: Bexar County, Buchanan Cave (Fort Hood)	1	JCC	AY633019–21, AY633023–24, AY633027–29
	TX009, 033	F	TX: Bell County, Triple J Cave (Fort Hood)	1	JCC	AY633020, AY633025
	TX034	F	TX: Bell County, Peep in the Deep Cave (Fort Hood)	1	JCC	AY633026
Cicurina loftini Cokendolpher	TX399	F	TX: Bexar County, SBC Cave	2	PP	AY633012
	TX400	imm	TX: Bexar County, SBC Cave	1	*post*	AY633013
Cicurina madla Gertsch	TX038	F	TX: Bexar County, PDH	1	JCC	AY633052
	TX383	imm	TX: Bexar County, PDH	2	*post*	AY633070
	TX241	M	TX: Bexar County, CBR	2	PP	AY633065
	TX283, 284	imm	TX: Bexar County, CRL	2	*post*	AY633068, AY633069
	TX277	imm	TX: Bexar County, SSC	2	*post*	AY633073
	TX216	F	TX: Bexar County, LHH	2	PP	AY633062
	TX397	imm	TX: Bexar County, Madla's Cave	2	*post*	AY633071
	TX221	imm	TX: Bexar County, CBH	2	*post*	AY633063
	TX245	imm	TX: Bexar County, CAL	2	*post*	AY633066
	TX248	imm	TX: Bexar County, YFF	2	*post*	AY633067
	TX137	F	TX: Bexar County, HPL	2	PP	AY633057
	TX192	imm	TX: Bexar County, LOS	2	*post*	AY633058
	TX199	imm	TX: Bexar County, NMF	2	*post*	AY633059
	TX203	imm	TX: Bexar County, RAS	2	*post*	AY633060
	TX210	F	TX: Bexar County, CVJ	2	PP	AY633061

Appendix *Continued*

Species name	Voucher number	Sex	Locality	Voucher location	ID	Genbank Accession no.
Cicurina madla Gertsch (*Continued*)	TX232	imm	TX: Bexar County, GWJ	2	*post*	AY633064
	TX267	F	TX: Bexar County, UEH	2	PP	AY633072
	TX072	imm	TX: Uvalde County, RAM	2	*post*	AY633053
	TX127	imm	TX: Bexar County, CQH	2	*post*	AY633056
Cicurina mixmaster Cokendolpher & Reddell	TX002	F	TX: Coryell County, mixmaster Cave	1	JCC	AY633014
Cicurina platypus Cokendolpher	TX145	imm	TX: Bexar County, Platypus Pit	2	*post*	AY633080
	TX060	F	TX: Bexar County, MARS Pit (Camp Bullis)	1	JCC	AY633077
Cicurina puentecilla Gertsch	TX041, 042	imm	TX: Bexar County, B-52 Cave	2	*post*	AY633075, AY633076
	TX261	F	TX: Bexar County, Black Cat Cave	2	PP	AY633081
	TX123	imm	TX: Comal County, Natural Bridge Caverns	2	*post*	AY633079
	TX121	F	TX: Comal County, Natural Bridge Caverns	2	PP	AY633078
Cicurina reddelli Gertsch	TX281	imm	TX: Travis Co., Cottrell Cave	2	*post*	AY633099
Cicurina reyesi Gertsch	TX092	F	TX: Travis County, Airman's Cave	2	PP	AY633082
	TX093	imm	TX: Travis County, Airman's Cave	2	*post*	AY633083
Cicurina troglobia Cokendolpher	TX070 holotype	F	TX: Bell County, Seven Mile Mountain Cave	1	JCC	AY633015
Cicurina vespera Gertsch	TX104, 105	imm	TX: Bexar County, Government Canyon Bat Cave	2	*post*	AY633054, AY633055
Cicurina vibora Gertsch	TX133, 135	imm	TX: Williamson County, Temples of Thor Cave	2	*post*	AY633089, AY633090
	TX251, 252	imm	TX: Bexar County, Sunless City Cave	2	*post*	AY633091, AY633092
Cicurina sp.	TX118	imm	TX: Travis County, Lakeline Cave	2	*post*	AY633086
Eyed species						
Cicurina arcuata Keyserling	TX366	F	Canada: QC, Missisquoi, Saint-Armand	2	PP	AY633100
	TX367	M	Canada: QC, Missisquoi, Saint-Armand	2	PP	AY633101
Cicurina breviaria Bishop & Crosby	TX159	M	NC: Macon County, S of Wayah Bald	2	PP	AY633096
	TX410	F	NC: Haywood County, Hwy 276, 1 mi. N of BRP	2	PP	AY633097
Cicurina bryantae Exline	TX158	M	NC: Clay County, Tusquitee Mountains, Fires Creek	2	PP	AY633094
	TX160	F	NC: Clay County, Tusquitee Mountains, Fires Creek	2	PP	AY633095
Cicurina pacifica Chamberlin & Ivie	TX353	imm	CA: Kern County, Greenhorn Summit	2	PP	AY633102
Cicurina pallida Kayserling	TX363	F	Canada: QC, Missisquoi, Saint-Armand	2	PP	AY633103
	TX365	M	Canada: QC, Missisquoi, Saint-Armand	2	PP	AY633104
Cicurina pampa Chamberlin & Ivie	TX157	F	TX: Bexar County, UP the Creek Cave (Camp Bullis)	2	JCC	AY633093
Cicurina placida Banks	TX360	M	ME: York County, near Waterboro	2	PP	Ay633105
	TX361	F	ME: York County, near Waterboro	2	PP	AY633106

Appendix *Continued*

Species name	Voucher number	Sex	Locality	Voucher location	ID	Genbank Accession no.
Cicurina varians Gertsch & Mulaik	TX011	F	TX: Bexar County, Low Priority Cave	2	JCC	AY633030
	TX227	imm	TX: Bexar County, CBH	2	*post*	AY633047
	TX012	F	TX: Coryell County, Dangerfield	2	JCC	AY633031
	TX141	F	TX: Bexar County, Platypus Pit	2	JCC	AY633042
Cicurina varians Gertsch & Mulaik	TX271	imm	TX: Bexar County, UEH	2	*post*	AY633046
	TX215	imm	TX: Bexar County, CVJ	2	*post*	AY633048
	TX233	imm	TX: Bexar County, GWJ	2	*post*	AY633049
	TX405	M	TX: Bexar County, UTSA Feature #43	2	PP	AY633051
	TX136	F	TX: Bexar County, HPL	2	PP	Ay633041
	TX074	imm	TX: Uvalde County, RAM	2	*post*	AY633037
	TX108	imm	TX: Bexar County, Government Canyon Bat Cave	2	*post*	AY633040
	TX013, 014	M	TX: Coryell County, Tippit Cave (Fort Hood)	2	JCC	AY633032, AY633033
	TX036	M	TX: Bexar County, Hold Me Back Cave (Camp Bullis)	1	JCC	AY633035
	TX053	F	TX: Bexar County, Cross the Creek Cave	1	JCC	AY633036
	TX398	F	TX: Bexar County, Madla's Cave	2	PP	AY633050
	TX258	imm	TX: Bexar County, Sunless City Cave	2	*post*	AY633043
	TX086	M	TX: Travis County, Airman's Cave	2	PP	AY633038
	TX087	imm	TX: Travis County, Airman's Cave	2	*post*	AY633039
	TX035	M	TX: Bell County, Rugger's Rift Cave (Fort Hood)	1	JCC	AY633034
	TX262	F	TX: Bell County, Black Cat Cave	2	PP	AY633042
	TX264	imm	TX: Bell County, Black Cat Cave	2	PP	AY633045
Yorima sp.	TX351, 352	imm	CA: Glenn Co., Grindstone Road	2	PP	AY633107, AY633108

Voucher location information: 1 = body with JCC, legs at SDSU; 2 = entire specimen at SDSU. Identification: JCC = James Cokendolpher, PP = Pierre Paquin, *post = a posteriori*, based on phylogenetic evidence. To protect the identity of unpublished cave locations for the federally listed *C. madla*, we have used three-letter codes (*these codes were also used for populations of *C. varians* occurring in these same caves). Cave names and locations associated with these codes are available upon request from the authors.

