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Up high and down low: Molecular systematics and insight into the diversification of the ground beetle genus *Rhadine* LeConte ☆

R. Antonio Gómez [a,d,*], James Reddell [b], Kipling Will [c], Wendy Moore [d]

[a] Graduate Interdisciplinary Program in Entomology and Insect Science, 1040 E. 4th Street, PO Box 210077, Tucson, AZ 85721-0077, USA
[b] Department of Integrative Biology, The University of Texas at Austin, 3001 Lake Austin Centre, Austin, TX 78705-5730, USA
[c] Essig Museum of Entomology, University of California, Berkeley, CA 94720, USA
[d] Department of Entomology, University of Arizona, Tucson, AZ 85721-0036, USA

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ABSTRACT

Rhadine LeConte is a Nearctic genus of flightless ground beetles that is poorly studied despite its relevance to evolutionary studies of subterranean fauna. Adults are notable for their slender and leggy habitus and the wide variety of habitat preferences among species, with several known only from mountaintops while others are restricted to caves or more general subterranean habitats. In central Texas, USA there are several cave endemics relevant to conservation. Here we present the first phylogenetic hypothesis for the overall structure of the genus with an emphasis on the troglobites in central Texas. We infer the phylogeny of *Rhadine* from ~2.4-kb of aligned nucleotide sites from the nuclear genes, 28S rDNA and CAD, and the mitochondrial gene COI. These data were obtained for 30 species of *Rhadine* as well as from members of their putative sister group, *Tanystoma* Motschulsky. Results reveal that *Rhadine* is polyphyletic, and morphological characters that have been traditionally used to classify the genus into species groups are shown to be convergent in many cases. *Rhadine* aside from two species of uncertain placement is composed of two major clades, Clades I and II that both include epigean and subterranean species in very unequal proportions. Clade I is primarily composed of subterranean species, and Clade II includes many epigean species and high altitude montane endemics.

A clade of troglobitic, cave-restricted species in Texas includes several species of large-eyed cave *Rhadine*. The slender habitus typical of some species [e.g., *R. exilis* (Barr and Lawrence), *R. subterranea* (Van Dyke), *R. austinica* Barr] evolved independently at least three times. Major biogeographic and evolutionary patterns based on these results include: troglobitic species north of the Colorado River in Texas (that also lack lateral pronotal setae) are found to comprise a monophyletic group, beetles in caves south of the Colorado River likely form another monophyletic group, and the "species pairs" of troglobitic *Rhadine* known to occur in the same caves are not resolved as each other's sister group, suggesting that these caves were colonized independently by more than one lineage of *Rhadine*. Our divergence time estimates support a Miocene age for the split between Clade I and II *Rhadine* and indicate that all subterranean Clade I *Rhadine* began diversifying in the late Miocene–early Pliocene, contemporary with cave formation in the Balcones Escarpment.

1. Introduction

Cave life has inspired biologists for more than one hundred years beginning with the discovery of the first known cave animal, the blind salamander *Proteus anguinus* Laurenti. Biologists have been interested in cave fauna because they are the products of long-term evolutionary experiments (Poulson and White, 1969). They represent some of the most striking examples of morphological convergence ever known (Culver et al., 1990), and while convergence poses problems for phylogenetic inference, it is a great teacher of selection and exaptation (Wake et al., 2011). In the past two decades there have been many studies (see review by Juan et al., 2010) framing cave animals not as evolutionary dead-ends with little diversification potential (e.g., Barr and Holsinger, 1985; Darwin, 1859; Poulson and White, 1969) but as notable and diverse branches of the tree of life. Recent studies are also finding that subterranean organisms can possess key innovative

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* Corresponding author at: Department of Integrative Biology, 3029 Cordley Hall, Oregon State University, Corvallis, OR 97331, USA.
E-mail address: gomezr@science.oregonstate.edu (R.A. Gómez).

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features and can even show higher diversification rates than their surface-dwelling (epigean) relatives (e.g., Cieslak et al., 2014). Nevertheless, many long-standing ecological and evolutionary questions remain in studies of cave organisms such as the roles of dispersal and vicariance, the scenarios that give rise to cave specialists (Barr, 1968; Desutter-Grandcolas and Grandcolas, 1996), the diversification potential and dispersal power of cave organisms (Rizzo et al., 2013), etc. There is also increasing evidence for the recognition of caves as lying on a spectrum of subterranean environments (Giachino and Vailati, 2010), and species known to occur from a variety of surface and subsurface habitats are interesting for evaluating the steps involved in the evolution of subterranean life.

Beetles (Coleoptera) are a prominent clade of animals with many terrestrial and freshwater subterranean species that make for valuable case studies on the evolution of subterranean diversity (e.g., Leys et al., 2003; Faille et al., 2010, 2014; Ribera et al., 2010; Rizzo et al., 2013). Beetles in the North American ground beetle genus *Rhadine* LeConte (Carabidae: Platynini) share many of the useful features found in other animals that have been used as case studies and that have revealed patterns of subterranean evolution. What has been lacking prior to the present study is a rigorously tested phylogenetic hypothesis for relationships within *Rhadine* that allows for tracing the history of morphological and ecological character evolution.

Rhadine includes approximately 50 species distributed from Oaxaca, Mexico to Canada, with most species known from the American Southwest and only a few species known from the eastern United States (Barr, 1960; Bousquet, 2012; Lorenz, 2005). Among carabids *Rhadine* as a whole is notable for its wide variety of habitat preferences ranging from high altitude mountaintops to mesic forests (Lindroth, 1966), rodent burrows and subsurface habitats (Barr, 1974; Van Dyke, 1949), caves (Barr, 1960, 1974) as well as cellars and mine shafts (Barr, 1960, 1974), while a given species may be narrowly endemic to a single habitat and elevation. To add to the complexity of the group's habitat preferences, some typically surface dwelling species are frequently collected within caves (e.g., *R. caudata* (LeConte) Fig. 1C; Barr, 1964). The pattern of ecological preferences in the history of the genus is unknown, and so we made predictions for relationships among these different lineages (Fig. 2A–C). Perhaps there are many isolated lineages of subterranean *Rhadine* (Fig. 2A), which could be the result of a scenario where relatively few surface dwelling ancestors independently colonized subterranean habitats. However, perhaps all subterranean lineages share a more ancient common ancestor (Fig. 2B), or extended further, those more specialized, troglobitic species may be a natural group (Fig. 2C) similar to the discovery of clades of exclusively troglobitic beetles from Pyrenean caves (Faille et al., 2013; Ribera et al., 2010).

Rhadine adults possess a distinctive, graceful habitus (Fig. 1) being long-legged and slender, often lightly pigmented, with strongly rounded elytral humeri and short metepisterna as commonly found in flightless carabids (e.g., Darlington, 1936). They lack flight wings (Barr, 1982) and are likely to have limited dispersal abilities. The morphological diversity of the genus is perhaps most readily appreciated among subterranean species (Fig. 1B–F) some of which possess 'aphaenopsian' features typical of the troglobitic form such as reduced compound eyes, long sensory setae and appendages, are depigmented and lightly sclerotized (Barr and Holsinger, 1985; Culver et al., 1990; Jeannel, 1943). The sister group of *Rhadine* is hypothesized to be *Tanystoma* Motschulsky, a genus of epigean beetles from the Mediterranean California ecoregions that includes species that are flight wing polymorphic (Liebherr, 1985, 1986). The characteristics of cave *Rhadine* and their putative relatives are similar to those of insular subterranean biotas that have been studied to address biogeographical questions formulated from oceanic island animals (e.g., Cooper et al., 2007). The genus, therefore, is an appealing group for studying regressive evolution (the reduction or total loss of traits over time) and biogeography. Evaluating the relationship between *Rhadine* and *Tanystoma* is also relevant to understanding the role of subterranean modifications in species diversification as *Rhadine* has ten times as many species, a much broader range of forms, and is also known from more habitat types.

Most of the troglobitic *Rhadine* species are known from limestone caves in the Balcones Escarpment and Edwards Plateau of central Texas (Barr, 1974; Bousquet, 2012), which are estimated to have become available approximately 5.3 million years ago (Ward, 2006; White et al., 2009; Wilson, 1956). The caves of the Balcones Fault Zone and Edwards Plateau are home to a spectacular diversity of cave adapted species from throughout Metazoa including: flatworms, leeches, amphipods, spiders, pseudoscorpions, millipedes and centipedes, crustaceans, harvestmen, collembolans, cave crickets, beetles, salamanders, and catfish (see Reddell (1994) for a review of the cave fauna of this region and Mitchell and Reddell (1971) for a review of the invertebrate cave fauna). Among subterranean beetles, troglobitic species from this karst region are known in Carabidae (thus far only *Rhadine*), pselaphine Staphylinidae in the genera *Batrisodes* Reitter and *Texamaurops* Barr and Steeves (Chandler, 1992), and Curculionidae with *Lymantes nadinae* Anderson (Paquin and Anderson, 2009). The region and the evolutionary history of its subterranean fauna have been the focus of several recent studies (e.g., Bryson et al., 2014; Miller et al., 2013; Taylor et al., 2007; Wiens et al., 2003), but ours is the first to investigate relationships within the genus *Rhadine*.

Individual troglobitic *Rhadine* species have restricted ranges, but they are often known from more than a single cave (Barr, 1974) and fit the description of narrow-range endemics (Harvey, 2002; Harvey et al., 2011). Three species are red-listed and threatened or endangered (Bousquet, 2012). Some caves are known to contain more than one species of troglobitic *Rhadine*, and in each case, the two species have markedly different body sizes and shapes (Barr, 1974; Fig. 1B + C, E + F). Robust phylogenetic frameworks, which include both members of such species pairs, and good taxon sampling in general, can reveal patterns of diversification, colonization, and morphological modifications (Juan et al., 2010). Previous studies of other arthropod taxa including such species pairs have found evidence for sympatric speciation post colonization from a single common ancestor (Arnedo et al., 2007; Leys et al., 2003; Leys and Watts, 2008).

The most recent major treatment of *Rhadine* was done by Barr (1974) whose efforts focused primarily on the microphthalmous subterranean species, but he also divided the entire genus into six species groups. Later, Barr (1982) revised the species known to occur in Mexico, but aside from isolated descriptions of new troglobitic species in the *subterranea*-group from Texas (Reddell and Cokendolpher, 2001, 2004; Reddell and Dupérré, 2009), the genus has received very little attention. Representative *Rhadine* species have only been included in previous phylogenies aimed at resolving higher-level relationships of Harpalinae (Ober and Maddison, 2008) or North American Platynini (Liebherr, 1986) based on DNA sequence data and adult morphological character data respectively. Barr (1960, 1974) presented intuitive trees (Fig. 2D and E) of the *subterranea*-group species with little discussion of deeper relationships of the genus as a whole. He observed that some species of cave-restricted *Rhadine* are extremely slender-bodied (Fig. 1C and F) whereas others are more robust (Fig. 1B and E), and he proposed that this might be phylogenetically informative (Fig. 2D; Barr, 1960). Later he also offered an alternative hypothesis that the slender forms may have evolved multiple times and be the result of convergence (Fig. 2E; Barr, 1974). However these hypotheses assume that all troglobites are in a single clade (Fig. 2C).

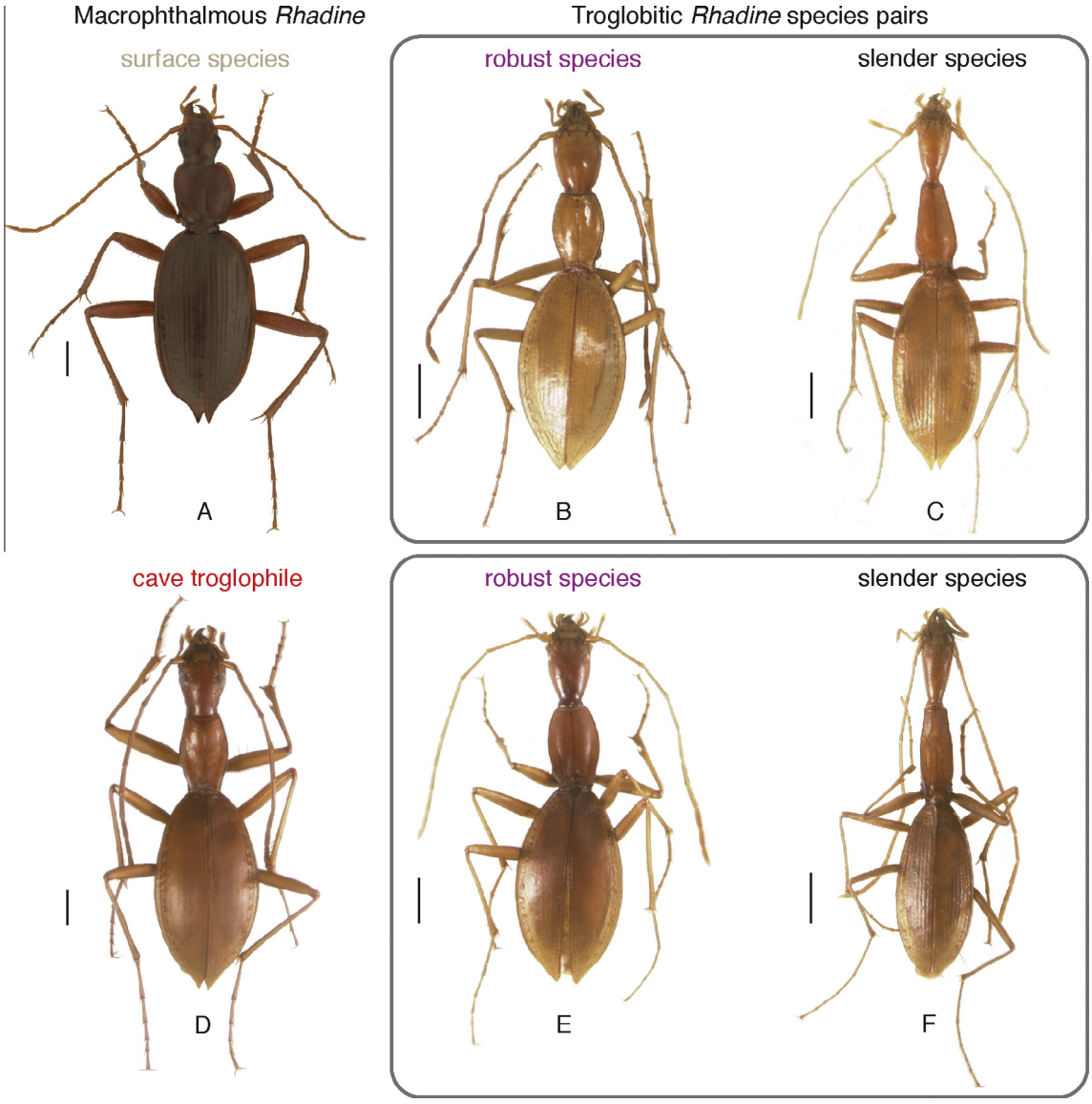

Fig. 1. Dorsal habitus of *Rhadine* adults. A. – *R. caudata* (photograph shared under creative commons by Terry Erwin); B. – *R. persephone*; C. – *R. subterranea*; D. – *R.* cf. *babcocki*; E. – *R. infernalis*; F. – *R. exilis*. Beetles bounded by gray lines are examples of troglobitic species pairs, B and C live in the same cave north of the Colorado River in Texas, E and F live in the same cave south of the Colorado River. Scale bars = 1 mm.

Barr (1960, 1974) also proposed a climatic relict hypothesis (e.g., Banarescu, 1975; Barr, 1968; Barr and Holsinger, 1985; Peck and Finston, 1993; Sbordoni, 1982) for the origin of troglobitic species in the genus. He hypothesized that the troglobitic species constitute a monophyletic group that descended from populations of a more facultative, troglophilic ancestor that became isolated in caves during transitions from cool, moist glacial periods to warm, dry interglacial periods (Fig. 2F; Barr, 1974). An alternative possible scenario for the origin of troglobites, the adaptive shift hypothesis (Desutter-Grandcolas and Grandcolas, 1996; Rivera et al., 2002; Rouch and Danielopol, 1987) posits that speciation occurs following an adaptive shift and that surface dwelling descendants of their most recent common ancestor still occur in the same geographic area (Fig. 2G).

This study presents the first in-depth attempt at inferring the phylogeny of *Rhadine* based on formal phylogenetic analyses and the first investigation of the group's phylogeny using molecular sequence data. Barr (1974) presented intuitive trees, and these did not seek to address the relationships within *Rhadine* as a whole. We aim to infer the phylogeny of *Rhadine* based on molecular sequence data by sampling exemplars across the known morphological diversity of the group to address (1) what are the relationships among epigean and subterranean *Rhadine*, and what can they tell us about the evolution of subterranean life?, (2) are Barr's species groups natural?, (3) what is the geographic structure of cave *Rhadine*, and is the general habitus of troglobites homoplastic or not?, and (4) are troglobitic species pairs monophyletic, and is there evidence for sympatric speciation? We also build a preliminary time-calibrated tree and use model comparison to evaluate alternative hypotheses for the timing of the origin of troglobitic life histories in *Rhadine*.

2. Materials and methods

2.1. Taxon sampling

Sequence data from 19 Platynini species were included in the analyses (Table S1) as outgroups. These data were collected as part

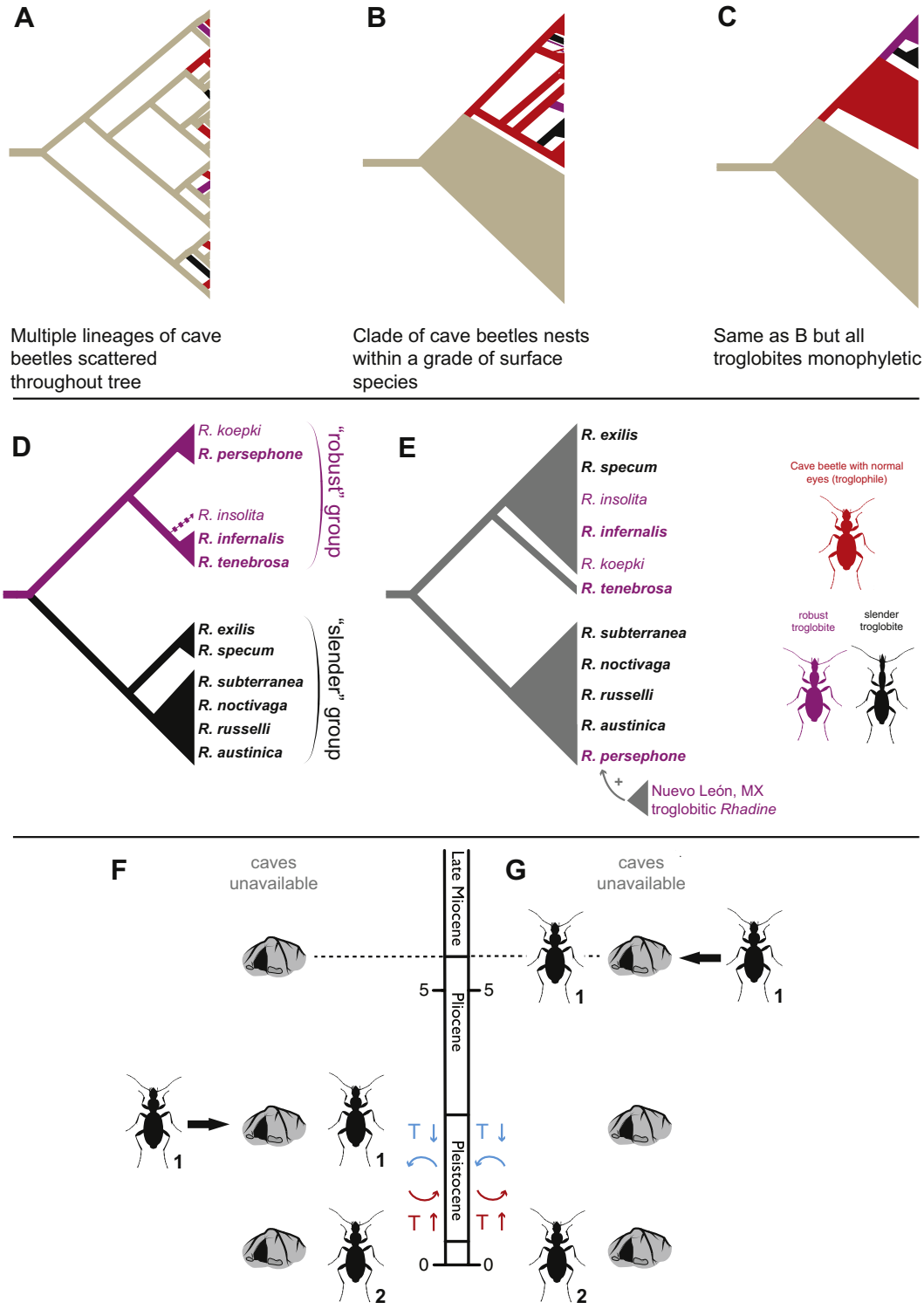

Fig. 2. A–C: Three alternative predictions of the evolutionary relationships of surface and subterranean *Rhadine*, A–C. Previous published hypotheses for relationships among troglobitic *Rhadine* (D and E): D. – intuitive tree presented by Barr (1960, 1974) showing two monophyletic groups: a "slender" and a "robust" group each containing only slender-bodied species and robust-bodied species respectively. E. – alternative hypothesis by Barr (1974) based on geographic and taxonomic distances among species (see Fig. 1 and Section 1) that depicts a tree in which the slender habitus is homoplastic, and there are slender- and robust-bodied *Rhadine* beetles in each major clade; the placement of Mexican troglobitic *Rhadine* (*R. chipinque* and *R. eliotti*) with *R. persephone* was proposed later (Barr, 1982). Taxon names are colored by habitus of the species; purple for robust-bodied species and black for slender-bodied species. Bolded taxon names are those species for which we have sequenced DNA as part of this study. F and G: Simplistic representations of two prominent hypotheses for the origin of troglobitic species: F – climatic relict hypothesis; G – adaptive shift hypothesis. Subscript "1" next to drawings refers to the epigean ancestral lineage of a troglobitic descendant indicated with subscript "2" that colonized a cave system in response to climate change (F) or that colonized a cave system around when it became available as a novel niche (G). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the Beetle Tree of Life (BToL) project (Maddison et al., unpublished), from ongoing projects of Platynini molecular phylogenetics (Will et al., unpublished), and from GenBank and the Barcode of Life Database. Outgroup taxa were selected based on the most recent comprehensive phylogeny of Harpalinae (Ober and Maddison, 2008). This study placed *Atranus* LeConte as sister to all remaining sampled platynine taxa, and for this reason, we rooted our phylogenies with *Atranus*.

We also sequenced species of *Tanystoma*, the putative sister group of *Rhadine* (Liebherr, 1986). In addition, we sampled a member of the *Rhadine–Tanystoma* lineage of uncertain placement that we tentatively identified as *Rhadine–Tanystoma* lineage gen. indet. sp. nr. *T. diabolicum* Liebherr (voucher nos. 3018, 3064, 3314, 3354) based on its combination of characters and on phylogenetic studies of the *Rhadine–Tanystoma* lineage (Liebherr, 1986, 1989b). The *Rhadine–Tanystoma* lineage is also thought to include an eastern Palearctic genus, *Paranchodemus* Habu (Liebherr, 1989a,b), but this relationship remains untested as we were unable to obtain DNA-grade material of *Paranchodemus*.

Sixty-five specimens were sequenced from 30 species of *Rhadine* (Tables 1 and S2), representing all of Barr's species groups. Thirty six vouchers represented 12 troglobitic species, including all three red-listed species. Most species are classified in one of three species groups: the *dissecta*-group (4 of 11 species), the *perlevis*-group (10 of 12 species), and the *subterranea*-group (12 of 18 species). We were unable to sequence *R. larvalis* (LeConte), the type species of the genus. However we included *R. caudata* (LeConte), a *larvalis*-group species thought to be closely related to the type species.

Because there is no monographic revision of *Rhadine*, voucher specimens could not always be identified to species with a high degree of confidence. Whenever possible, multiple specimens were sequenced of a particular species. Because most *subterranea*-group species are known from several, closely adjacent caves, our taxon sampling also emphasized sampling these troglobitic species from throughout their ranges whenever possible. Vouchers are currently held in the University of Arizona Insect Collection (UAIC, W. Moore) and will be deposited in the collections of the loaning institutions at the end of this study (Table S2).

Species identifications with 'cf.' preceding the specific epithet indicate uncertainty in the determination. Identifications with 'sp. nr.' preceding the specific epithet indicate the specimen does

Table 1
Rhadine–Tanystoma lineage species sampled in this study. Species group membership is based on Barr (1974). The numbers in brackets to the right of the species groups are the number of species sampled and the total number of species in the species group respectively. Ecological data are primarily based on the following: Barr (1960, 1968, 1974), Casey (1913), Liebherr (1985), and Van Dyke (1949). The Vouch. column refers to the number of specimens sequenced per species, and the Sites column refers to the total number of localities sampled per species. Additional molecular and locality sampling data are reported in Table S2.

	Vouch.	Sites	General dist. of vouchers	Eye red.	Ecological aspects
Rhadine					
dissecta-group [4/11]					
R. cf. *anthicoides* Casey	1	1	Northern Arizona, USA		Mammal burrows and high elevation surface
R. cf. *dissecta* LeConte	3	3	Southern Arizona, USA		Mammal burrows and mixed elevation surface
R. cf. *rubra* (Barr)	2	1	Northern Texas, USA		Cave troglophile
R. dissecta-group sp. 1	1	1	Southern New Mexico, USA		Cave troglophile
jejuna-group [1/2]					
R. jejuna (LeConte)	1	1	Eastern California, USA		High elevation surface
larvalis-group [1/4]					
R. caudata (LeConte)	1	1	West Virginia, USA		Surface and cave accidental
nivalis-group [1/3]					
R. cf. *umbra* Casey	2	1	Northern New Mexico, USA		High elevation surface
perlevis-group [10/12]					
R. cf. *albamontana* Dajoz	1	1	Eastern California, USA		Surface
R. cf. *babcocki* (Barr)	2	2	Central Texas, USA		Cave troglophile
R. cf. *howdeni* (Barr and Lawrence)	2	2	Central Texas, USA		Cave troglophile
R. cf. *myrmecodes* (Horn)	1	1	Utah, USA		Surface
R. cf. *perlevis* Casey	1	1	Southern Arizona, USA		High elevation surface
R. longicollis Benedict	2	1	Southern New Mexico, USA		Cave troglophile
R. perlevis-group sp. 1	1	1	Southern Arizona, USA		Cave troglophile
R. perlevis-group sp. 2	1	1	Eastern California, USA		Cave troglophile
R. sp. nr. *longicollis* Benedict	1	1	Southern Arizona, USA		Cave troglophile
R. spec. nov. 1	2	1	Western Texas, USA		Cave troglophile
subterranea-group [12/18]					
R. austinica Barr	6	5	Central Texas, USA	✓	Cave troglobite
R. cf. *elliotti* Barr	1	1	Nuevo León, México	✓	Cave troglobite
R. exilis (Barr and Lawrence)	4	3	Central Texas, USA	✓	Cave troglobite
R. infernalis (Barr and Lawrence)	6	5	Central Texas, USA	✓	Cave troglobite
R. noctivaga Barr	5	4	Central Texas, USA	✓	Cave troglobite
R. persephone Barr	3	3	Central Texas, USA	✓	Cave troglobite
R. russelli Barr	1	1	Central Texas, USA	✓	Cave troglobite
R. sp. nr. *austinica* Barr	1	1	Central Texas, USA	✓	Cave troglobite
R. sp. nr. *chipinque* Barr	1	1	Nuevo León, México	✓	Cave troglobite
R. specum (Barr)	2	1	Central Texas, USA	✓	Cave troglobite
R. subterranea (Van Dyke)	6	6	Central Texas, USA	✓	Cave troglobite
R. tenebrosa (Barr)	2	1	Central Texas, USA	✓	Cave troglobite
Unplaced to species group					
R. sp. 1 nr. *nivalis*-group	2	1	Southern Arizona, USA		High elevation surface
Tanystoma					
T. maculicolle (Dejean)	1	1	Southern California, USA		Surface
T. striatum (Dejean)	1	1	Northern California, USA		Surface
Unplaced to genus					
Gen. indet. sp. nr. *T. diabolicum* Liebherr	4	4	Southern California, USA		Surface

not belong to that species but is suspected to be a close relative. Those specimens that could not be identified beyond species group were simply identified as "sp. 1" such as *R. perlevis*-group sp. 1.

2.2. Molecular data collection: DNA extraction, gene selection, amplification, and sequencing

Total genomic DNA was extracted from one mid-leg following the ATL protocol in the Qiagen DNeasy kit (Valenica, CA). Initial extractions included tissue maceration, but we found we were able to obtain satisfactory results without grinding the leg, which allowed us to re-associate it with the rest of the voucher specimen after extraction (Gilbert et al., 2007).

Gene fragments from cytochrome c oxidase subunit I (COI), 28S ribosomal DNA (28S or 28S rDNA), and carbamoylphosphate synthetase domain of the rudimentary gene (CAD) were amplified using the Polymerase Chain Reaction (PCR) on an Eppendorf Mastercycler Thermal Cycler with Invitrogen Platinum Taq DNA Polymerase (Carlsbad, CA). The primers, details of the PCR protocols and cycling conditions are provided in the Appendix. The amplified products were cleaned, quantified, normalized and sequenced at the University of Arizona's Genomic and Technology Core Facility using an Applied Biosystems 3730 DNA Analyzer or a 3730 XL Applied Biosystems automatic sequencer.

Simultaneous contig assembly and initial base calls were performed using the Phred (Green and Ewing, 2002) and Phrap (Green, 1999) programs as implemented in Mesquite 2.71 (Maddison and Maddison, 2009b) in combination with the Chromaseq package (Maddison and Maddison, 2009a). Final base calls were made after manual inspection of individual sequences in Chromaseq; universal ambiguity, IUPAC, codes were used when multiple peaks were present at individual sites.

Sequences obtained for all *Rhadine–Tanystoma* lineage taxa sampled in this study were deposited in GenBank with accession numbers KM986120 through KM986319 (Table S2).

2.3. Multiple sequence alignment

Fragments of COI and CAD were not length variable and were manually aligned in Mesquite (Maddison and Maddison, 2009b), and the resulting matrices were 559 bp and 648 bp respectively. Longer COI and CAD sequences were submitted to GenBank for some taxa, but these sites were trimmed prior to analyses if they were not present in 50% of the taxa. 28S rDNA sequences were aligned using an online version of MAFFT 7 (http://maftft.cbrc.jp/alignment/server; Katoh and Standley, 2013) employing a Q-INS-i search strategy that accounts for RNA secondary structure (Katoh and Toh, 2008). The alignment was then inspected in Mesquite and obviously misaligned blocks were corrected manually.

2.4. Phylogenetic reconstruction

Phylogenetic trees were inferred using model-based methods on individual gene matrices as well as a concatenated matrix of all three gene fragments ("Total data", ~2.4 kb). Prior to tree building, optimal models and partitioning schemes were selected using PartitionFinder (Lanfear et al., 2012) and the Bayesian Information Criterion. PartitionFinder selected the GTR+I+G model for each gene separately excluding CAD, for which the invariant option was not selected. The following abbreviations are used when reporting support values for particular groups: maximum likelihood bootstrap (MLB) and posterior probabilities (pp).

Maximum likelihood (ML) heurisitic searches were conducted using RAxML 8.0.9 (Stamatakis, 2006, 2014) on CIPRES (Miller et al., 2010). ML searches based on single gene matrices included 1000 alternative runs. Searches based on the concatenated matrix included 1000 alternative runs repeated twice from different starting seeds, keeping the highest likelihood tree of the three sets of runs. Non-parametric bootstrap analyses were performed separately from the ML tree searches. Bootstrap values (bs) were inferred from 500 and 1000 bootstrap replicates for single gene and combined data matrices respectively. The partitioning scheme and models selected as optimal for RAxML were used when inferring trees, including the estimate of the proportion of invariant sites +I when selected by PartitionFinder.

Bayesian analyses were performed with MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003). The concatenated dataset was analyzed using GTR+G for CAD as well as +I for COI and 28S with 1 cold chain and 3 heated chains starting from different random points in treespace. Reconstructions were run for 50 million generations logging every 1000 generations. Rather than selecting the burn-in fraction of trees a priori, we evaluated the posterior parameter values for proper mixing and convergence using Tracer 1.6 (Rambaut et al., 2014) and then summarized the tree files after discarding the appropriate burn-in fraction of trees to produce a 50% (posterior probability) consensus tree using SumTrees 3.3.1, a program within DendroPy 3.12.0 (Sukumaran and Holder, 2010). Separate Bayesian analyses were run on CIPRES Science Gateway V. 3.3 (www.phylo.org; Miller et al., 2010).

2.5. Divergence time estimation

Commonly used tree annotation methods incorporate absolute ages on a phylogenetic tree (Rutschmann, 2005). However, there are a large number of pitfalls associated with divergence time estimations, due to the use of imperfect or incomplete fossils (Christin et al., 2013); too few constraints (Saquet et al., 2011); the use of stem age versus crown age of a fossil constraint (Magallón, 2004); sensitivity of model choice, including site-to-site variation (Near and Sanderson, 2004); the rate of evolution of different markers and lineages (Brandley et al., 2011); the reconstruction method or algorithm (Mulcahy et al., 2012), etc. Given all of these well-documented concerns, we undertook divergence time estimation with caution, and we are explicit with our methods and priors. We employed two strategies to estimate the divergence times: (1) using a fossil constraint and (2) using this constraint in conjunction with the mutation rate of COI (our mitochondrial marker) based on previous studies (Contreras-Diaz et al., 2007; Gómez-Zurita et al., 2000; Ruiz et al., 2009).

In general, Platynini fossils are not well studied, and taxonomic identities claimed in publications for most of them are suspect (Klebs, 1910). A notable exception is a recently discovered Baltic amber specimen of *Limodromus*, a modern genus of Platynini (Schmidt, 2015). Since we did not include this genus in our taxon sampling, we used this fossil to constrain the root node of Platynini. One disadvantage of this conservative approach is that we may have underestimated divergence times. Since Baltic amber dates to the Eocene (Grimaldi and Engel, 2005; Ritzkowski, 1997), and may be as young as the Priabonian (33.8–38 Ma; Archibald et al., 2006) we constrained the root node using a lognormal distribution, offset by 35 million years before present with a standard deviation of 1.5 and a mean of 2.0.

Mutation rates in beetles, particularly for mitochondrial markers have been a subject of interest with regard to molecular clocks (Pons and Vogler, 2005; Pons et al., 2010), and studies thus far have rate estimates that vary from 0.0038 substitutions per site per million years per lineage (subs/s/my/l) for Chrysomelidae (Gómez-Zurita et al., 2000) to as high as 0.0861 subs/s/my/l for Adephaga (Pons et al., 2010). For ground beetles, Contreras-Diaz et al. (2007) recovered a rate of 0.0152 subs/s/my/l for the genus *Trechus* Clairville, and Ruiz et al. (2009) estimated a slower rate of 0.0046 subs/s/my/l for the Sphodrini, which are somewhat closely

related to the Platynini. Pons et al. (2010) summarized the variability in estimates of the mutation rate of the cytochrome oxidase I gene in Coleoptera between individual studies and urged caution when using a single marker, particularly COI, to estimate deep divergences. In this study we used a lognormal distribution with a mean of 0.0046 truncated to 0.0152 subs/s/my/l as the upper bound and 0.0038 subs/s/my/l as a lower bound following the methods employed by Schmidt et al. (2012).

Divergence time estimates were conducted using an uncorrelated relaxed clock in BEAST 1.8.0 (Drummond and Rambaut, 2007; Suchard and Rambaut, 2009) using the nucleotide models and partitions selected by BIC and PartitionFinder (Lanfear et al., 2012) using the 'search = beast' option, which were identical to those chosen and used for MrBayes searches with one exception. We initially selected GTR+I+G for our COI subset as recommended by PartitionFinder, and the initial BEAST log files revealed ESS values below 50 for one of the relative rate parameters. We re ran the analysis using HKY+I+G for the COI partition (following methods in Bryson et al., 2014), which resulted in ESS values >200 in final analyses. The XML input files for these analyses were prepared using BEAUti v1.8.0. (Drummond and Rambaut, 2007). We ran each analysis for 150 million generations, sampling every 5000 with either a Yule tree prior or a birth–death process prior.

After comparing the results (see Section 2.6; Table 2), runs with optimal settings were repeated two additional times from different starting seeds. Individual log files were inspected with Tracer 1.6 to ensure proper mixing and adequate sampling as indicated by high ESS values. After discarding the burn-in trees, tree files were combined using LogCombiner 1.8.0 (Drummond et al., 2012) and summarized using TreeAnnotator v.1.8.0 (Drummond and Rambaut, 2007). Mean divergence time estimates with 95% highest posterior density (HPD) error bars were mapped onto the maximum clade credibility tree from BEAST.

2.6. Hypothesis testing in a phylogenetic framework

We used the likelihood ratio test to evaluate (1) support against Barr's (1974) hypothesis of a natural group of Texas troglobites (e.g., Fig. 2D and E) and (2) whether sympatric troglobitic species are each other's sister groups. We tested the support for these hypotheses in RAxML by providing the program a constraint file with the '-g' argument. We constrained tree searches by forcing the troglobitic *Rhadine* from Texas (*subterranea*-group *sensu* Barr, 1974) and the troglobitic species pairs to be monophyletic (see Discussion). We made 3 different constraint files for the two species pairs we sampled, one for each pair independent of the other, along with a third file where both pairs were clades. We compared hypotheses using model fit and AIC values (Arnold, 2010; Burnham and Anderson, 2004) (Table S5).

Table 2
Model comparison based on Log Bayes Factors from marginal likelihood estimations using path sampling in BEAST 1.8.0. Rows refer to separate analyses and are sorted from highest to lowest marginal likelihood. The analysis with a birth–death tree prior, the fossil calibration, and the mtDNA rate prior was chosen as optimal and is shown in Fig. 3.

	Tree prior	Additional constraint	PS
1. Fossil + rate priors	Birth–death	2.58 Ma (H_1)	−18597.8157
2. Fossil + rate priors	Birth–death	5.3 Ma (H_2)	−18598.53698
3. Fossil prior	Birth–death	5.3 Ma (H_2)	−18600.9752
4. Fossil prior	Birth–death	2.58 Ma (H_1)	−18604.05425
5. Fossil + rate priors	Birth–death	–	−18641.0831
6. Fossil prior	Birth–death	–	−18646.9670
7. Fossil + rate priors	Yule	–	−18662.2275
8. Fossil prior	Yule	–	−18668.4900

Second, we evaluated two different evolutionary scenarios for the timing of the origin of troglobitic *Rhadine* by constraining the divergence time of the clade that includes all troglobitic *Rhadine* from Texas caves (node 5 in Fig. 3) and comparing models in BEAST. Hypothesis testing in BEAST (set 2) was conducted using model comparison based on marginal likelihood estimates. Marginal likelihood scores were estimated using path sampling/stone stepping as implemented in BEAUti using the code presented by Baele et al. (2012). Marginal likelihood was estimated from separate runs from different starting seeds totaling 150 power posteriors with 10 million generations per step. The two input files separately specified 100 and 50 power posteriors, and each chain was set to run sufficiently beyond the burn-in stage before estimating the marginal likelihood. In addition to the unconstrained runs performed as specified above, we also ran additional analyses constraining the crown-group age of the clade that includes all Texas troglobitic *Rhadine*. We constrained this clade (node 5 in Fig. 3) with two different normal priors: H_1: mean = 2.58, std = 0.5 and H_2: mean = 5.3, std = 0.5. These priors were chosen in order to test the fit of a model where the most recent common ancestor of these troglobitic species began diversifying when the climate changed dramatically during the Quaternary glaciation (H_1; Barr (1974)) with a model where the crown-group age closely follows the ages of the caves of the Balcones Escarpment (H_2) that formed near the boundary of the Miocene and Pliocene epochs (Ward, 2006; White et al., 2009; Wilson, 1956).

3. Results

3.1. Phylogenetic reconstruction

The highest likelihood tree based on the concatenated matrix partitioned by gene under GTR+I+G is shown in Fig. 4. Support values for these clades in our single gene ML analyses are also reported on this tree. A majority-rule consensus tree built using Bayesian inference for this same dataset and partitioning scheme is shown in Fig. S5.

Support values for most of the nodes along the backbone of the tree are generally high with most of the topological uncertainty limited to shallower nodes, mostly within species (Fig. S4). There is limited disagreement in topology between trees built with likelihood and Bayesian inference (Figs. 4, S4, and S5), and the discordance between reconstruction methods in support values for these arrangements is mostly limited to poorly supported nodes.

The *Rhadine–Tanystoma* lineage is monophyletic with high support (>95 pp, >90 MLB) across datasets and inference methods (Figs. 4, S1–S5). This clade also includes the *Rhadine–Tanystoma* lineage gen. indet. sp. nr. *T. diabolicum* as part of the basal grade. *Rhadine* is not recovered as monophyletic because the *Tanystoma* species are more closely related to some, but not all *Rhadine* species (node 5, Fig. 4). The support values for these basal grade relationships of the *Rhadine–Tanystoma* lineage are somewhat low, and their arrangement varies greatly between individual gene analyses (Figs. 4, S1–S3), suggesting the need for additional data to further resolve these relationships. However, *Rhadine* aside from two troglobitic beetles from northern Mexican caves is well supported in concatenated analyses and is consistently recovered in single gene searches (Figs. 4, S1–S3). This larger *Rhadine* clade (node 5, Fig. 4) is composed of two clades, hereafter referred to as Clade I and Clade II.

Clade I is well supported and was recovered in all analyses, including single gene searches (Fig. 4). It includes members of four of Barr's (1974) six species groups: *dissecta*-, *larvalis*-, *subterranea*-, and *perlevis*-groups. Of these, the *dissecta*-, *perlevis*-, and *subterranea*-groups are all polyphyletic (e.g., Fig. 4). *Rhadine caudata*,

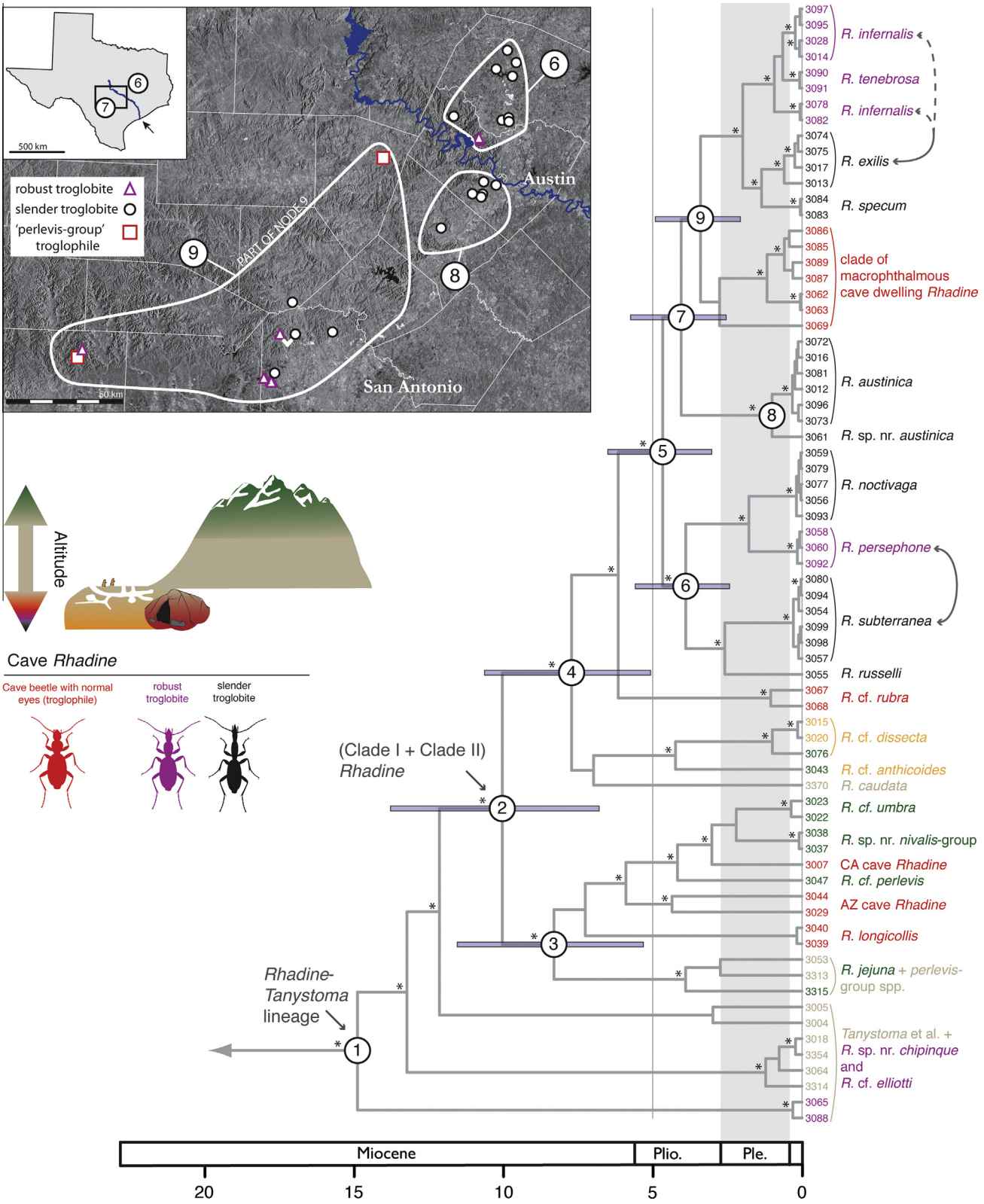

Fig. 3. Time calibrated maximum clade credibility tree annotated with mean ages estimated using a relaxed lognormal clock, a fossil constraint, a birth–death tree prior, and rate priors for our mtDNA with BEAST. Node bars correspond to 95% highest posterior density of divergence time estimates from BEAST, and not all are shown for the sake of legibility. Nodes with error bars are also indicated by circled numbers that correspond to divergence time estimates from both analyses that are reported in Table 3. Four-digit numbers to the right of the terminal branches are extraction codes for separate voucher specimens. Four-digit codes are colored by habitat from which the specimen was collected that corresponds to the cartoon graphics above the scale bar. Additional colors are used to separate troglophiles from the troglobites. Taxon names and clade names are colored based on habitat preferences and morphological aspects of the taxon or taxa. Gray double arrows indicate species pairs. The inset shows the geographic distribution of all of the sampled troglobites in central Texas from node 5 but excludes certain samples of macrophthalmous cave *Rhadine* from node 9 that occur outside of the indicated area. Asterisks above branches indicate nodes supported by over 95 posterior probability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

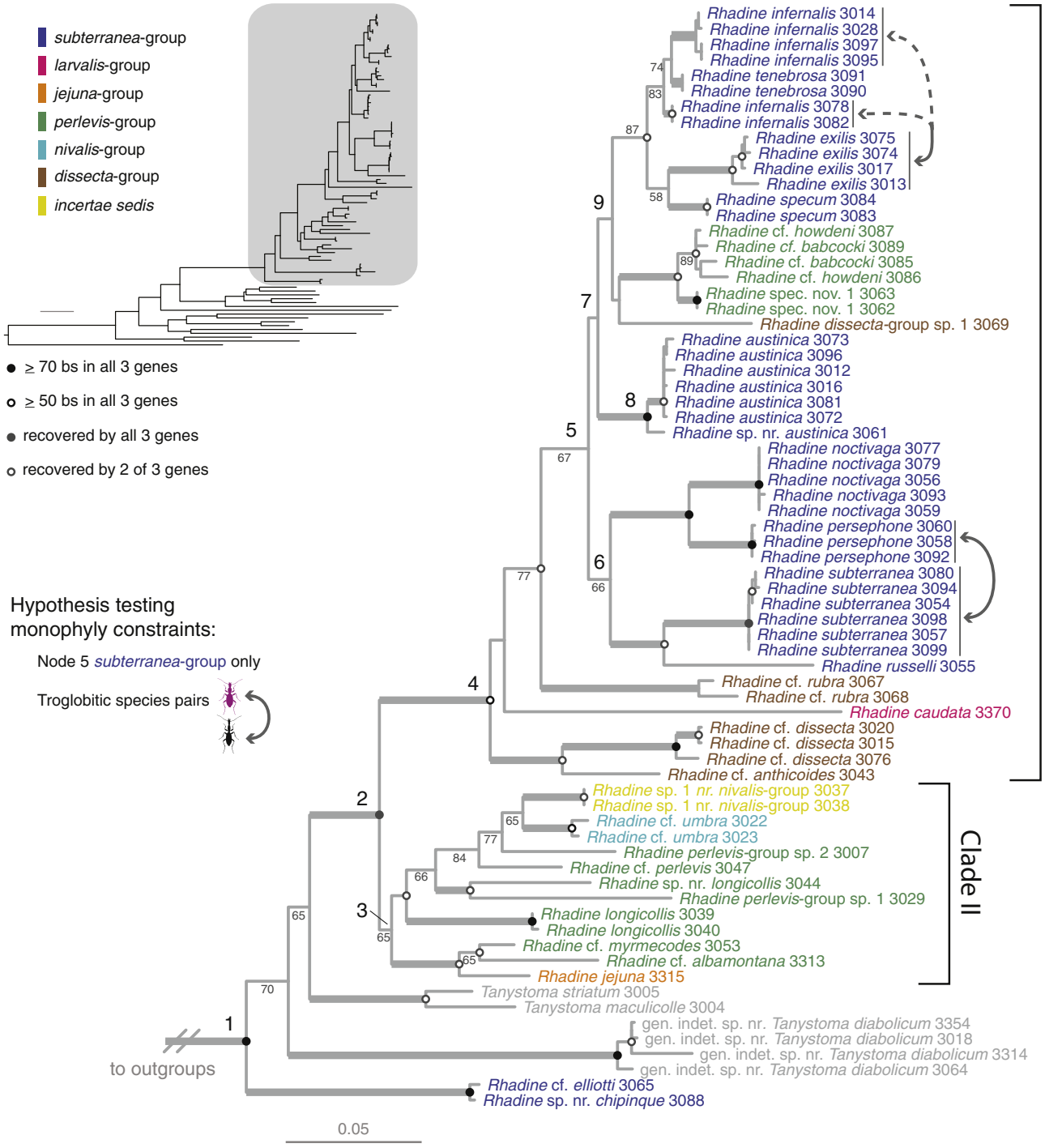

Fig. 4. Highest likelihood tree of concatenated "All data" matrix partitioned by gene under GTR+I+G. Non *Rhadine*–*Tanystoma* lineage taxa are not shown, but the inset in the upper-left depicts the entire phylogram. Branches are thickened if present with over 90 MLB. Numbers below branches are MLB values when present below 90 but above 50. Values of some shallower nodes are not shown due to limited space. Branches and taxon names are colored according to placement within Barr's (1974) species groups or not if placement is ambiguous. Gray double arrows indicate species pairs. Scale bar = 0.05 expected substitutions per site as reconstructed by RAxML. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

our sole representative of the *larvalis*-group, is placed as sister to Clade I cave *Rhadine*. One of three clades of *dissecta*-group species was recovered as sister to all other Clade I taxa. The *dissecta*-group is not monophyletic with respect to *R.* cf. *rubra* Barr and an unidentified subterranean *dissecta*-group species, which share a more recent common ancestor with the troglobitic species from Texas caves (Fig. 4; Table 1). The most diverse clade within Clade I contains several lineages of troglobitic *Rhadine* and a clade of macrophthalmous *Rhadine* in the *dissecta*- and *perlevis*-groups from caves in Texas and New Mexico, USA. The deeper relationships among these lineages are somewhat equivocal between gene trees (Figs. S1–S3), but in concatenated analyses the clade of macrophthalmous cave dwelling *Rhadine* nests within a grade of troglobitic species (Fig. 4).

Clade II principally includes epigean species in the *perlevis*-group *sensu* Barr (1974) as well as the *jejuna*- and *nivalis*-groups

(Fig. 4). Additionally, the *nivalis*-group nests well within a grade of *perlevis*-group species. Clade II is not recovered in COI and CAD single gene analyses, and the basal split within Clade II is equivocal (Figs. S1–S3). Clade II also includes large eyed, cave-dwelling species from New Mexico, Arizona, and California that are not recovered as a clade.

3.2. Divergence time estimates

The chronogram resulting from the BEAST analyses using an uncorrelated, relaxed lognormal clock, a birth–death tree prior, and mtDNA mutation rate priors (Fig. 3) was favored based on model comparison of Log Bayes Factors (Table 2). When mtDNA rate priors are excluded, a birth–death tree prior is also optimal (Table 2). Table 3 summarizes the divergence time estimates from both of these analyses for the following notable clades: the *Rhadine–Tanystoma* lineage, Clade I + Clade II *Rhadine*, Clade I, Clade II, and *Rhadine* north and south of the Colorado river in Texas. The age of the *Rhadine–Tanystoma* lineage is estimated to be approximately Miocene or slightly older. The mean divergence time estimate of the node subtending Clade I and II *Rhadine* is ~10 Ma with 95% highest posterior density between approximately 7 Ma to 14 Ma in the late Miocene (Fig. 3; Table 3). The divergence times of the basal node of Clades I and II are similar, though the latter is estimated to be slightly older. Clade I species that occur in caves in central Texas are estimated to have begun diversifying within the past 4–5 million years, an estimate which is compatible with the ages of the limestone caves in the Balcones Escarpment and Edwards Plateau of Texas. We note that the error bars on our estimates are large (Fig. 3) likely due to the small number of priors. In general, the estimates based upon the analyses using only the primary outgroup fossil constraint were older than those with both the fossil constraint and the substitution rate priors for COI, but the estimates were not significantly different from one another (Table 3). The topology shared by the BEAST maximum clade credibility trees is well supported with most nodes receiving over 95 pp and differs little from the time-free phylogenetic reconstructions (e.g., Figs. 4, S4 and S5). The basal split of Clade I places *R. caudata* as sister to a clade of *dissecta*-group species, which is a notable difference from the arrangement in the time-free phylogenetic reconstruction (Fig. 4).

The estimated mean mutation rate for COI for all platynine taxa included in the study ranged from 0.0113 substitutions/site/MY to a slightly faster estimate of 0.0125 substitutions/site/MY when using the published substitution rate priors as well as the fossil calibration prior. These estimates are very close to recent molecular clock studies from more distantly related beetle lineages (Andújar et al., 2012; Papadopoulou et al., 2010).

3.3. Hypothesis testing

Tables 2 and S5 summarize the results of our hypothesis tests. For every relationship that we tested, our preferred tree (Fig. 3) fits the data significantly better than models with the alternative hypotheses we tested. Based on our data, Barr's (1974) hypothesis of a monophyletic *subterranea*-group from Texas caves is unsupported, and models that do not constrain the troglobitic species pairs to be sister species are strongly favored compared to constrained models (Table S5; $\Delta AIC > 10$).

The constrained BEAST runs based on hypothesis tests received the highest marginal likelihood scores among all analyses performed. In addition, the analyses without our mtDNA priors that constrained the crown group age of node 5 (Fig. 3) to fall within a normal distribution with a mean of 5.3 and a standard deviation of 0.5 fit the data better than a more recent calibration (mean = 2.58, std = 0.5). When we included the mtDNA rate priors, a model with a more recent calibration of node 5 is only marginally better fitting (Table 2).

4. Discussion

4.1. Limits of Rhadine and the Rhadine–Tanystoma lineage

When Barr (1982) revised the species of cavernicolous *Rhadine* from Mexico he described two unusual, small-bodied, microphthalmous, cave species from Nuevo León (*R. elliotti* Barr and *R. chipinque* Barr) that he thought were closely related to *R. persephone* due to the absence of setae on their pronota, the similarity in dorsal habitus, and their somewhat larger eye rudiments (Fig. 2E). Our results indicate that *Rhadine* exclusive of these troglobitic beetles from Nuevo León is monophyletic with strong support (Fig. 4). In light of these results, but given the modest nodal support along the basal grade of our trees, we have chosen to place *R. chipinque* and *R. elliotti* as *Rhadine incertae sedis* (Table S6) until such a time as we can more confidently place them.

Support for the circumscription of *Rhadine* is significant. There are no obvious and unambiguous morphological synapomorphies for the genus, but there are putative morphological synapomorphies for the *Rhadine* + *Tanystoma* clade (e.g., Liebherr, 1986) and for *Tanystoma* (Liebherr, 1989b). However, none of our analyses support *Tanystoma* monophyly. The sister of *Rhadine* Clade I and II is a paraphyletic *Tanystoma*, with moderate support in ML analyses based on the "Total data" matrix, and this is also recovered with high support in the Bayesian analyses of the same dataset (Figs. 4, S4 and S5), corroborating previous cladistic analyses of morphological data (Liebherr, 1986). Furthermore, the epigean *Rhadine–Tanystoma* lineage gen. indet. sp. nr. *T. diabolicum* is con-

Table 3
Divergence time estimates from separate sets of combined BEAST runs for crown ages associated with nodes indicated in Fig. 3. These analyses were chosen as best fitting the data based on model comparison using path sampling (see Table 2) for those analyses that didn't include an additional constraint on node 5 (see Fig. 3 and text). HPD = highest posterior density.

Node number	Clade	Calibration + birth–death tree prior Mean height (95% HPD)	Calibration and mtDNA rate + birth–death tree prior Mean height (95% HPD)
1	*Rhadine–Tanystoma* lineage	16.72 (8.56, 26.51)	14.88 (9.99, 20.50)
2	Clade I + Clade II	11.33 (5.81, 18.08)	10.04 (6.79, 13.78)
3	Clade I	8.71 (4.44, 13.96)	7.71 (5.07, 10.63)
4	Clade II	9.39 (4.78, 15.27)	8.31 (5.31, 11.54)
5	*subterranea*-group species and a clade of macropthalmous cave *Rhadine*	5.32 (2.66, 8.65)	4.66 (3.02, 6.50)
6	*subterranea*-group clade north of the Colorado river	4.44 (2.11, 7.31)	3.89 (2.42, 5.58)
7	Clade south of the Colorado river	4.63 (2.21, 7.57)	4.04 (2.54, 5.74)
8	*R. austinica* and *R.* sp. nr. *austinica*	1.12 (0.35, 2.13)	1.00 (0.37, 1.77)
9	Same as 7, excluding *R. austinica* and *R.* sp. nr. *austinica*	3.89 (1.84, 3.88)	3.40 (2.06, 4.91)

sistently placed as sister to *Rhadine* Clades I and II and a paraphyletic grade of *Tanystoma*. Based on these results, excluding *Rhadine* from *Tanystoma* would render it paraphyletic, and for now we defer to a future treatment on the systematics and classification of the entire generic complex that includes better sampling and makes use of integrative taxonomic methods (Will et al., 2005).

4.2. Phylogenetic relationships within Rhadine

As currently circumscribed, *Rhadine* is non monophyletic, but aside from two troglobitic species from northern Mexico, all *Rhadine* are part of a single clade. This large clade (node 2, Fig. 4) contains two main clades, both of which contain surface and subterranean species (nodes 3 and 4, Fig. 3). All troglobitic Texas *Rhadine* are contained within Clade I (node 4, Figs. 3 and 4), and the troglobitic form (see Section 1) evolved within the *Rhadine–Tanystoma* lineage at least three times. Clade II (node 3, Figs. 3 and 4) includes subterranean species with normally developed eyes and morphological features considered to be less specialized from caves in New Mexico, Arizona, and California (Table 1) that are not a clade. Altogether this indicates that *Rhadine* beetles have colonized subterranean habitats multiple times, and aspects of all three of our predictions for the phylogeny of *Rhadine* match our results.

In addition to diversifying in subterranean habitats, *Rhadine* species have also colonized high altitude habitats. Similar to Clade I containing mostly cave species, Clade II includes many mountaintop endemics and epigean species (Fig. 3). This imbalance in species composition between Clade I and Clade II indicates that subterranean habitats and mountaintops have both been key strategies in the evolutionary history of *Rhadine*. These habitats have also been important to the diversification of other insects such as rock-crawlers (Jarvis and Whiting, 2006; Schoville and Kim, 2011). Restriction to mountaintops and to caves could be parallel responses to the warming and drying of the climate in the Pliocene and to large fluctuations in climate during the Pleistocene. Based on these results, it seems likely that the ecological preferences of *Rhadine* may be an important factor behind its diversity relative to *Tanystoma*. *Rhadine* species often have narrow geographic distributions, occur in more diverse habitats, and are ten times more diverse than *Tanystoma*. *Tanystoma* species are flight wing polymorphic unlike *Rhadine*, are all surface dwelling, and they form a grade in which Clade I and Clade II *Rhadine* nest. This suggests that these *Rhadine* species are descendants of a *Tanystoma*-like epigean ancestor.

Barr (1974) used many of the morphological features typical of subterranean species and their absence in epigean species to classify the genus into different groups, which poses problems when many of these characters are the result of convergence (e.g., Wiens et al., 2003). These morphological features include the degree of eye development, general habitus (see Section 4.3), length and shape of the pronotum, and pubescent pits on the mentum (Barr, 1974). Three out of six of Barr's (1974) species groups were found to be non-monophyletic, and two more nest within larger supraspecific groups. For instance, the *perlevis*-group is partly defined based on an elongated pronotum (Barr, 1974), and cave *Rhadine* from both major clades possess elongated bodies and appendages similar to other subterranean fauna (Culver et al., 1990).

Barr (1974) hypothesized that the *perlevis*-group was closely related to his exclusively troglobitic *subterranea*-group due to similar habits, body form, and presence of densely packed scales on the endophallus. Despite favoring this hypothesis, he also noted that adults of only the *dissecta*- and *subterranea*-groups possess deep, pubescent foveae on the mentum, and he suggested that this character might be evidence for a sister group relationship between these groups (Barr, 1974). Members of both groups are closely related to the troglobitic species from Texas caves. Part of a polyphyletic *dissecta*-group is sister to all other Clade I species, but the remaining *dissecta*-group species share more recent ancestry with cave *Rhadine*. These relationships are relevant to understanding the evolution of habitat preferences and morphological modifications in the genus as other *dissecta*-group species are known troglophiles (Table S2; Barr, 1964) while many other beetles with the same morphological character combination are frequent inquilines of mammal burrows (Barr, 1974).

One of the more surprising results of the present study is that Texas troglobitic *Rhadine* are rendered paraphyletic without the inclusion of a clade of macrophthalmous subterranean *Rhadine* (so far known from caves in Texas and New Mexico). As the macrophthalmous condition is generally considered plesiomorphic (Culver et al., 1990), it was anticipated that the troglobitic taxa would be scattered throughout the tree (Fig. 2A) or would be derived from a basal grade of macrophthalmous species (Fig. 2C). Our results show that the pattern of nesting of macrophthalmous species within a clade of troglobitic taxa is a statistically better fitting model as indicated by ΔAIC values larger than 10 (Arnold, 2010; Table S5). None of the predictions for relationships between epigean and subterranean species (Fig. 2A–C) perfectly match our results, but our findings fit well with other studies showing that there exist large clades of predominately subterranean fauna (Fig. 2B,C) as exemplified here by Clade I *Rhadine*.

Based on our molecular results, we redefine the supraspecific classification of the genus by restricting the boundaries of Barr's (1974) groups or sinking those that nest within larger species group (Table S6) as appropriate. We present a key to these groups in the Appendix. As additional insights into the phylogeny of *Rhadine* are gained, with expanded taxon sampling, more detailed morphological character data, and additional DNA data, the current classification will become more refined.

4.3. The evolution and biogeography of troglobitic Rhadine

Previous research on subterranean fauna has shown that correspondence between geographic distribution and phylogeny is a very common pattern (e.g., Foulquier et al., 2008; Juan et al., 2010; Leys et al., 2003; Ribera et al., 2010). The monophyly of troglobitic *Rhadine* occurring north of the Colorado River in Texas is an example of this pattern apparent in our study (Fig. 3). Five troglobitic *Rhadine* occur north of the Colorado River, which has presumably been a major barrier to dispersal since the Eocene (Veni, 1994), and Barr (1974) suspected that these species were closely related. We included four of these species, which form a clade (Figs. 3 and 4). A similar result was also observed in Clade I subterranean *Rhadine* south of the Colorado River (Fig. 3) albeit with lower support values. This north-south split has also been observed in plethodont salamanders (Wiens et al., 2006) and *Ceuthophilus* cave crickets (Taylor et al., 2007).

There are three instances of two troglobitic species that are sympatric: *R. specum* and *R. koepkei* (not sampled here); *R. infernalis* and *R. exilis*; and *R. subterranea* and *R. persephone*. In each of these pairs, one of the species is distinctly slender-bodied and the other is more robust (Fig. 1; Barr, 1974). This is similar to the pattern of sympatric pairs or triplets of stygobitic diving beetles in Australia (Leys et al., 2003; Leys and Watts, 2008) and spiders of the genus *Dysdera* in the Canary Islands (Arnedo et al., 2007). The series of studies on stygobitic diving beetles have discovered 12–13 cases of sister species that are inferred to be the result of sympatric speciation (Cooper et al., 2002; Leys et al., 2003; Leys and Watts, 2008). We sequenced two of the three pairs, and in neither case were the species pairs found to be monophyletic (Figs. 3 and 4), which contradicts a hypothesis of sympatric speciation.

Based on our analysis, *R. infernalis* is paraphyletic (Figs. 3 and 4, S1–S3). *Rhadine infernalis* is one of five species of troglobitic *Rhadine* with named subspecies and evident morphological variation between populations (Barr, 1960, 1974; Bousquet, 2012). Though the status of *R. infernalis* taxa is uncertain, both clades of *R. infernalis* are more closely related to other troglobites that do not occur in the same caves. Although we did not sample nominal species where sympatric in the case of *R. persephone* and *R. subterranea*, we did sample both *R. infernalis* and *R. exilis* from the same cave, Helotes Blowhole Cave (Fig. 3; Table S2). These taxa are not each other's sister but are part of a larger clade of troglobites. Constraining the search to find the optimal tree that includes the species pairs as monophyletic results in a dramatically poorer fit to the data regardless of whether both or only one of these pairs are constrained to be monophyletic (Table S5).

Two hypotheses can be proposed based on these findings: (1) these caves that contain sympatric troglobitic species were colonized independently, (2) the diversity of these caves was much higher in the past and through extirpation and extinction, these non-monophyletic species-pairs remain as relicts. Hypothesis 1 requires positing divergence prior to cave colonization or dispersal between caves (Rizzo et al., 2013), but these seem more plausible than the specific pattern of extinction required under hypothesis 2. Whatever the mechanism, the distinctly slender habitus typical of several species is homoplastic in our trees (Fig. 3). Barr's (1960) hypothesis for the evolutionary relationships of the species is rejected, and aspects of his alternative hypothesis are supported (Barr, 1974; Fig. 2E). The repeated evolution of the slender habitus (particularly the extremely elongate pronotum) is likely an example of convergent evolution given the phylogenetic history of these lineages (Fig. 3), the similarity in habitat preferences, and possibly, the similarity in feeding preferences (i.e., if all of the species prey on cave cricket eggs; Mitchell, 1971; Reddell, 1994; Taylor et al., 2007). Altogether this suggests that the primary drivers of this convergent form are habitat and history.

4.4. An approximate dated tree of Rhadine: origin of troglobitic species in the late Tertiary

The preliminary results from our preferred BEAST analysis using a relaxed lognormal clock recover a mean crown group age of ~15 Ma for the *Rhadine–Tanystoma* lineage (Fig. 3). The age of the split between Clade I and II *Rhadine* was estimated to be mid-late Miocene, a period in which global temperatures were much lower than temperatures of the mid to early Miocene (Zachos et al., 2001), and both Clade I and Clade II *Rhadine* have similar divergence time estimates that occur shortly thereafter (Fig. 3; Table 3). In addition to this, members of each clade are rather homogeneous in terms of their habitat preferences. Clade I species are almost entirely species that are known inquilines of mammal burrows, troglophiles, and troglobites (Barr, 1960,1964,1974). This result is similar to the recently documented clades of exclusively troglobitic beetles in the Pyrenees (Faille et al., 2010; Ribera et al., 2010) though on a much larger scale than in *Rhadine*. Clade II species, on the other hand, are largely surface-dwelling species some of which are only known from high altitude habitats with only a few lineages being cavernicolous. Our results indicate that geographic distribution is correlated with monophyly as well as habitat preference (Fig. 3).

The pattern of habitat preferences along the tree suggests that subsurface habitats are important stepping-stones to invading subterranean habitats (Giachino and Vailati, 2010; Culver and Pipan, 2008; Pipan and Culver, 2012). This result suggests that ecological niches have been conserved within clades though it could be that this pattern is instead due to widespread convergence. We favor a hypothesis that *Rhadine* like many lineages with cryptic diversity and young ages, displays high ecological niche conservatism (Wiens et al., 2010). This presupposes that species with similar habitat preferences are closely related, and this is borne out in the sister relationship between the *dissecta*-group *sensu* Barr (1974) species that are frequently collected in mammal burrows and all other Clade I species, most of which occur in caves. Also, the species that render Barr's (1974) group non monophyletic have similar character combinations, but are found in caves not in burrows as would be predicted. *Rhadine caudata*, though typically found on the surface in leaf litter, is also frequently collected in caves (Barr, 1964), and it is part of the basal grade of Clade I (Figs. 3 and 4).

When ecological niches are conserved it is expected that species will be susceptible to extirpation or extinction when subjected to habitat disturbance (Wiens et al., 2010). Therefore, understanding the role of ecological niche conservatism in *Rhadine* is directly relevant to conservation policies for managing the cave fauna of the Edwards Plateau. The foremost concern for the conservation of these troglobites is urban sprawl. The World Wildlife Fund and US Fish and Wildlife have listed three of the species in the genus as endangered (the Endangered Species Act; Bousquet, 2012) because these caves are located in the 'urban corridor' of Texas, which is a region that has one of the highest population growth rates in the state. This creates a challenge for protecting troglobitic *Rhadine* given that these species are so intricately associated with these caves and have strongly conserved ecological niches. Any dramatic changes to their environment are likely not to be tolerated and could result in extirpation or extinction. As this is the case, it is crucial to have a well-founded understanding of which lineages are phylogenetically distinct in order to incorporate that into decisions that bear on policy and management practices. Further sampling within the *subterranea*-group can improve our understanding of the group's evolutionary history, which can shed light on the limits of species hypotheses and help guide future conservation strategies (Paquin and Hedin, 2004).

Past climate change has been proposed as an explanation for the current distribution of troglobitic *Rhadine*. Barr (1960,1974) hypothesized that troglobitic *Rhadine* were descended from one (or more) troglophilic species that became restricted to caves with the onset of regionalized warming and drying during interglacial periods (e.g., Denton, 2000; Fig. 2F). Our estimates date the divergence events within the clade that includes all troglobitic *Rhadine* from Texas caves to have occurred within the past 4–5 million years (95% HPD ranging from 3 to 6.5 Ma). Because many *Rhadine* troglobites are known from more than one cave, it seems likely that troglobitic *Rhadine* dispersed periodically, and there is no immediate reason to accept that there is a one to one match between the occurrence of a troglobitic species in a cave and an independent colonization event (Rizzo et al., 2013). It is, however, unknown how many lineages independently colonized caves during this time.

Most of the caves in the Balcones Escarpment were formed at the Miocene-Pliocene boundary (Ward, 2006; White et al., 2009; Wilson, 1956), which is compatible with our divergence time estimates (i.e., these lineages are not estimated to be significantly older than the formation of the karst features in the Balcones fault zone). Recent molecular phylogenetic studies of other cave beetles have inferred that most of the colonization events occurred prior to the Last Pleistocene Glacial cycle (e.g., Faille et al., 2010,2013,2014; Ribera et al., 2010). Dramatic fluctuations in temperature during glacial cycles define the start of the Quaternary period (~2.58 Ma), which postdates our divergence time estimates for this clade (Fig. 3; Table 3). Barr (1974) suggested that desiccation (during warm interglacials) might have had a strong influence on the group, noting that these species are almost all known from moist caves. This conclusion would support the hypothesis that caves

of the Balcones Escarpment served as Pleistocene refugia (Bryson et al., 2014). Alternatively, the sensitivity of troglobites to desiccation may simply be one consequence of specialization following an adaptive shift and may not necessarily have been a primary mechanism behind the origin of troglobitic *Rhadine*.

Model comparison using path sampling of these separate hypotheses indicates that model fit varies between analyses. When we exclude mutation rate priors for COI, a model based on the adaptive shift hypothesis (H_2; Fig. 2G) has a higher likelihood. When we include mutation rate priors for COI, the H_1 model is preferred, but the difference in Log Bayes Factors is small and not decisive (Table 2). Kass and Raftery (1995), for example, used the threshold of 3 log likelihood units for strong support of a more parameter-rich model over a reduced model.

The adaptive shift hypothesis for the origin of troglobites predicts that colonization and subsequent speciation occur shortly after the caves first become available (Desutter-Grandcolas and Grandcolas, 1996; Rivera et al., 2002; Rouch and Danielopol, 1987; Fig. 2G). If this hypothesis is applied to troglobitic *Rhadine* from caves in central Texas, we would expect the temporal pattern of the colonization of these caves to reflect non-simultaneous adaptive shifts. If the climactic relict hypothesis is invoked, then we would predict coincident divergence events. However, divergence timing is not clearly correlated aside from the estimates at deeper nodes 3 and 4 (Fig. 3). Methods used here may not be able to detect climate driven, cave colonization given that speciation may not necessarily coincide with colonization of caves. One facet of the adaptive shift hypothesis is the expectation that epigean relatives that share a recent common ancestor with a troglobite occur in a similar geographic area (see Section 1). This particular prediction was not supported by our results because the closest non-troglobitic relatives of the troglobites are other cave dwelling species. However, these closely related macrophthalmous species occur in nearby caves in central Texas (Figs. 1D and 3; Table S2), and the more distantly related mammal burrow dwelling species are also diverse in Texas and the Great Plains (Barr, 1974). If the ancestor of cave *Rhadine* in Clade I was generally subterranean, then the occurrence of these closely related, macrophthalmous beetles in more or less the same geographic space and habitats could be evidence that an adaptive shift in a generally subterranean ancestor led to the loss of certain features typical of troglobitic species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.01.018.

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