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Molecular Phylogenetics and Evolution 42 (2007) 435-448

www.elsevier.com/locate/ympev

Molecular taxonomy in the dark: Evolutionary history, phylogeography, and diversity of cave crayfish in the subgenus *Aviticambarus*, genus *Cambarus*

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Received 20 February 2006; revised 9 July 2006; accepted 12 July 2006 Available online 27 July 2006

Abstract

Freshwater crayfish species in the subgenus *Aviticambarus* (Cambaridae: *Cambarus*) are restricted to caves along the Cumberland Plateau, the Sequatchie Valley, and the Highland Rim which extend along the Tennessee River in southcentral Tennessee and northern Alabama. Historically, three stygobitic species, *Cambarus jonesi, Cambarus hamulatus*, and *Cambarus veitchorum*, comprise this subgenus. We examine species' boundaries and phylogeographic structure of this imperiled Southern Appalachian assemblage to shed light on patterns of cave colonization. We also provide estimates of genetic diversity for conservation status assessment. Using geologic evidence, phylogeographic analyses, and sequence data from five gene regions (two nuclear: Histone H3 and GAPDH and three mitochondrial: 12S, 16S, and CO1 totaling almost 2700 base pairs), we identify two well-supported cryptic species in addition to the three currently recognized taxa. Four of these taxa exhibit low levels of genetic variation both currently and historically, which may indicate local extirpation events associated with geological and river basin changes. Our results also support other recent findings that pre-Pleistocene paleodrainages may best explain the current patterns of aquatic faunal biodiversity in the Southern Appalachians. © 2006 Elsevier Inc. All rights reserved.

Keywords: Nested clade analysis; Phylogeography; Southern Appalachians; Stygobite; Population genetics; Crustacean; Conservation

1. Introduction

The freshwater crayfish genus *Cambarus* (Erichson, 1846) is one of the largest genera of crayfish in the world, with approximately 100 species (of the 600 global species) and a distribution across the eastern United States. This large genus is comprised of species with varying life history traits including inhabitation of streams, burrows, big rivers, lakes, and caves. Within *Cambarus*, only 11 species are restricted to caves, and these species are distributed in karst (limestone) areas of the Ozarks Plateau, the Greenbrier region of West Virginia, and Cumberland Plateau of the

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1055-7903/\$ - see front matter © 2006 Elsevier Inc. All rights reserved. doi:10.1016/j.ympev.2006.07.014

Southern Appalachians (Hobbs and Barr, 1960). In the Southern Appalachians, the subgenus *Aviticambarus* is currently comprised of three obligate cave-dwelling species (stygobites) with ranges restricted to southcentral Tennessee and northern Alabama. This subgenus is a monophyletic group within the genus *Cambarus* (Sinclair et al., 2004; Buhay and Crandall, unpublished data) and each of these groundwater species exhibits troglomorphisms, including albinistic morphology and reduced eyes without pigment.

Subterranean biomes are currently regarded as highly endangered ecosystems, with 95% of obligate cave-dwelling species (including aquatic and terrestrial) in the United States considered to be "vulnerable" or "imperiled" by the Nature Conservancy (Culver et al., 2000). Yet, none of the *Aviticambarus* species were previously evaluated for global extinction risk, and hence, remain unlisted on the IUCN Red List of Threatened Species (World Conservation Union, www.redlist.org). However, all three species are of "High Conservation Concern" according to the state of Alabama's Department of Conservation and Natural Resources, while Taylor et al. (1996) evaluated national conservation status, considering only *Cambarus veitchorum* to be "Endangered," with *Cambarus hamulatus* and *Cambarus jonesi* regarded as "Currently Stable."

Cambarus hamulatus (Cope and Packardi, 1881) inhabits caves in the Sequatchie Valley, Tennessee south to the headwaters of the Black Warrior River, Alabama. In the most recent survey, Hobbs et al. (1977) reported that C. hamulatus is known from 22 caves, with the greatest concentration of localities in Jackson County, Alabama, and Marion County, Tennessee. The type locale for the species is Nickajack Cave in Marion County, Tennessee which is now flooded due to dams and lakes built on the Tennessee River. C. hamulatus has a somewhat patchy distribution, occurring in valleys not inhabited by Orconectes australis australis, another obligate cave-dwelling crayfish which ranges along the western escarpment of the Cumberland Plateau (Buhay and Crandall, 2005). The Southern portion of the Cumberland Plateau and its western escarpment end in northern Alabama, which makes it difficult to identify whether Orconectes australis australis or C. hamulatus occurs at a particular cave without capturing males for species-level diagnosis. Both species are known to inhabit caves in northern Alabama, but they have not been found to co-occur in the same cave. Prior to this study, surveys had not been conducted to determine which caves in the northern Alabama mountains are inhabited by C. hamulatus and large gaps occur between the 22 reported C. hamulatus sites from Hobbs et al. (1977).

Cambarus jonesi (Hobbs and Barr, 1960) was previously recorded from 14 sites along the Highland Rim region of northern Alabama. This distribution encompasses six counties along both sides of the Tennessee River channel and overlaps with the ranges of other obligate cave crayfish species. Unlike C. hamulatus, C. jonesi is known to co-occur with other obligate cave-dwelling crayfish species. C. jonesi co-occurs with Orconectes australis australis and Orconectes sheltae at Shelta Cave in Madison County, which is in the eastern part of the C. jonesi range. In the western part of its range, C. jonesi co-occurs with Procambarus pecki. Additionally, C. jonesi is found with Cambarus veitchorum (Cooper and Cooper, 1997) at White Spring Cave in Limestone County, which is the only currently known cave site for C. veitchorum. C. veitchorum was last seen in 1968 and only seven individuals of the species have ever been seen and collected (Cooper and Cooper, 1997). Morphological differences separate C. jonesi and C. veitchorum at White Spring Cave. C. veitchorum is a small species, with the maximum carapace length recorded as 16.7 mm, and the second through fifth tail segments have a spine. C. jonesi is the larger species with a maximum carapace length of 28.9 mm and it lacks tail spines (Cooper and Cooper, 1997).

A recent study on the evolutionary history and phylogeography of obligate cave crayfish in the genus Orconectes along the western escarpment of the Cumberland Plateau found that current surface drainage patterns are not reflective of the species' boundaries between cave crayfish (Buhay and Crandall, 2005). Moreover, ancient drainage basin events appear to have played major roles in the speciation patterns of other cave animals in the Southern Appalachians as well (spiders: Hedin, 1997a; Hedin, 1997b; Hedin and Wood, 2002; beetles: Barr, 1969; amphipods: Holsinger, 1969), yet, the physical barriers (e.g., ridges or rivers) that once separated the species are no longer apparent or present on the surface (Kane et al., 1992). Therefore, determining species' boundaries and geographic limits for subterranean fauna must be approached using a thorough sampling scheme across entire distributional ranges and incorporate high resolution genetic data because of the inherent difficulties of relying on a solely morphologically-based taxonomy (Marmonier et al., 1993; Wiens et al., 2003; Finston and Johnson, 2004; Buhay and Crandall, 2005). Appropriately, Proudlove and Wood (2003) in their "Blind Leading the Blind" article called for "DNA taxonomy" to shed light on cryptic subterranean species, particularly for freshwater crustaceans, and to accurately assess biodiversity in the dark which is poorly understood and understudied. Thus, the objectives of our study were to: (1) determine species' boundaries within the cave crayfish subgenus Aviticambarus using phylogenetic and geologic information, and (2) assess the phylogeographic structure, genetic diversity, and conservation status of each lineage using inferences from nested clade analysis and information about demographic and historical events.

2. Materials and methods

2.1. Tissue and data collection

We collected tissue samples (claw or leg which are regenerated) from 130 cave crayfish individuals from 27 caves spanning the entire previously known ranges of C. hamulatus and C. jonesi, and we discovered new localities that extended the geographic range of the subgenus (Table 1, Fig. 1). C. veitchorum was not found at White Springs Cave (type locality and only known locality for this species), but C. jonesi was collected at that locale. Individuals were captured by hand or by using small aquarium nets, and then returned to the place of capture immediately after removing the tissue sample which was placed in a vial containing 95% ethanol. In some cases, whole adult specimens were taken to serve as vouchers for caves added to the distribution after the most recent listing by (Hobbs et al. (1977) and Table 1). Voucher individuals were placed in 95% ethanol and are stored at the Monte L. Bean Museum at Brigham Young University, Provo, Utah.

Genomic DNA was extracted using standard methods and the 16S mtDNA gene, which shows variation within and between crayfish populations (Buhay and Crandall, 2005; Crandall and Fitzpatrick, 1996), was amplified for all Table 1

Cambarus (subgenus Aviticambarus) taxa, cave names with locations and cave survey numbers, and abbreviated cave names

ChomadataArcon TolkristYesTN: CumberlandTCD62Arcon of ChomadatasChomadatasGuror CoveNoTN: ScamberlandTCD62Run to the MillChamadatasGuror SpringYesTN: FranklinTFR199GarnerChamadatasLittle Crow CreekNoTN: FranklinTFR158—ChamadatasBubb SpringYesTN: MarionTMN160—ChamadatasBubb SpringYesTN: MarionTMN160—ChamadatasButterflyNoTN: MarionTMN160—ChamadatasGourdneckNoTN: MarionTMN160—ChamadatasGourdneckNoTN: MarionTMN160—ChamadatasLost Pje'NoTN: MarionTMN20—ChamadatasLost Pje'NoTN: MarionTMN20—ChamadatasNickisjack' (typs)No"TN: MarionTMN175OwenChamadatasShighYesTN: MarionTMN175OwenChamadatasShighYesTN: MarionTMN176—ChamadatasShighYesTN: MarionTMN175DiakeragChamadatasShighYesTN: MarionTMN186—ChamadatasShighYesTN: MarionTMN176DwenChamadatasShighYesTN: MarionTMN178DiakeragChamadatasShighNoTN: MarionTMN186—ChamadatasShigh </th <th>Species</th> <th>Cave</th> <th>Sampled</th> <th>State: county</th> <th>Cave survey No.</th> <th>Abbreviation</th>	Species	Cave	Sampled	State: county	Cave survey No.	Abbreviation
C. hamudataRun To The MailYesTN: CamberlandTCD62Ranto the MailC. hamudatasGarner SpringYesTN: FranklinTFR13—C. hamudatasLittle CrowCeekNoTN: FranklinTFR15—C. hamudatasBibbs SpringYesTN: FranklinTFR15—C. hamudatasBibbs SpringYesTN: MarionTMN140—C. hamudatasBibbs SpringYesTN: MarionTMN160—C. hamudatasDruin SpringYesTN: MarionTMN164—C. hamudatasDours ParisNoTN: MarionTMN164—C. hamudatasHoneycuttaNoTN: MarionTMN264—C. hamudatasNickrighet' (type)NoTN: MarionTMN179—C. hamudatasNickrighet' (type)NoTN: MarionTMN179—C. hamudatasSignal Light PftYesTN: MarionTMN179—C. hamudatasSignal Light PftYesTN: MarionTMN262—C. hamudatasSignal Light PftYesTN: MarionTMN262—C. hamudatasSignal Light PftYesTN: MarionTMN262—C. hamudatasSignal Light PftYesTN: MarionTMN484WhitesideC. hamudatasSignal Light PftYesTN: MarionTMN484WhitesideC. hamudatasSignal Light PftYesTN: MarionTMN484—C. hamudatasSignal Lig	C. hamulatus	Aaron Tolletts ^a	Yes	TN: Bledsoe	TBD1	Aaron
C hamidatas Ganer Spring Yes TN: Franklin TFR3 — — C hamidatas Litle Crow Creek No TN: Franklin TFR159 Garner C hamidatas Litle Crow Creek No TN: Franklin TFR158 — — C hamidatas Bayes Spring Yes TN: Kranklin TFR158 Payne Bayes The Spring Yes TN: Kranklin TFR158 Payne Data Spring Yes TN: Kranklin TFR158 Payne C hamidatas Buterfly No TN: Marion TMN160 — — C hamidatas Gourincek No TN: Marion TMN160 — — C hamidatas Los Lygrey No TN: Marion TMN160 — — C hamidatas Los Lygrey No TN: Marion TMN160 — — C hamidatas Court of the Spring Yes TN: Marion TMN260 — — C hamidatas Nickajack (type) No TN: Marion TMN260 — — C hamidatas Shakrong Yes TN: Marion TMN270 — — C hamidatas Shakrong Yes TN: Marion TMN176 Owen C hamidatas Shakrong Yes TN: Marion TMN176 — — C hamidatas Shakrong Yes TN: Marion TMN176 — — C hamidatas Shakrong Yes TN: Marion TMN370 — Signal C hamidatas Shakrong Yes TN: Marion TMN370 — — C hamidatas Shakrong Yes TN: Sequatchie TSO15 K exphole C hamidatas Goreve Yes TN: Sequatchie TSO15 K exphole C hamidatas Goreve Yes AL: Bakason AJK190 — — C hamidatas Graves Yes AL: Bakason AJK191 — — C hamidatas Graves Yes AL: Bakason AJK292 — Winoth C hamidatas Graves Yes AL: Bakason AJK293 — — C hamidatas Shakrong Yes AL: Bakason AJK293 — — C hamidatas Shaft Nevé Yes AL: Bakason AJK293 — — C hamidatas Shaft Nevé Yes AL: Bakason AJK293 — — C hamidatas Shaft Nevé Yes AL: Bakason AJK294 — — C hamidatas Shaft Nevé Yes AL: Bakason AJK294 — — C hamidatas Shaft Nevé Yes AL: Ba	C. hamulatus	Run To The Mill	Yes	TN: Cumberland	TCD62	Run to the Mill
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$ \begin{array}{llllllllllllllllllllllllllllllllllll$	C. hamulatus	Owen ^a	Yes	TN: Marion	TMN176	Owen
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$ \begin{array}{llllllllllllllllllllllllllllllllllll$	C. hamulatus	Shakerag	Yes	TN: Marion	TMN371	Shakerag
C hamulatusSignal Light PitYesTh: MarionTMN40SignalC hamulatusSpeegle Saltpeter*NoTh: MarionTMN46 $$ C hamulatusWhiteside*YesTh: MarionTMN48WhitesideC hamulatusWine*YesTh: MarionTMN48WhitesideC hamulatusWine*YesTh: SequatchicTSQ5WimothC hamulatusWimothYesTh: SequatchicTSQ5WimothC hamulatusGravesYesAL: BlountABA14 $$ C hamulatusRickwood*YesAL: BlountABA236RickwoodC hamulatusBidr RiverYesAL: JacksonAJK2800BluffC hamulatusGriger*YesAL: JacksonAJK074 $$ C hamulatusGreger*YesAL: JacksonAJK074 $$ C hamulatusGreger*YesAL: JacksonAJK280BluffC hamulatusHorseskull*NoAL: JacksonAJK289 $-$ C hamulatusJess Eliott*NoAL: JacksonAJK289 $-$ C hamulatusJesselliott*NoAL: JacksonAJK289 $-$ C hamulatusKylesNoAL: JacksonAJK289 $-$ C hamulatusKileve*YesAL: JacksonAJK24 $-$ C hamulatusKileve*NoAL: JacksonAJK24 $-$ C hamulatusSalt River*YesAL: JacksonAJK24 $-$ <t< td=""><td>C. hamulatus</td><td>Ship^a</td><td>No</td><td>TN: Marion</td><td>TMN39</td><td>_</td></t<>	C. hamulatus	Ship ^a	No	TN: Marion	TMN39	_
C hamulatusSnake WellNoTh: MarionTMN262C hamulatusWhitesideYesTh: MarionTMN46C hamulatusWhitesideYesTh: MarionTMN48WhitesideC hamulatusWine*YesTh: SequatchicTSQ15KeyholeC hamulatusKeyholeYesTh: SequatchicTSQ15KeyholeC hamulatusGravesYesAL: BlountABA1200GravesC hamulatusRackwood*YesAL: BlountABA236RickwoodC hamulatusRickwood*YesAL: JacksonAJK2800BluffC hamulatusBluff RiverYesAL: JacksonAJK230GeigerC hamulatusGeiger*YesAL: JacksonAJK323C hamulatusHorseskul*NoAL: JacksonAJK613C hamulatusHorseskul*NoAL: JacksonAJK613C hamulatusHorseskul*NoAL: JacksonAJK613C hamulatusHorseskul*NoAL: JacksonAJK166C hamulatusRussel*NoAL: JacksonAJK166C hamulatusRussel*NoAL: JacksonAJK232C hamulatusRussel*NoAL: JacksonAJK166C hamulatusRussel*NoAL: JacksonAJK166C hamulatusRussel*NoAL: JacksonAJK323C hamulatusRusse	C. hamulatus	Signal Light Pit	Yes	TN: Marion	TMN40	Signal
C hamulatusSpeegle Saltpeter ⁴ NoTN: MarionTMN46—C hamulatusWine ⁴ YesTN: MarionTMN48WhitesideC hamulatusWine ⁴ YesTN: SequatchieTSQ15KeyholeC hamulatusWimothYesTN: SequatchieTSQ5WimothC hamulatusGravesYesAL: BlountABA1200GravesC hamulatusRadolph ⁴ No**AL: BlountABA144—C hamulatusRickwood ⁴ YesAL: BlountABA144—C hamulatusRickwood ⁴ YesAL: BlountABA144—C hamulatusCrow Creek ³ NoAL: JacksonAJK613—C hamulatusCrow Creek ³ NoAL: JacksonAJK613—C hamulatusGeiger ⁴ YesAL: JacksonAJK613—C hamulatusGeiger ⁴ NoAL: JacksonAJK289—C hamulatusJess Elliott ⁴ NoAL: JacksonAJK289—C hamulatusJess Elliott ⁴ NoAL: JacksonAJK284TalleyC hamulatusTaleNoAL: JacksonAJK248TalleyC hamulatusTaleNoAL: JacksonAJK248TalleyC hamulatusTaleNoAL: JacksonAJK244—C hamulatusTaleNoAL: JacksonAJK248TalleyC hamulatusTaleNoAL: JacksonAJK248TalleyC hamulatus<	C. hamulatus	Snake Well	No	TN: Marion	TMN262	_
C hamulatusWhiteside*YesTN: MarionTMN144WhitesideC hamulatusKeyholeYesTN: SequatchieTSQ15KeyholeC hamulatusKeyholeYesTN: SequatchieTSQ5WilmothC hamulatusGravesYesAL: BlountABA1200GravesC hamulatusRardolph*No**AL: BlountABA236Rickwood*C hamulatusRickwood*YesAL: JacksonAJK490BluffC hamulatusBluff RiverYesAL: JacksonAJK497—C hamulatusGeiger*YesAL: JacksonAJK459GeigerC hamulatusGeiger*YesAL: JacksonAJK459—C hamulatusHorseskull*NoAL: JacksonAJK459—C hamulatusHorseskull*NoAL: JacksonAJK459—C hamulatusJes Elliott*NoAL: JacksonAJK280—C hamulatusSalt River*YesAL: JacksonAJK28SaltC hamulatusRusself*NoAL: JacksonAJK29—C hamulatusSalt River*YesAL: JacksonAJK29—C hamulatusTatley Ditch*YesAL: JacksonAJK248TalleyC hamulatusTatley Ditch*YesAL: JacksonAJK23—C hamulatusBuds*No*AL: JacksonAJK248—C hamulatusBuds*No**AL: JacksonAJK244—C hamul	C. hamulatus	Speegle Saltpeter ^a	No	TN: Marion	TMN46	_
$ \begin{array}{llllllllllllllllllllllllllllllllllll$	C. hamulatus	Whiteside ^a	Yes	TN: Marion	TMN48	Whiteside
C. hamulatusKeyholeYesTN: SequatchieTS()1KeyholeC. hamulatusGravesYesAL: BlountABA1200GravesC. hamulatusRandolpha"No**AL: BlountABA414C. hamulatusRandolpha"No**AL: BlountABA236RickwoodC. hamulatusBluff RiverYesAL: JacksonAJK2800BluffC. hamulatusBluff RiverYesAL: JacksonAJK1074C. hamulatusGeiger*YesAL: JacksonAJK1074C. hamulatusGeiger*YesAL: JacksonAJK613C. hamulatusHorseskull*NoAL: JacksonAJK323C. hamulatusHorseskull*NoAL: JacksonAJK289C. hamulatusKylesNoAL: JacksonAJK289C. hamulatusKyles*NoAL: JacksonAJK289C. hamulatusTalley Ditcha*YesAL: JacksonAJK289C. hamulatusTalley Ditcha*YesAL: JacksonAJK248TalleyC. hamulatusTalley Ditcha*No***AL: JacksonAJK171C. hamulatusTalley Ditcha*No***AL: JacksonAJK171C. hamulatusTalley Ditcha*No***AL: MarshallAMS135C. hamulatusTalley Ditcha*No***AL: MarshallAMS135C. hamulatusBuds*No***AL: Marshall <td>C. hamulatus</td> <td>Wine^a</td> <td>Yes</td> <td>TN: Marion</td> <td>TMN141</td> <td>Wine</td>	C. hamulatus	Wine ^a	Yes	TN: Marion	TMN141	Wine
C. hamulatusWilmothYesTN: SequatchieTSQ5WilmothC. hamulatusGravesYesAL: BlountABA1200GravesC. hamulatusRickwood*YesAL: BlountABA236RickwoodC. hamulatusBilf RiverYesAL: JacksonAJK2300BluffC. hamulatusGriger*YesAL: JacksonAJK290GrigerC. hamulatusGriger*YesAL: JacksonAJK459GrigerC. hamulatusGriger*YesAL: JacksonAJK323C. hamulatusHorseskull*NoAL: JacksonAJK230C. hamulatusJess Elliot*NoAL: JacksonAJK289C. hamulatusRussell*NoAL: JacksonAJK214C. hamulatusRussell*NoAL: JacksonAJK214C. hamulatusTatley Ditch*YesAL: JacksonAJK244C. hamulatusTatley Ditch*YesAL: JacksonAJK324C. hamulatusTatley Ditch*No*AL: JacksonAJK324C. hamulatusTatley Ditch*No**AL: MarshallAMS1155C. hamulatusTumbling Rock*No**AL: MarshallAMS135C. hamulatusKing School*No**AL: MarshallAMS135C. hamulatusKing School*No**AL: MarshallAMS135C. jonesiRockhouse*NoAL: MarshallA	C. hamulatus	Keyhole	Yes	TN: Sequatchie	TSQ15	Keyhole
C. hamulatusGravesYesAL: BlountABA1200GravesC. hamulatusRandolphaNo**AL: BlountABA14—C. hamulatusRickwood*YesAL: BlountABA14—C. hamulatusBluff RiverYesAL: JacksonAJK2800BluffC. hamulatusGrigger*YesAL: JacksonAJK1974—C. hamulatusGrigger*YesAL: JacksonAJK1974—C. hamulatusGeiger*YesAL: JacksonAJK459GeigerC. hamulatusHorseskull*NoAL: JacksonAJK289—C. hamulatusHesselliott*NoAL: JacksonAJK289—C. hamulatusRussell*NoAL: JacksonAJK289—C. hamulatusSalt River*YesAL: JacksonAJK248TalleyC. hamulatusTalley Ditch*YesAL: JacksonAJK248TalleyC. hamulatusTatbeNoAL: JacksonAJK373—C. hamulatusTatbeNo*AL: JacksonAJK374—C. hamulatusTatbeNo*AL: MarshallAMS135—C. hamulatusTumbling Rock*No*AL: MarshallAMS135—C. hamulatusBuds*No*AL: LacksonAJK374—C. hamulatusBuds*No*AL: MarshallAMS135—C. hamulatusBuds*No*AL: LacksonAJK248—C. hamulatusBuds*	C. hamulatus	Wilmoth	Yes	TN: Sequatchie	TSQ5	Wilmoth
C hamulatusRandolph"No"AL: BlountABA414—C hamulatusRickwood"YesAL: BlountABA226RickwoodC hamulatusBluff RiverYesAL: JacksonAJK2800BluffC hamulatusCrow Creek"NoAL: JacksonAJK1074—C hamulatusGeiger"YesAL: JacksonAJK459GeigerC hamulatusHorseskull"NoAL: JacksonAJK23—C hamulatusJess Elliott"NoAL: JacksonAJK23—C hamulatusJess Elliott"NoAL: JacksonAJK29—C hamulatusSalt River"YesAL: JacksonAJK21SaltC hamulatusRussell"NoAL: JacksonAJK248TalleyC hamulatusTalley Ditch"YesAL: JacksonAJK248TalleyC hamulatusTalley Ditch"YesAL: JacksonAJK248TalleyC hamulatusTalley Ditch"No"AL: MarshallAMS30—C hamulatusRing Socol"No"AL: Marshal	C. hamulatus	Graves	Yes	AL: Blount	ABA1200	Graves
C. hamulatusRickwoodYesAL: BlountABA236RickwoodC. hamulatusBluff RiverYesAL: JacksonAJK2800BluffC. hamulatusGeiger ^a NoAL: JacksonAJK479GeigerC. hamulatusGeiger ^a YesAL: JacksonAJK459GeigerC. hamulatusHorseskull ^a NoAL: JacksonAJK453C. hamulatusHorseskull ^a NoAL: JacksonAJK233C. hamulatusKylesNoAL: JacksonAJK29C. hamulatusRussell ^a NoAL: JacksonAJK29C. hamulatusRussell ^a NoAL: JacksonAJK21SaltC. hamulatusTalley Ditch ^a YesAL: JacksonAJK248TalleyC. hamulatusTalley Ditch ^a YesAL: JacksonAJK11C. hamulatusTalley Ditch ^a YesAL: JacksonAJK171C. hamulatusTalley Ditch ^a NoAL: JacksonAJK171C. hamulatusTumbling Rock ^a NoAL: JacksonAJK171C. hamulatusBuds ^a No ^{***} AL: MarshallAMS135C. hamulatusBuds ^a No ^{****} AL: ColbertACE46C. hamulatusBuds ^a No ^{****} AL: LauderdaleALD99KeyC. jonesiRockhouse ^a NoAL: LauderdaleALD932C. jonesiRockhouse ^a NoAL: Madi	C. hamulatus	Randolph ^a	No**	AL: Blount	ABA414	_
C. hamulatusBluff RiverYesA.L: JacksonAJK 2800BluffC. hamulatusCrow Creek ^a NoA.L: JacksonAJK 1074C. hamulatusGeiger*YesA.L: JacksonAJK 459GeigerC. hamulatusHorseskull*NoA.L: JacksonAJK 323C. hamulatusJess Elliot*NoA.L: JacksonAJK 289C. hamulatusKylesNoA.L: JacksonAJK 289C. hamulatusRussell*NoA.L: JacksonAJK 289C. hamulatusSalt River*YesA.L: JacksonAJK 248TalleyC. hamulatusTalley Ditch*YesA.L: JacksonAJK 248C. hamulatusTauley Ditch*NoA.L: JacksonAJK 211C. hamulatusTauber*NoA.L: JacksonAJK 248C. hamulatusTauber*NoA.L: JacksonAJK 211C. hamulatusTumbling Rock*NoA.L: JacksonAJK 211C. hamulatusKing Schol*No*A.L: MarshallAMS135C. jonesiMcKinney Pit*No*A.L: MarshallAMS135C. jonesiRockhouse*No*A.L: LimestoneALM 22WhiteC. jonesiRockhouse*NoA.L: MadisonAMD158C. jonesiBobcat*NoA.L: MadisonAMD1283C. jonesiBobcat*No*A.L: MadisonAMD6	C. hamulatus	Rickwood ^a	Yes	AL: Blount	ABA236	Rickwood
C hamulatusCrow CreekªNoAL: JacksonAJK 1074C hamulatusGeiger³YesAL: JacksonAJK459GeigerC hamulatusHorseskull³NoAL: JacksonAJK613C hamulatusHorseskull³NoAL: JacksonAJK233C hamulatusKylesNoAL: JacksonAJK289C hamulatusRussell³NoAL: JacksonAJK289C hamulatusRussell³NoAL: JacksonAJK248TalleyC hamulatusTalley Ditch³YesAL: JacksonAJK248C hamulatusTateNoAL: JacksonAJK171C hamulatusTateNoAL: JacksonAJK171C hamulatusBuds³No**AL: MarshallAMS1135C hamulatusBuds³No**AL: MarshallAMS135C hamulatusKing School³No*AL: MarshallAMS135C hamulatusKing School³No*AL: MarshallAMS135C jonesiMcKinney Pit³No*AL: LauderdaleALD99KeyC jonesiRockhouse³NoAL: LimestoneALM312C jonesiBarclayNoAL: MadisonAMD1908C jonesiBorcla*NoAL: MadisonAMD1908C jonesiBorcla*NoAL: MadisonAMD1908C jonesiBorcla*NoAL:	C. hamulatus	Bluff River	Yes	AL: Jackson	AJK2800	Bluff
C hamulatusGeigerYesAL: JacksonAJK459GeigerC hamulatusHorseskulläNoAL: JacksonAJK613C hamulatusJess ElliottäNoAL: JacksonAJK323C hamulatusKylesNoAL: JacksonAJK29C hamulatusRussellaNoAL: JacksonAJK169C hamulatusSalt RiveråYesAL: JacksonAJK248TalleyC hamulatusTalley DitchåYesAL: JacksonAJK248TalleyC hamulatusTateNoAL: JacksonAJK324C hamulatusTateNoAL: JacksonAJK324C hamulatusTambling RockåNoAL: JacksonAJK37C hamulatusBudsåNo***AL: MarshallAMS1135C hamulatusBudsåNo***AL: ColbertACE46C jonesiMcKinney PitåNo*AL: LauderdaleALD99KeyC jonesiRockhouse*NoAL: LiamestoneALM242WhiteC jonesiBarclayNoAL: MadisonAMD1283C jonesiBarclayNoAL: MadisonAMD1283C jonesiBarclayNoAL: MadisonAMD1283C jonesiBarclayNoAL: MadisonAMD1283C jonesiByrd Spring*NoAL: MadisonAMD1283C jonesiBarclayNo*AL:	C. hamulatus	Crow Creek ^a	No	AL: Jackson	AJK1074	_
C. hamulatusHorseskull ^a NoAL: JacksonAJK 323C. hamulatusJess Elliott ^a NoAL: JacksonAJK 323C. hamulatusKylesNoAL: JacksonAJK 289C. hamulatusSalt River ^a YesAL: JacksonAJK 210SaltC. hamulatusSalt River ^a YesAL: JacksonAJK 221SaltC. hamulatusTalley Ditch ^a YesAL: JacksonAJK 244TalleyC. hamulatusTateNoAL: JacksonAJK 171C. hamulatusTumbling Rock ^a NoAL: JacksonAJK 171C. hamulatusBuds ^a No***AL: MarshallAMS 39C. hamulatusKing School ^a No***AL: ColbertACE46C. jonesiKey ^a YesAL: LimestoneALM 312C. jonesiRockhouse ^a NoAL: LimestoneALM 312C. jonesiRockhouse ^a NoAL: MadisonAMD 55C. jonesiBoclat ^a NoAL: MadisonAMD 23C. jonesiBoclat ^a No*AL: MadisonAMD 44 <tr< td=""><td>C. hamulatus</td><td>Geiger^a</td><td>Yes</td><td>AL: Jackson</td><td>AJK459</td><td>Geiger</td></tr<>	C. hamulatus	Geiger ^a	Yes	AL: Jackson	AJK459	Geiger
C hamulatusJess Elliott ^a NoAL: JacksonAJK 233 $ C$ hamulatusKylesNoAL: JacksonAJK 289 $ C$ hamulatusSalt River ^a YesAL: JacksonAJK 169 $ C$ hamulatusSalt River ^a YesAL: JacksonAJK 221Salt C hamulatusTalley Ditch ^a YesAL: JacksonAJK 248Talley C hamulatusTateNoAL: JacksonAJK 248 $ C$ hamulatusTumbling Rock ^a NoAL: JacksonAJK 171 $ C$ hamulatusBuds ^a No****AL: MarshallAMS1135 $ C$ hamulatusBuds ^a No****AL: MarshallAMS39 $ C$ jonesiMcKinney Pit ^a No*AL: ColbertACE46 $ C$ jonesiRockhouse ^a NoAL: LauderdaleALD99Key C jonesiRockhouse ^a NoAL: LimestoneALM22White C jonesiArrowoodNo**AL: MadisonAMD1908 $ C$ jonesiBarclayNoAL: MadisonAMD23 $ C$ jonesiBarclayNoAL: MadisonAMD23 $ C$ jonesiByrd Spring ^a NoAL: MadisonAMD23 $ C$ jonesiBuckata*No*AL: MadisonAMD23 $ C$ jonesiBuckata*No*AL: MadisonAMD23 $ C$ jonesiBuckata*No*AL: MadisonAMD4	C. hamulatus	Horseskull ^a	No	AL: Jackson	AJK613	_
C. hamulatusKylesNoAL: JacksonAJK289C. hamulatusRussell ^a NoAL: JacksonAJK169C. hamulatusSalt River ^a YesAL: JacksonAJK221SaltC. hamulatusTalley Ditch ^a YesAL: JacksonAJK248TalleyC. hamulatusTateNoAL: JacksonAJK171C. hamulatusBuds ^a No***AL: JacksonAJK171C. hamulatusBuds ^a No****AL: MarshallAMS1135C. hamulatusKing School ^a No*AL: MarshallAMS399C. jonesiMcKinney Pit ^a No*AL: ColbertACE46C. jonesiMcKinney Pit ^a No*AL: LimestoneALM312C. jonesiRockhouse ^a NoAL: LimestoneALM242WhiteC. jonesiArrowoodNo***AL: MadisonAMD1908C. jonesiBarclayNoAL: MadisonAMD23C. jonesiByd Spring ^a NoAL: MadisonAMD23C. jonesiByd Spring ^a No*AL: MadisonAMD4C. jonesiBobcat ^a No*AL: MadisonAMD4C. jonesiByd Spring ^a No*AL: MadisonAMD4C. jonesiShelta ^a No*AL: MadisonAMD4C. jonesiShelta ^a No*AL: MarshallAMS326CaveC. jonesi <td>C. hamulatus</td> <td>Jess Elliott^a</td> <td>No</td> <td>AL: Jackson</td> <td>AJK323</td> <td>_</td>	C. hamulatus	Jess Elliott ^a	No	AL: Jackson	AJK323	_
C hamulatusRussell®NoAL: JacksonAJK169C hamulatusSalt RiverªYesAL: JacksonAJK21SaltC hamulatusTalley DitchªYesAL: JacksonAJK248TalleyC hamulatusTateNoAL: JacksonAJK324C hamulatusTumbling RockªNoAL: JacksonAJK171-C hamulatusTumbling RockªNoAL: JacksonAJK171-C hamulatusKing SchoolªNo***AL: MarshallAMS39C hamulatusKing SchoolªNo**AL: ColbertACE46C jonesiMcKinney PitªNo*AL: LauderdaleALD99KeyC jonesiRockhouse*NoAL: LimestoneALM312C jonesiArrowoodNo**AL: MadisonAMD1908C jonesiBarclayNoAL: MadisonAMD1908C jonesiBarclayNoAL: MadisonAMD23C jonesiByrd SpringªNoAL: MadisonAMD606C jonesiByrd SpringªNo*AL: MorganAMG63CaveC jonesiButhewa*No*AL: MarshallAMS23C jonesiBocat*NoAL: MadisonAMD23C jonesiBorda*No*AL: MadisonAMD23C jonesiCave Spring*YesAL: MorganAMG47C jonesiTalcah*No*AL: Mo	C. hamulatus	Kyles	No	AL: Jackson	AJK289	_
C. hamulatusSalt River ^a YesA.L: JacksonAJK221SaltC. hamulatusTalley Ditch ^a YesA.L: JacksonAJK248TalleyC. hamulatusTateNoA.L: JacksonAJK324C. hamulatusTumbling Rock ^a NoA.L: JacksonAJK171C. hamulatusBuds ^a No***A.L: MarshallAMS1135C. hamulatusKing School ^a No**A.L: MarshallAMS39C. jonesiMcKiney Pit ^a No*A.L: ColbertACE46C. jonesiKey ^a YesA.L: LauderdaleALD99KeyC. jonesiRockhouse ^a NoA.L: LimestoneALM312C. jonesiRockhouse ^a NoA.L: MadisonAMD1908C. jonesiBarclayNoA.L: MadisonAMD1908C. jonesiBocat ^a NoA.L: MadisonAMD1283C. jonesiByd Spring ^a NoA.L: MadisonAMD23C. jonesiByd Spring ^a NoA.L: MadisonAMD666C. jonesiByd Spring ^a No*A.L: MadisonAMD4C. jonesiShelta ^a No*A.L: MadisonAMD4C. jonesiCave Spring ^a (type)YesA.L: MadisonAMD666C. jonesiCave Spring ^a (type)YesA.L: MarshallAMG334CaveC. jonesiCave Spring ^a (type)YesA.L: Morgan	C. hamulatus	Russell ^a	No	AL: Jackson	AJK169	_
C. hamulatusTalley Ditch ^a YesA.L: JacksonAJK248TalleyC. hamulatusTateNoA.L: JacksonAJK324C. hamulatusBuds ^a No***A.L: MarshallAMS1135C. hamulatusBuds ^a No***A.L: MarshallAMS39C. hamulatusBuds ^a No***A.L: MarshallAMS39C. hamulatusKing School ^a No*A.L: ColbertACE46C. jonesiKey ^a YesA.L: LimestoneALM312C. jonesiRockhouse ^a NoA.L: LimestoneALM242WhiteC. jonesiWhite Spring ^a YesA.L: MadisonAMD1908C. jonesiBobcat ^a NoA.L: MadisonAMD123C. jonesiBobcat ^a NoA.L: MadisonAMD606C. jonesiByrd Spring ^a NoA.L: MadisonAMD633C. jonesiByrd Spring ^a NoA.L: MadisonAMD606C. jonesiByrd Spring ^a NoA.L: MadisonAMD633C. jonesiByrd Spring ^a No*A.L: MadisonAMD4C. jonesiShelta ^a No*A.L: MarshallAMS633CaveC. jonesiShelta ^a No*A.L: MarshallAMS633CaveC. jonesiShelta ^a No*A.L: MarshallAMS634C. jonesiTalucah ^a No*A.L: MarshallAMS633Cave </td <td>C. hamulatus</td> <td>Salt River^a</td> <td>Yes</td> <td>AL: Jackson</td> <td>AJK221</td> <td>Salt</td>	C. hamulatus	Salt River ^a	Yes	AL: Jackson	AJK221	Salt
C. hamulatusTateNoA.L: JacksonAJK 324C. hamulatusTumbling Rock ^a NoAL: JacksonAJK 171C. hamulatusBuds ^a No***AL: MarshallAMS1135C. hamulatusKing School ^a No*AL: MarshallAMS39C. jonesiMcKinney Pit ^a No*AL: ColbertACE46C. jonesiKey ^a YesAL: LauderdaleALD99KeyC. jonesiRockhouse ^a NoAL: LimestoneALM312C. jonesiWhite Spring ^a YesAL: LimestoneALM242WhiteC. jonesiArrowwoodNo**AL: MadisonAMD1908C. jonesiBarclayNoAL: MadisonAMD55C. jonesiBochat ^a NoAL: MadisonAMD606C. jonesiByrd Spring ^a NoAL: MadisonAMD4C. jonesiMatthews ^a No*AL: MadisonAMD4C. jonesiShelta ^a No*AL: MadisonAMD4C. jonesiShelta ^a No*AL: MorganAMG333LaconC. jonesiShelta ^a No*AL: MorganAMG3343LaconC. jonesiTalucah ^a No*AL: MorganAMG3343LaconC. jonesiShelta ^a YesAL: MorganAMG3343LaconC. jonesiTalucah ^a No*AL: MorganAMG3343LaconC. jonesi<	C. hamulatus	Talley Ditch ^a	Yes	AL: Jackson	AJK248	Talley
C. hamulatusTumbling Rock ^a NoA.L: JacksonAJK171—C. hamulatusBuds ^a No***A.L: MarshallAMS1135—C. hamulatusKing Schol ^a No*A.L: MarshallAMS39—C. jonesiMcKinney Pit ^a No*A.L: ColbertACE46—C. jonesiKey ^a YesA.L: LauderdaleALD99KeyC. jonesiRockhouse ^a NoA.L: LimestoneALM312—C. jonesiWhite Spring ^a YesA.L: MatisonAMD1908—C. jonesiBarclayNoA.L: MatisonAMD155—C. jonesiBockat ^a NoA.L: MatisonAMD55—C. jonesiBoctat ^a NoA.L: MatisonAMD606—C. jonesiByrd Spring ^a NoA.L: MatisonAMD23—C. jonesiBoctat ^a NoA.L: MatisonAMD23—C. jonesiBotcat ^a No*A.L: MatisonAMD406—C. jonesiBotcat ^a No*A.L: MatisonAMD606—C. jonesiBotcat ^a No*A.L: MatisonAMD606—C. jonesiBotcat ^a No*A.L: MatisonAMD606—C. jonesiBotcat ^a No*A.L: MatisonAMD606—C. jonesiShelta ^a No*A.L: MatisonAMD633CaveC. jonesiTalucah ^a No*A.L: MatisonAMG47—C. jonesiTalucah ^a	C. hamulatus	Tate	No	AL: Jackson	AJK324	_
C. hamulatusBudsåNo****AL: MarshallAMS1135C. hamulatusKing SchoolaNo*AL: MarshallAMS39C. jonesiMcKinney PitaNo*AL: ColbertACE46C. jonesiKeyaYesAL: LauderdaleALD99KeyaC. jonesiRockhouseaNoAL: LimestoneALM312C. jonesiRockhouseaNo**AL: LimestoneALM242WhiteC. jonesiArrowoodNo**AL: MadisonAMD1908C. jonesiBarclayNoAL: MadisonAMD1283C. jonesiBobcataNoAL: MadisonAMD0606C. jonesiByd SpringaNoAL: MadisonAMD23C. jonesiMatthewsaNo*AL: MadisonAMD4C. jonesiSheltaaNo*AL: MorganAMG53CaveC. jonesiSheltaaNo*AL: MorganAMG47C. jonesiTalucahaNo*AL: MorganAMG3343LaconC. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2Porche SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS693PorchesC. sp. nov. 2Becch Spring ⁴ No*AL: MarshallAMS1710CherryC. sp. nov. 2Becch Spring ⁴ No*AL: MarshallAMS347 <t< td=""><td>C. hamulatus</td><td>Tumbling Rock^a</td><td>No</td><td>AL: Jackson</td><td>AJK171</td><td>_</td></t<>	C. hamulatus	Tumbling Rock ^a	No	AL: Jackson	AJK171	_
C. hamulatusKing School ^a No*AL: MarshallAMS39C. jonesiMcKinney Pit ^a No*AL: ColbertACE46C. jonesiKey ^a YesAL: LauderdaleALD99KeyC. jonesiRockhouse ^a NoAL: LimestoneALM312C. jonesiWhite Spring ^a YesAL: LimestoneALM242WhiteC. jonesiArrowoodNo**AL: MadisonAMD1908C. jonesiBarclayNoAL: MadisonAMD55C. jonesiBobcat ^a NoAL: MadisonAMD1283C. jonesiByd Spring ^a NoAL: MadisonAMD23C. jonesiByd Spring ^a NoAL: MadisonAMD406C. jonesiMatthews ^a NoAL: MadisonAMD4C. jonesiShelta ^a No*AL: MadisonAMD4C. jonesiShelta ^a No*AL: MorganAMG63CaveC. jonesiTalucah ^a No*AL: MorganAMG3343LaconC. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS347C. sp. nov. 2Reech Spring ^a No*AL: MarshallAMS347C. sp. nov. 2Reech Spring ^a No*AL: MarshallAMS347<	C. hamulatus	Buds ^a	No***	AL: Marshall	AMS1135	_
C. jonesiMcKinney Pit ^a No*AL: ColbertACE46—C. jonesiKey ^a YesAL: LauderdaleALD99KeyC. jonesiRockhouse ^a NoAL: LimestoneALM312—C. jonesiWhite Spring ^a YesAL: LimestoneALM242WhiteC. jonesiArrowwoodNo**AL: MadisonAMD1908—C. jonesiBarclayNoAL: MadisonAMD155—C. jonesiBobcat ^a NoAL: MadisonAMD1066—C. jonesiByrd Spring ^a NoAL: MadisonAMD606—C. jonesiByrd Spring ^a NoAL: MadisonAMD23—C. jonesiMatthews ^a No*AL: MadisonAMD4—C. jonesiShelta ^a No*AL: MorganAMG53CaveC. jonesiShelta ^a No*AL: MorganAMG47—C. jonesiTalucah ^a No*AL: MorganAMG3343LaconC. sp. nov. 1Lacon ExitYesAL: MarshallAMS266KellersC. sp. nov. 2Kellers ^a YesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS347—C. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. sp. nov. 2Beech Spring ^a No*AL: LimestoneALM242—	C. hamulatus	King School ^a	No*	AL: Marshall	AMS39	_
C. jonesiKeyaYesAL: LauderdaleALD99KeyC. jonesiRockhouseaNoAL: LimestoneALM312—C. jonesiWhite SpringaYesAL: LimestoneALM242WhiteC. jonesiArrowwoodNo**AL: MadisonAMD1908—C. jonesiBarclayNoAL: MadisonAMD55—C. jonesiBobcataNoAL: MadisonAMD606—C. jonesiByd SpringaNoAL: MadisonAMD606—C. jonesiByd SpringaNoAL: MadisonAMD23—C. jonesiMatthewsaNoAL: MadisonAMD4—C. jonesiSheltaaNo*AL: MorganAMG47—C. jonesiCave Springa (type)YesAL: MorganAMG3343LaconC. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2KellersaYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS633PorchesC. sp. nov. 2Beech SpringaNo*AL: MarshallAMS347—C. sp. nov. 2Beech SpringaNo*AL: MarshallAMS347—C. sp. nov. 2Beech Spring (type)No*AL: MarshallAMS347—C. sp. nov. 2Beech SpringaNo*AL: MarshallAMS347—C. sp. nov. 2Beech SpringaNo*AL: LimestoneALM242—	C. jonesi	McKinney Pit ^a	No*	AL: Colbert	ACE46	_
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C. jonesiWhite Spring ^a YesAL: LimestoneALM242WhiteC. jonesiArrowwoodNo**AL: MadisonAMD1908C. jonesiBarclayNoAL: MadisonAMD55C. jonesiBobcat ^a NoAL: MadisonAMD606C. jonesiByrd Spring ^a NoAL: MadisonAMD606C. jonesiByrd Spring ^a NoAL: MadisonAMD606C. jonesiMatthews ^a NoAL: MadisonAMD23C. jonesiShelta ^a No*AL: MadisonAMD4C. jonesiShelta ^a No*AL: MorganAMG63CaveC. jonesiTalucah ^a No*AL: MorganAMG477C. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347C. veitchorumWhite Spring (type)No*AL: LimestoneALM242	C. jonesi	Rockhouse ^a	No	AL: Limestone	ALM312	_
C. jonesiArrowoodNo**AL: MadisonAMD1908—C. jonesiBarclayNoAL: MadisonAMD55—C. jonesiBobcat ^a NoAL: MadisonAMD1283—C. jonesiByrd Spring ^a NoAL: MadisonAMD606—C. jonesiMatthews ^a NoAL: MadisonAMD23—C. jonesiMatthews ^a No*AL: MadisonAMD4—C. jonesiShelta ^a No*AL: MorganAMG53CaveC. jonesiCave Spring ^a (type)YesAL: MorganAMG47—C. jonesiTalucah ^a No*AL: MorganAMG3343LaconC. sp. nov. 1Lacon ExitYesAL: MarshallAMS326KellersC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. sp. nov. 2Beech Spring (type)No*AL: MarshallAMS347—C. svitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. jonesi	White Spring ^a	Yes	AL: Limestone	ALM242	White
C. jonesiBarclayNoAL: MadisonAMD55—C. jonesiBobcat ^a NoAL: MadisonAMD1283—C. jonesiByrd Spring ^a NoAL: MadisonAMD606—C. jonesiMatthews ^a NoAL: MadisonAMD23—C. jonesiMatthews ^a No*AL: MadisonAMD4—C. jonesiShelta ^a No*AL: MorganAMG53CaveC. jonesiCave Spring ^a (type)YesAL: MorganAMG47—C. jonesiTalucah ^a No*AL: MorganAMG47—C. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2Kellers ^a YesAL: MarshallAMS326KellersC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. veitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. jonesi	Arrowwood	No**	AL: Madison	AMD1908	_
C. jonesiBobcat ^a NoAL: MadisonAMD1283—C. jonesiByrd Spring ^a NoAL: MadisonAMD606—C. jonesiMatthews ^a NoAL: MadisonAMD23—C. jonesiShelta ^a No*AL: MadisonAMD4—C. jonesiShelta ^a No*AL: MorganAMG53CaveC. jonesiCave Spring ^a (type)YesAL: MorganAMG47—C. jonesiTalucah ^a No*AL: MorganAMG47—C. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2Kellers ^a YesAL: MarshallAMS326KellersC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. veitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. jonesi	Barclay	No	AL: Madison	AMD55	_
C. jonesiByrd Spring ^a NoAL: MadisonAMD606—C. jonesiMatthews ^a NoAL: MadisonAMD23—C. jonesiShelta ^a No*AL: MadisonAMD4—C. jonesiCave Spring ^a (type)YesAL: MorganAMG53CaveC. jonesiTalucah ^a No*AL: MorganAMG47—C. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2Kellers ^a YesAL: MarshallAMS326KellersC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. veitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. jonesi	Bobcat ^a	No	AL: Madison	AMD1283	_
C. jonesiMatthews ^a NoAL: MadisonAMD23—C. jonesiShelta ^a No*AL: MadisonAMD4—C. jonesiCave Spring ^a (type)YesAL: MorganAMG53CaveC. jonesiTalucah ^a No*AL: MorganAMG47—C. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2Kellers ^a YesAL: MarshallAMS326KellersC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. veitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. jonesi	Byrd Spring ^a	No	AL: Madison	AMD606	_
C. jonesiShelta ^a No*AL: MadisonAMD4—C. jonesiCave Spring ^a (type)YesAL: MorganAMG53CaveC. jonesiTalucah ^a No*AL: MorganAMG47—C. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2Kellers ^a YesAL: MarshallAMS326KellersC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. veitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. jonesi	Matthews ^a	No	AL: Madison	AMD23	_
C. jonesiCave Spring ^a (type)YesAL: MorganAMG53CaveC. jonesiTalucah ^a No*AL: MorganAMG47—C. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2Kellers ^a YesAL: MarshallAMS326KellersC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. veitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. jonesi	Shelta ^a	No*	AL: Madison	AMD4	_
C. jonesiTalucahaNo*AL: MorganAMG47—C. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2KellersaYesAL: MarshallAMS326KellersC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech SpringaNo*AL: MarshallAMS347—C. veitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. jonesi	Cave Spring ^a (type)	Yes	AL: Morgan	AMG53	Cave
C. sp. nov. 1Lacon ExitYesAL: MorganAMG3343LaconC. sp. nov. 2Kellers ^a YesAL: MarshallAMS326KellersC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. veitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. jonesi	Talucah ^a	No*	AL: Morgan	AMG47	
C. sp. nov. 2KellersaYesAL: MarshallAMS326KellersC. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech SpringaNo*AL: MarshallAMS347—C. veitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. sp. nov. 1	Lacon Exit	Yes	AL: Morgan	AMG3343	Lacon
C. sp. nov. 2Porches SpringYesAL: MarshallAMS693PorchesC. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. veitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. sp. nov. 2	Kellers ^a	Yes	AL: Marshall	AMS326	Kellers
C. sp. nov. 2Cherry HollowYesAL: MarshallAMS1710CherryC. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347—C. veitchorumWhite Spring (type)No*AL: LimestoneALM242—	C. sp. nov. 2	Porches Spring	Yes	AL: Marshall	AMS693	Porches
C. sp. nov. 2Beech Spring ^a No*AL: MarshallAMS347-C. veitchorumWhite Spring (type)No*AL: LimestoneALM242-	C. sp. nov. 2	Cherry Hollow	Yes	AL: Marshall	AMS1710	Cherry
C. veitchorum White Spring (type) No* AL: Limestone ALM242 —	C. sp. nov. 2	Beech Spring ^a	No*	AL: Marshall	AMS347	_
	C. veitchorum	White Spring (type)	No*	AL: Limestone	ALM242	—

^a Records from Hobbs et al., 1977.
 * Cave visited but species not found.

** Cave impounded by dam.

*** Cave destroyed.



Fig. 1. Distribution of each *Cambarus* (subgenus *Aviticambarus*) species. Dots in the middle of the symbols represent sampled caves and open symbols are not included in this study. The subgenus is currently known from 58 cave sites. Areas referred to in the text are labeled for geographic reference.

sampled individuals (Table 2) during PCR. Two other mitochondrial genes, 12S (Mokady et al., 1999) and CO1 (Folmer et al., 1994) and two nuclear genes, Histone H3 (Colgar et al., 1998) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (M. Schultz, pers. comm.) were amplified for one individual from every sampled cave and three outgroup taxa (Cambarus gentryi, Cambarus brachydactylus, and Cambarus friaufi) (Table 3). Cycle-sequencing reactions were run with purified PCR products (Millipore Montage PCR₉₆ plate cleanups) and the Big Dye Ready-Reaction kit on a Perkin-Elmer Thermocycler. Reactions were sequenced using an Applied Biosystems 3730 XL automated DNA sequencer. Sequences were edited and aligned by eye using BioEdit (Hall, 1999) and were deposited into GenBank as Accession Nos. DQ411711-DQ411808 (Table 3). No indels were found in the protein-coding gene sequences.

2.2. Phylogenetic analyses

Unique haplotypes of the 16S gene were analyzed using the Maximum Likelihood approach in PhyML (Guindon and Gascuel, 2003; http://atgc.lirmm.fr/phyml/) and the Bayesian approach in MrBayes v3.04b (Ronquist and Huelsenbeck, 2003) to determine monophyletic lineages and species' relationships. PhyML was run for 500 bootstrap replicates using the ModelTest 3.06 (Posada and Crandall, 1998) parameters: number of substitution types (nst)=2, invariable sites (I)=0, transition/transversion ratio (Tratio)=2.8493, model=HKY, and γ distribution (G)=estimated at 0.035 (shape). The Bayesian analysis was run for 20 million generations over 10 chains (9 heated, 1 cold) with nst=2 and rates= γ as the starting parameters determined by ModelTest with 1/1000 trees sampled. Tracer (http://evolve.zoo.ox.ac.uk/software/tracer/) was used to determine the burnin and a consensus tree was estimated from the remaining trees. Multiple independent Bayesian and ML runs were performed to ensure convergence on similar results.

Data from the five gene regions were combined into one sequence (totaling 2686 bp) representing one individual from every cave sampled along with three outgroups. The Bayesian analysis was run for 20 million generations over 10 chains (9 heated, 1 cold) with nst = 2 and $rates = \gamma$ as the starting parameters determined by ModelTest. Every 1000th tree was sampled and the burnin determined by Tracer was discarded. The remaining trees were used to make a consensus tree. PhyML was run for 500 bootstrap replicates using the parameters nst=2, G=estimated, Tratio = estimated, I = 0.7355 with the HKY model determined by ModelTest and the initial tree determined by Neighbor-joining. Similar topologies and likelihood scores were found with repeated identical runs in both MrBayes and PhyML. The nuclear gene portions showed very little variation and were not analyzed separately, but the combined mtDNA sequences (12S + 16S + CO1) were run separately to check for similar results. ModelTest parameters for the various datasets examined are available from the authors upon request.

We consider bootstrap support (BS) 70% and higher and Bayesian posterior probability (PP) 95% and higher to be significant support for a clade (Felsenstein, 1985; Hillis and Bull, 1993; Huelsenbeck and Ronquist, 2001; Wilcox et al., 2002).

2.3. Phylogeographic analyses

We used nested clade analysis (NCA: Templeton et al., 1995; Templeton, 1998; Templeton, 2001) to test for signifi-

16S

haplotype

Table 2 (continued)

Species

C. hamulatus

Individual No.

JC2256

Table 2 Cambarus individuals with voucher numbers, locality, and 16S haplotype

Locality

Individual No.

JC353

JF2784

JF2785

JC752

JC753

JF2835

JF2836

JF2837

JF2838

JF2839

JF2840

JF2841

JF2842

JF2843

JF2844

JF2845

JF2846

JF2847

JF2848

JF2849

JF2850

JF2851

JF2852

JF2853

JC844

JF2786

JF2787

JF2788

JF2789

JC1513

JC1515

JC1549

JC1550

JC1551

JC1552

JC1553

JC1554

JC1555

JC809

JF3338

JF3339

JC2415

JC2416

JC2417

JC2418

JC2419

JC2245

JC2246

JC2247

JC2248

JC2249

JC2250

JC2251

JC2252

JF2790

JC1627

JC1628

JC1548

JC2421

JC2422

JC2423

JC2424

JC2255

Species

C. hamulatus

Aaron Tolletts Cave	11	JC2257	C. hamulatus	Run to the Mill Cave	9
Bible Spring Cave	6	JC2258	C. hamulatus	Run to the Mill Cave	9
Bible Spring Cave	6	JC2259	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	8	JC2260	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	JC2261	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	JC2262	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	JC2263	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	JC2264	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	JC2265	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	JC2266	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	JC2267	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	JC2268	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	JC2269	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	JC2270	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	JC2273	C. hamulatus	Run to the Mill Cave	9
Bluff River Cave	6	KC713	C. hamulatus	Salt River Cave	6
Bluff River Cave	6	JC2228	C. hamulatus	Shakerag Cave	6
Bluff River Cave	6	JC2229	C. hamulatus	Shakerag Cave	10
Bluff River Cave	6	JC1966	C. hamulatus	Signal Light Pit	4
Bluff River Cave	6	JC1967	C. hamulatus	Signal Light Pit	4
Bluff River Cave	6	JC1968	C. hamulatus	Signal Light Pit	4
Bluff River Cave	6	JC2425	C. hamulatus	Talley Ditch Cave	6
Bluff River Cave	6	JC2426	C. hamulatus	Talley Ditch Cave	6
Bluff River Cave	6	JC2427	C. hamulatus	Talley Ditch Cave	6
Druin Spring Cave	10	JC2428	C. hamulatus	Talley Ditch Cave	6
Druin Spring Cave	10	JC2429	C. hamulatus	Talley Ditch Cave	6
Druin Spring Cave	10	JC2430	C. hamulatus	Talley Ditch Cave	6
Druin Spring Cave	10	JC2431	C. hamulatus	Talley Ditch Cave	6
Corner Spring Cave	10	JC2432 IC2432	C. hamulatus	Talley Ditch Cave	0
Garner Spring Cave	7	JC2435	C. hamulatus	Talley Ditch Cave	6
Garner Spring Cave	10	JC2434	C. hamulatus	Talley Ditch Cave	6
Garner Spring Cave	10	JC2455	C. hamulatus	Whiteside Cave	5
Garner Spring Cave	10	JC2059	C. hamulatus	Whiteside Cave	4
Garner Spring Cave	10	JC2000	C. hamulatus	Wilmoth Cave	11
Garner Spring Cave	6	JC2242 JC2243	C. hamulatus	Wilmoth Cave	11
Garner Spring Cave	6	JC2245	C. hamulatus	Wilmoth Cave	11
Garner Spring Cave	10	IC831	C hamulatus	Wine Cave	10
Geiger Cave	6	JE2791	C hamulatus	Wine Cave	10
Geiger Cave	6	01 2771	er mannaria		10
Geiger Cave	6	KC1916	C. jonesi	Cave Spring Cave	20
Graves Cave	1	JC1643	C. jonesi	Key Cave	19
Graves Cave	2	JC778	C. jonesi	White Spring Cave	14
Graves Cave	1	JC779	C. jonesi	White Spring Cave	14
Graves Cave	3	JC781	C. jonesi	White Spring Cave	16
Graves Cave	3	JC783	C. jonesi	White Spring Cave	14
Keyhole Cave	11	JC785	C. jonesi	White Spring Cave	13
Keyhole Cave	11	JC786	C. jonesi	White Spring Cave	13
Keyhole Cave	11	JC787	C. jonesi	White Spring Cave	15
Keyhole Cave	11	JC788	C. jonesi	White Spring Cave	18
Keyhole Cave	11	JC789	C. jonesi	White Spring Cave	17
Keyhole Cave	11	JC790	C. jonesi	White Spring Cave	13
Keyhole Cave	11	JC791	C. jonesi	White Spring Cave	18
Keyhole Cave	11	JC/92	C. jonesi	White Spring Cave	18
Owen Spring Cave	10	JC2436	C. sp. nov. 1	Lacon Exit Cave	22
Payne Spring Cave	6	JC2437	C. sp. nov. 1	Lacon Exit Cave	22
Payne Spring Cave	10	JC2438	C. sp. nov. 1	Lacon Exit Cave	21
Pryor Cave Spring	10	JC2439	C. sp. nov. 1	Lacon Exit Cave	23
Rickwood Caverns	12	JC2540	C. sp. nov. 1	Lacon Exit Cave	21
Rickwood Caverns	12	102440	C	Kallana C	26
Rickwood Caverns	12	JC2440 IC2441	C. sp. nov. 2	Kellers Cave	20 26
Rickwood Caverns	12	JC2441 IC2442	C. sp. nov. 2	Kellers Cave	20
Run to the Mill Cave	9	JC2442	C. sp. nov. 2	Keners Cave	20 n nort nao -)
				(continued o	п пехт page)

16S

9

haplotype

Locality

Run to the Mill Cave

Table 2 (continued)

Individual No.	Species	Locality	16S haplotype		
JC2287	C. sp. nov. 2	Kellers Cave	25		
JC2288	C. sp. nov. 2	Kellers Cave	25		
JC2289	C. sp. nov. 2	Kellers Cave	25		
JC2227	C. sp. nov. 2	Porches Spring Cave	25		
JC2412	C. sp. nov. 2	Cherry Hollow Cave	24		
Outgroups					
JF2508	C. gentryi	Williams Branch, Dick	son Co. TN		
JF2543	C. friaufi	Salt Lick Ck, Monroe	Co. KY		
JF2579	C. brachydactylus	Blue Ck., Humphreys Co. TN			

cant associations between geographic and genetic information to elucidate historical and contemporary evolutionary processes and patterns. We first used the program TCS (Clement et al., 2000) with our 16S data to build the haplotype network that illustrates mutational step distances between unique sequences. GEODIS (Posada et al., 2000) was then used to test for significant relationships between geographic locations (cave sites recorded as latitude–longitude coordinates at the entrance) and genetic distances for 5000 random permutations. Clade distances (D_c) represent geographic range for the respective clade level, while nested clade distances (D_n) represent the average distance of samples with a certain haplotype compared to the geographic center of the clade. The 2005 inference key, available from http://darwin.uvigo/es/software/ geodis.html, was used to determine which historical processes might have lead to the current evolutionary patterns.

2.4. Genetic diversity, effective population sizes, and demographics

Current genetic diversity and recent historical diversity estimates were obtained from the program DNASP 4.0 (Rozas et al., 2003) using 16S sequence data. Current diversity estimates (θ_{π} ; Nei, 1987 equations 10.5 or 10.6 and the standard error, equation 10.7) are based on pairwise base differences between sequences, while historical diversity estimates (θ_{W} ; Watterson, 1975) are based on the number of segregating sites among the sequences. These two estimates used together provide insight into recent declines or expansions in genetic diversity and effective population sizes ($\theta = 2Ne_{(f)}\mu$ for mitochondrial DNA where $Ne_{(f)} = effective$ population size for maternal lineages and $\mu =$ mutation rate)

Table 3

Cambarus species, cave names, specimen voucher numbers, and GenBank accession numbers for gene sequences included in this study

Species	Cave	Specimen No.	16S ^a	12S ^b	CO1 ^c	GAPDH ^d	Histone H3 ^e
C. hamulatus	AARON	JC353	DQ411744	DQ411711	DQ411760	na	DQ411794
C. hamulatus	BIBLE	JF2785	DQ411739	DQ411712	DQ411761	DQ411786	DQ411794
C. hamulatus	BLUFF	JF2835	DQ411739	DQ411713	DQ411762	DQ411786	DQ411795
C. hamulatus	DRUIN	JC844	DQ411743	DQ411714	DQ411763	DQ411787	DQ411794
C. hamulatus	GARNER	JC1554	DQ411739	DQ411713	DQ411764	DQ411787	DQ411796
C. hamulatus	GEIGER	JC809	DQ411739	DQ411713	DQ411765	na	DQ411797
C. hamulatus	GRAVES	JC2418	DQ411736	DQ411715	DQ411766	DQ411788	DQ411794
C. hamulatus	KEYHOLE	JC2245	DQ411744	DQ411716	DQ411767	na	DQ411794
C. hamulatus	OWEN	JF2790	DQ411743	DQ411717	DQ411768	na	DQ411798
C. hamulatus	PAYNE	JC1627	DQ411739	DQ411713	DQ411764	DQ411787	DQ411794
C. hamulatus	PRYOR	JC1548	DQ411743	DQ411714	DQ411769	DQ411787	DQ411794
C. hamulatus	RICKWOOD	JC2424	DQ411745	DQ411718	DQ411770	DQ411786	DQ411794
C. hamulatus	RUNTOTHEMILL	JC2265	DQ411742	DQ411719	na	DQ411789	DQ411794
C. hamulatus	SALT	KC713	DQ411739	DQ411720	DQ411771	DQ411787	DQ411795
C. hamulatus	SHAKERAG	JC2229	DQ411743	DQ411717	DQ411772	DQ411786	DQ411794
C. hamulatus	SIGNAL	JC1967	DQ411737	DQ411721	DQ411773	DQ411786	DQ411795
C. hamulatus	TALLEY	JC2434	DQ411739	DQ411722	DQ411774	DQ411787	DQ411799
C. hamulatus	WHITESIDE	JC2059	DQ411738	DQ411723	DQ411775	na	DQ411794
C. hamulatus	WILMOTH	JC2242	DQ411744	DQ411714	DQ411776	DQ411786	DQ411794
C. hamulatus	WINE	JC831	DQ411743	DQ411717	DQ411768	DQ411786	DQ411800
C. jonesi	CAVE	KC1916	DQ411753	DQ411724	DQ411777	DQ411790	DQ411805
C. jonesi	KEY	JC1643	DQ411752	DQ411725	DQ411778	DQ411791	DQ411806
C. jonesi	WHITE	JC781	DQ411749	DQ411726	DQ411779	DQ411790	DQ411805
C. sp. nov. 2	CHERRY	JC2412	DQ411757	DQ411727	DQ411780	na	DQ411808
C. sp. nov. 2	KELLERS	JC2442	DQ411759	DQ411727	DQ411781	DQ411792	DQ411807
C. sp. nov. 2	PORCHES	JC2227	DQ411758	DQ411727	DQ411781	DQ411792	DQ411807
C. sp. nov. 1	LACON	JC2436	DQ411755	DQ411728	DQ411782	DQ411793	DQ411801
C. gentryi	na	JF2508	AY853664	DQ411731	DQ411783	na	DQ411804
C. friaufi	na	JF2543	DQ411733	DQ411730	DQ411784	na	DQ411803
C. brachydactylus	na	JF2579	DQ411732	DQ411729	DQ411785	na	DQ411802

na, not available.

^a 16S primers: 16sf-cray: 5' GACCGTGCKAAGGTAGCATAATC 3' and 16s-1492r: 5' GGTTACCTTGTTACGACTT 3'.

^b 12S primers: 12sf: 5' GAAACCAGGATTAGATACCC 3' and 12sr: 5' TTTCCCGCGAGCGACGGGCG 3'.

^c CO1 primers: HCO2198: 5' TAAACTTCAGGGTGACCAAAAAATCA 3' and LCO1490: 5' GGTCAACAAATCATAAAGATATTG 3'.

^d GAPDH primers: G3PCq157F: 5' TGACCCCTTCATTGCTCTTGACTA 3' and G3PCq981R: 5' ATTACACGGGTAGAATAGCCAAACTC 3'.

e Histone H3 primers: H3af: ATGGCTCGTACCAAGCAGACVGC 3' and H3ar: 5' ATATCCTTRGGCATRGTGAC 3'.

(Templeton, 1993; Yu et al., 2003; Buhay and Crandall, 2005). Using a rate of 2.2×10^{-8} substitutions per site per year (Cunningham et al., 1992), effective population sizes were calculated using ten year generation times for obligate cave-dwelling crayfish species and equal sex ratios (Cooper, 1975).

Sample sizes were low for *C. jonesi* (n = 14 from 3 caves), C. sp. nov. 1 (n=5 from 1 cave), and C. sp. nov. 2 (n=8from 3 caves) despite extensive fieldwork and range-wide coverage. Therefore, we only examined demographic history for C. hamulatus (n = 103 individuals from 20 caves). Tests for neutrality can be used to assess demographic history with significant negative D values of Tajima (1989) and F^* values of Fu and Li (1993) indicating population expansions. We also performed a mismatch analysis (which plots the distribution of the number of differences between pairs of haplotypes) for population growth (expansion) for C. hamulatus in DNASP (Rozas et al., 2003) using an initial $\theta = 0.$ $\theta = 1000$. with expansion final parameter $\tau = 2\mu t = 3.803$. Population expansion would appear as a "wave" in the mismatch distribution, while stable population sizes produce ragged multi-modal distributions (Rogers and Harpending, 1992; Harpending, 1994). The probability of obtaining values of r (raggedness) less than the observed ($P(r_{\text{expected}} < r_{\text{observed}})$) was calculated using the coalescent algorithm in DNASP over 1000 pseudoreplications with a random seed and no recombination.

3. Results

3.1. Phylogenetic analyses

Phylogenetic relationships among the cave crayfish species of the subgenus *Aviticambarus* were determined using Bayesian and Maximum Likelihood approaches for both the 16S haplotype dataset and the combined five gene dataset representing one individual from each sampled cave. For each of the Bayesian analyses, the first 2000 trees were discarded as burnin and the consensus tree was estimated using the remaining 18,000 trees.

Rather than two extant species (*C. hamulatus* and *C. jonesi*), we found evidence of four distinct lineages (Figs. 1-3) in addition to the unsampled *C. veitchorum*. Using only



Fig. 2. Phylogram of the relationships between 16S haplotypes for each of the species (Bayesian topology shown). Analyses done in PhyML are given below the nodes as bootstrap support (BS) percentages from 500 replicates (log likelihood = -1340.87). Bayesian support values are given above the nodes as posterior probability (PP) percentages (log likelihood = -1374.16). Support values are not shown for intra-specific groupings.

16S haplotypes (GenBank Nos. DQ411734–DQ411759), *Cambarus sp. nov. 2* (endemic to Marshall Co, Alabama) is sister to the other lineages with significant support (Fig. 2: 100% PP, 97% BS). *C. hamulatus* diverged from a common ancestor with *C. jonesi* and *C. sp. nov. 1* with 100% BS and PP support for the node. The distinctiveness of *C. jonesi* and *C. sp. nov. 1* was highly supported (100% PP and 99% BS and 100/100%, respectively).

The combined five gene dataset was analyzed using both ML and Bayesian approaches and revealed some similar trends to the 16S haplotype analysis. *C. sp. nov. 2* was recovered as basal to the other cave lineages with significant BS and PP support for the node (Fig. 3). In contrast to the haplotype analysis, the node separating *C. jonesi* from *C. hamulatus* and *C. sp. nov. 1* was significantly supported with both BS and PP, while the sister relationship between *C. sp. nov. 1* and *C. hamulatus* was not highly supported. This same topology was recovered using only mtDNA

combined sequence data of the three genes for each cave with similar nodal support values (not shown).

3.2. Nested clade analysis

The statistical parsimony network included 26 unique haplotypes within the subgenus *Aviticambarus* (Fig. 4). The Marshall County, Alabama (*C. sp. nov. 2*) samples fell out as a separate network (higher-level clades are marked with an 'A' and the network is shaded orange in Fig. 4) connected to haplotype 4 of *C. hamulatus* by 23 steps. Therefore, the main network contained *C. hamulatus* and *C. jonesi* connected by nine steps (=95% confidence limit), but the *C. sp. nov. 1* haplotypes were outside the limit with twelve steps. These three lineages were grouped together for the phylogeographic analysis (main network) and a separate analysis was done on the Marshall County network (Fig. 4: *C. sp. nov. 2*, shaded in orange).



Fig. 3. Phylogenetic relationships of the species with the subgenus *Aviticambarus* estimated from the combined dataset of three mtDNA genes (12S, 16S, and CO1) and two nuclear genes (Histone H3 and GAPDH) totaling 2686 bases. The first values are bootstrap support of 500 replicates in PhyML (log likelihood = -8026.43) and values after the slash represent posterior probabilities from Bayesian analysis (log likelihood = -6811.02) since both analyses yielded similar topologies (Bayesian topology shown). Support values are not shown for intra-specific groupings.



Fig. 4. Haplotype network depicting the nesting levels used to infer historical processes which gave rise to current genetic structuring. Haplotype circles are colored to represent the four lineages: *C. hamulatus* (haplotypes 1–12, red), *C. jonesi* (haplotypes 13–20, yellow), *C. sp. nov. 1* (haplotypes 21–23, pink), and *C. sp. nov. 2* (haplotypes 24–26, orange). Ancestral haplotypes are represented by squares and empty circles represent unsampled or extinct haplotypes. The significance level connecting the network was nine steps, and *C. sp. nov. 1* and *C. sp. nov. 2* were outside the 95% confidence limit in addition to the three outgroup taxa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

The main network contained twelve 1-step clades, seven 2-step clades, and four 3-steps clades in the total cladogram while the Marshall County network contained two 1-step clades and two 2-step clades in the total cladogram (Table 4). The outgroups C. gentryi and C. brachydactylus were connected to Haplotype 24 by 31 steps and C. friaufi was connected by 28 mutational steps to Haplotype 9 (Fig. 4). Eight clades differed significantly from random distributions (p < 0.05; Table 5). All clades with significantly large or small distances were examined using Templeton's 2005 inference key to elucidate historical and current processes which contribute to the genetic structuring of each lineage. Contiguous range expansion, long-distance colonization, past fragmentation, restricted gene flow, and isolation by distance were the inferred patterns.

3.3. Species' boundaries

Delineation of species' boundaries is a hotly-debated issue for systematists and conservation biologists, since spe-

cies are the fundamental units of biodiversity (Sites and Crandall, 1997; Agapow et al., 2004). We chose to define species with criteria specified for the Genealogical Concordance Species concept (Avise and Ball, 1990; Baum and Shaw, 1995), since we are investigating multiple independent characters (genetic; geographic; geologic). This lineage-based concept necessitates concordance among different characters and defines a "genealogical species" as a group of organisms more closely related to each other ("exclusivity") than to organisms outside its group (Baum and Shaw, 1995).

Phylogenetic analyses of 16S haplotypes (Fig. 2) revealed significant support for the monophyly of *C. hamulatus* (75% BS), *C. jonesi* (100/99% PP/BS), *C. sp. nov.* 1 (100/100% PP/BS), and *C. sp. nov.* 2 (100/100% PP/BS). The combined gene analyses also supported the distinction of *C. hamulatus*, *C. jonesi*, *C. sp. nov.* 1, and *C. sp. nov.* 2 as separate lineages (Fig. 3), although the sister relationship of *C. sp. nov.* 1 lacked significant support. Nested phylogeographic analyses supported the recognition of three separate lineages: *C. hamulatus*/*C. jonesi* which was grouped at

Table 4	
Results of the nested clade analysis of Aviticambarus 16S haplotypes based on 5000 permutations	

0-step clades			1-step clades		2-step clades			3-step clades			
Haplotype	D _c	D _n	Clade	$D_{\rm c}$	D _n	Clade	D _c	D _n	Clade	D _c	D _n
1			1-1	0.00s	96.18L	2-1	64.06L	62.10L	3–1	30.70s	30.44s
2											
3											
4	1.66s	1.87s	1–2	1.87s	47.00s						
5	0.00	1.87									
I - T	1.66s	0.00s	I - T	1.87	-48.18s						
6	13.15	13.15	1-3			2–2	13.03s	16.09s			
7	0.00	6.43									
8	0.00	13.04									
I - T	13.15	3.41				I - T	-51.02s	-46.00s			
9			1–4	0.00s	72.72L	2–3	33.48s	31.09s	3–2	45.45	37.41
10	10.06s	20.74	1-5	26.46	28.90						
11	24.43	37.06									
I - T	-14.36	-1631									
12			1–6			2-4	0.005	182.61L			
13			1-7			2-5	0.00s	23 73	3_3	37.22	11677I
14			- /			20	01000	20170	00	07122	1100772
15			1-11	0.00s	28 488	2-6	40.83	39.92			
16				01000	201100	2 0	10102	00002			
17											
18			1-8	0 00s	28 48s						
19			1_9	0.005	50.87L						
20			1-10	0.00	36.97						
20			I - T	0.00	-617	I - T	-40.83s	-1618			
21			1_12	0.00	0.17	2_7	10.055	10.10	3_4	0.00s	109 56
22			1 12			2 /			5 1	0.005	109.50
23											
25						I - T				15 59	14 99
24			1–2A			2_2A	0.00	746		15.55	14.99
25	1.80	2.03	1_1A			2_1A	0.00	7.46s			
26	0	2.03	1 1/1			2 1/1	0.00	7.405			
I - T	1 80	0.001									
	1.00	0.001									

Note. Clade (D_c) and nested clade (D_n) distances are given. An "s" indicates that the distance is significantly small at the 5% confidence level and an "L" indicates that the distance is significantly large. In clades with both tip and interior groups, the average I - T distance is provided. Interior clades with genetic-geographic differences are italicised.

the 95% confidence limit, C. sp. nov. 1, and C. sp. nov. 2 (Fig. 4 and Table 5).

Geologic evidence (province and district separations provided by the Geological Society of Alabama, S. McGregor) supported the recognition of each of the four lineages. C. hamulatus is restricted to the area around the Sequatchie Valley and the Jackson County, Alabama mountains district of the Cumberland Plateau province in Jackson County, while C. jonesi is endemic to the Tennessee Valley district of the Highland Rim province (Fig. 1). C. sp. nov. 1 is found in the Moulton Valley district of the Highland Rim province, while C. sp. nov. 2 is restricted to the Jackson County Mountain district of the Cumberland Plateau in Marshall County, Alabama. Based on concordance of multiple characters and exclusivity, there appears to be no less than five distinct genealogical species in the subgenus Aviticambarus. Although we chose to employ the Genealogical Concordance Species Concept a priori, we also recognize that these results fit nicely with criteria of the Phylogenetic Species Concept (de Queiroz and Donoghue, 1990), including monophyly and "exclusivity" (Baum and Donoghue,

1995). Likewise, the species are supported by the Cohesion Concept using the exchangeability criteria of Templeton (2001).

3.4. Genetic diversity and demography

Estimates of genetic variability are reported in Table 6 and the four lineages show low to moderate levels of diversity. These low levels of diversity are common among species thought to have undergone a bottleneck, but both current and historical estimates of population size are similar. Examining deviations from neutrality can help clarify past demographic events, as significant negative D (Tajima, 1989) and F^* (Fu and Li, 1993) values are often associated with bottlenecks followed by range expansions. In the case of *C. hamulatus*, using 16S haplotypes (n=12), we found D=-0.1814 (P>0.10) and $F^*=-0.52837$ (P>0.10). A unimodal mismatch distribution is predicted for populations having undergone expansion (which is indicated by the expected curve in Fig. 5), but our observed distribution shows a slightly ragged bimodal distribution, typical of

 Table 5

 Nested contingency results and inferred patterns

Clade	χ^2	Probability	Inference chain	Inferred pattern
1–2	1.8750	0.4022	1–2–11–12-No	CRE
1-3	11.3118	0.4276	NA	NA
1-5	27.0000	0.0000^{*}	1–2–11–12-No	CRE
2-1	10.0000	0.0076*	1-19-20-2-11-12-13-Yes	LDC
2-3	45.0000	0.0000^{*}	1–2–11–17–4-No	RGF w/ IBD
2-6	16.0000	0.0342*	1–2–11–12-No	CRE
3–1	54.0000	0.0000^{*}	1–2–11–12-No	CRE
3–2	49.0000	0.0000^{*}	1-2-11-12-13-Yes	LDC
3–3	1.7500	1.0000	1–2–11–12-No	CRE
Total	350.6975	0.0000*	1–3–5–15-No	PF and LDC
1–1A	0.8750	1.0000	1–2–11–17–4-No	RGF
Total A	0.0000	0.0000^{*}	1–2–11–17–4-No	RGF w/ IBD

Inferences were made using Templeton's 2005 key. Abbreviations for the inferences are: CRE, contiguous range expansion; NA, not applicable; LDC, long-distance colonization; RGF, restricted gene flow; IBD, isolation by distance; PF, past fragmentation.

Indicates significant at the P < 0.05 level.

Table 6

Current ($\theta_{\pi} \pm SE$) and historical-based (θ_{W}) estimates of genetic diversity and effective population sizes estimated using an equal sex ratio, ten-year generation time, and 2.2×10^{-8} substitution rate

Cave species	Current	Historical		
	θ_{π}	$N_{\rm e}$	$\theta_{\rm W}$	$N_{\rm e}$
C. hamulatus	0.00442 ± 0.00039	40182	0.00477	43364
C. jonesi	0.00452 ± 0.00085	41091	0.00585	53182
C. sp. nov. 1	0.00206 ± 0.00057	18727	0.00198	18000
C. sp. nov. 2	0.00369 ± 0.00183	33545	0.00478	43455



Fig. 5. Mismatch distribution for the 16S haplotypes for *Cambarus hamulatus*. The observed frequency is represented by the diamond and thick solid line, and the expected frequency under the expansion model is depicted by thin solid line connecting square symbols.

constant population sizes, not growth (raggedness = 0.036; *P* ($r_{\text{expected}} < r_{\text{observed}}$) = 0.15); (Rogers and Harpending, 1992; Harpending, 1994).

3.5. Conservation status assessment

Using categories and criteria to evaluate species for endangerment, we suggest that all five *Aviticambarus* lineages be considered for conservation measures and listing on the IUCN Red List (version 3.1 criteria found on www.redlist.org). According to the Preamble of the 2001 Categories and Criteria, the IUCN affords protection to "species or lower taxonomic levels, including forms that are not yet formally described" (www.redlist.org), and therefore, we recommend conservation status for each species based on the information available.

Cambarus veitchorum should receive the highest protection, critically endangered (CR), as only a total of seven individuals (six adults, 1 juvenile) have ever been documented from White Spring Cave which is the only known locale for the species despite search efforts by many biologists for the past three decades since the last sighting in 1968. *C. veitchorum* meets the CR category with the criteria of decline in occurrence, extent of occurrence estimated to be less than a 100 sq. km. area, only known from a single location (area of occupancy), and inferred decline in number of mature individuals (IUCN A2c, B1a, B1bi-v, B2a, C2i, D).

Cambarus jonesi is currently known from only twelve locations along both sides of the Tennessee River basin in northern Alabama. We recommend this species receive vulnerable (VU) status, as it meets criteria of a geographic extent of occurrence less than 20,000 sq. km., severely fragmented range, area of occupancy less than 2000 sq. km., and inferred decline in the quality of the cave habitat (IUCN B1a, B1biii, B2biii).

Cambarus sp. nov. 1 is currently only known from one cave locality in Alabama, with five individuals found, and we suggest that this species be afforded vulnerable (VU) status. The cave locality occurs on a major interstate highway, as the cave entrance was blasted open by the road construction. We feel that intensive field surveys might find a few new localities of this species, but the known caves in the vicinity have been extensively searched for cave crayfish for decades. This species meets the same vulnerable status criteria as *C. jonesi*, with additional criteria of a very small population size and a very restricted area of occupancy (IUCN B1a, B1biii, B2biii, D2).

C. sp. nov. 2 is currently only known from four cave localities in Marshall County, Alabama. Extensive field-work was conducted in the direct vicinity of the four known cave locations for this species, and we feel additional surveys might result in only a few more locations at best. We suggest that this species be considered vulnerable (VU) because it meets the criteria of extent of occurrence less than 20,000 sq. km., known to exist at less than ten locales, area of occupancy less than 2000 sq. km., and very small and restricted populations (IUCN B1a, B2a, D2).

Cambarus hamulatus is the most widespread species of the *Aviticambarus* assemblage, but the bulk of the known cave localities are clustered around the geographic center of its range. This species does not meet the criteria for vulnerable status, but we feel that its fragmented distribution coupled with a population size that does not appear to be expanding qualifies this species for "near threatened" (NT) status.

4. Discussion

Phylogeographic studies on the faunal biodiversity of the Southern Appalachian Mountains, an area among the highest in species richness in North America, are increasing in the literature for various animal groups (e.g., salamanders: Rissler and Taylor, 2003; Crespi et al., 2003; Kozak et al., 2006; Jones et al., 2006 spiders: Hedin, 1997a,b; Hedin and Wood, 2002 fish: Berendzen et al., 2003 insects: Schultheis et al., 2002 crayfish: Buhay and Crandall, 2005). The Cumberland Plateau and adjacent Highland Rim that surround the Nashville Basin, are disjunct topographic karst units off the main Southern Appalachian chain, and these areas also rank high for species richness in endemic fauna, particularly for freshwater mussels, snails, and crayfish. This area of the Southern Appalachians also lends itself to limestone (karst) cave development and ranks among the highest in cave density in the world with nearly 5000 caves located on the Cumberland Plateau province and 3500 caves on the Highland Rim (Aulenbach and Cressler, 1998). Following with this pattern of high cave density, Culver et al. (2000) found that the area of greatest diversity for terrestrial cave animals (troglobites) in the United States was the northeastern corner of Alabama, including Jackson, Madison, and Marshall Counties which is also the geographic center of the range of the cave crayfish subgenus Aviticambarus.

Over half of the animal species on the United States Natural Heritage List of imperiled and vulnerable taxa (http://www.natureserve.org) are terrestrial and aquatic subterranean species, which brings to light the need for science-based conservation assessments, status surveys, and "DNA taxonomy" to separate morphologically-cryptic taxa. Our study revealed two new cave crayfish lineages previously assigned to C. jonesi and found that all members of the subgenus Aviticambarus are indeed imperiled due to low genetic diversity and population size estimates, small geographic ranges, and few known locales. C. hamulatus, a cave crayfish species that spans seven counties in two states, showed stable, not expanding, populations with only a moderate level of genetic diversity. These results are in stark contrast to another cave crayfish assemblage (genus Orconectes, subgenus Orconectes) on the western escarpment of the Cumberland Plateau, which showed moderate to high levels of genetic diversity, larger ranges, more known locales, and extensive gene flow (Buhay and Crandall, 2005).

The complex, dynamic geologic and hydrologic history of the Sequatchie Valley appears to have played major roles in the distribution and current population structure of *C. hamulatus.* Run to the Mill Cave in Cumberland County, Tennessee (Fig. 1: northernmost locale) is a massive groundwater system at the head of the Sequatchie Valley and Sequatchie River, which flows south directly into the Tennessee River. On the extreme Southern end of the Sequatchie Valley is Rickwood Caverns in Blount County, Alabama (Fig. 1: Southernmost locale), which currently

drains south into the Black Warrior River of the Mobile Basin. But the next Southernmost site was Graves Cave in Blount County, Alabama (Fig. 4: Haplotypes 1, 2, 3) which was colonized during a different migration episode than its Southern neighbor Rickwood Caverns (Fig. 4: Haplotype 12). Perhaps, as the Southern portion of the Sequatchie Valley was widened by erosion and geologic activity, separate colonization events (wash-outs or long-distance migrations) expanded the range of C. hamulatus. The Sequatchie Valley (anticline) was formed by a geological uplift which has since weathered and eroded down to the current valley floor (Thomas, 1986), leaving a wide area of limestone (including Mississippian Limestone strata) exposed in the valley floor and slopes of the Sequatchie Valley. However, cave development along the Sequatchie Valley is limited due to the complex hydrogeologic history of the area, which may also have prohibited accumulation of genetic diversity or prevented population growth in C. hamulatus. It is interesting to note that while Orconectes australis australis migrated mainly southward in leading-edge expansion events along the Cumberland Plateau's western escarpment accumulating genetic diversity along the way (Buhay and Crandall, 2005), C. hamulatus originated in the center of its current range and expanded in both northward and southward directions from the area of the Alabama-Tennessee state line along the eroding Sequatchie Anticline.

Finally, this study supports previous findings that paleodrainages, specifically pre-Pleistocene water routes, played important roles in phylogeography and speciation processes of freshwater fauna in the southeastern United States. Ancient drainage changes may have lead to the extirpation of populations, which may be reflected as unsampled, possibly extinct haplotypes in the parsimony network. Current drainage basins do not reflect species' geographic boundaries or barriers between cave crayfish in the Southern Appalachians and even more importantly, biologists may be drastically underestimating biodiversity by relying on contemporary hydrologic delineations, physiographic boundaries, and convergent similar morphology. For example, a recent extensively-sampled study of the Eurycea bislineata complex in eastern North America identified 13 putative independent lineages rather than five taxonomically-recognized salamander taxa within the bislineata complex (Kozak et al., 2006). Our study supports five distinct lineages rather than three within the subgenus Aviticambarus, and highlights the need for integration of genetic, phylogeographic, and environmental (hydrological and geological) analyses in well-sampled studies of freshwater fauna, particularly obligate cavedwellers, to tease apart convergence and taxonomy and afford conservation and protection to these unique organisms.

Acknowledgments

Thank you to all the cavers of the Alabama and Tennessee grottos of the National Speleological Society, especially the Drake and Caudle families and Marion O. Smith and Jason Record, who provided new cave crayfish localities. Special thanks are due to Jim Godwin and Carl Scardina for permission to sample Rickwood Caverns State Park. This study would be impossible without fantastic field assistants, especially Heather Levy, Joe Douglas, Jonathan Griffith, Kristin Bobo, Kevin Toepke, Daniel Mann, Avis Moni, Lin Guy, Kenneth Rupil, Matt Niemiller, and some great landowners, including Mr. Wilson, the keeper of Run to the Mill Cave. Thank you to Stuart McGregor for help with geological provinces in Alabama, to Michael Gilbert for knowledge about Rickwood Caverns, and to Mark Schultz who provided primer sequences for the GAPDH gene. John Cooper, Tom Barr, Brice Noonan, and two anonymous reviewers kindly commented on earlier drafts which greatly improved this manuscript. This study was funded by NSF DDIG (DEB 0508580) to J.E.B. and K.A.C. and by the Cave Conservancy Foundation and Brigham Young University Graduate School and Integrative Biology Department to J.E.B.

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