TAXONOMIC REVISION OF CAVE CRAYFISHES IN THE GENUS *ORCONECTES*, SUBGENUS *ORCONECTES* (DECAPODA: CAMBARIDAE) ALONG THE CUMBERLAND PLATEAU, INCLUDING A DESCRIPTION OF A NEW SPECIES, *ORCONECTES BARRI*

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ABSTRACT

Historically, three obligate cave-dwelling species of *Orconectes* comprise an assemblage along the Cumberland Plateau of the southeastern United States, including *Orconectes australis* (with two subspecies, *australis* and *packardi*), *O. incomptus*, and *O. sheltae*. Using genetic data from three mitochondrial genes (cytochrome oxidase subunit I (COI), 12S, and 16S), we present evidence for four cryptic lineages excluding *O. sheltae*. The subspecies of *Orconectes australis* represent distinct taxonomic units with non-sister phylogenetic relationships and will be each recognized as separate species. *Orconectes* (*Orconectes*) *barri* is a new species of subterranean crayfish with a small distribution around the Kentucky - Tennessee state border in Mississippian limestone. This species was uncovered during a previous thoroughly-sampled phylogeographic survey of the southern Appalachians. Additionally, we assign genetic barcodes (COI sequences) and conservation status to each species for management direction and identification of newly discovered populations.

KEY WORDS: bar coding, cave fauna, crayfish, cryptic species, molecular systematics, *Orconectes*

INTRODUCTION

Crayfishes are the largest and most conspicuous obligate macroinvertebrates in cave ecosystems across the southeastern United States, inhabiting karst (limestone) geographic regions including: the Cumberland Plateau of the southern Appalachians of eastern Kentucky, eastern Tennessee, and northern Alabama; the Interior Lowlands of southern Indiana, western Kentucky, and northwestern Tennessee; the Greenbrier Valley of West Virginia; the Ozark Plateau of southwestern Missouri, northern Arkansas, and eastern Oklahoma; and the Florida Lime Sinks (Hobbs et al., 1977; Peck, 1998). In addition to the monotypic genus *Troglocambarus*, each of the three largest genera of freshwater crayfishes (*Orconectes*, *Cambarus*, and *Procambarus*) contains stygobitic (obligate cave-dwelling) species, totaling 34 species and subspecies in the contiguous United States (Hobbs III, 2001).

Within the freshwater crayfish genus *Orconectes*, there are historically six stygobitic species and four subspecies: *O. pellucidus* (Tellkampf, 1844), *O. inermis inermis* (Cope, 1872), *O. inermis testii* (Hay, 1891), *O. australis australis* (Rhoades, 1941), *O. australis packardi* (Rhoades, 1944), *O. incomptus* (Hobbs and Barr, 1972), *O. sheltae* (Cooper and Cooper, 1997) and *O. stygocanei* (Hobbs III, 2001). Within the genus, these species were assigned to the subgenus *Orconectes* (Fitzpatrick, 1987) and these eight taxa were hypothesized to be descendants of a wide-ranging surface species related to *O.* (*Faxonius*) *limosus* (Rafinesque, 1817). *O. inermis inermis* was designated as the type species of the genus (Cope, 1872). All stygobitic members show greatly reduced morphological features of reproductive anatomy and visual systems, loss of body and eye pigment, and extension of sensory organs compared to surface-dwelling species of the genus.

Within the subgenus *Orconectes*, there is an assemblage of obligate subterranean taxa found across the Cumberland Plateau of the southern Appalachians. Historically, *Orconectes australis australis* was stated to occur in Mississippian limestone caves along the eastern escarpment of the Cumberland Plateau, ranging from northern Alabama to the state boundary between Tennessee and Kentucky (Hobbs et al., 1977). This boundary area was thought to be void of suitable cave habitat, and hence, was the northern geographic limit of *O. a. australis*. On the north side of the state border, *O. a. packardi* was located in Mississippian caves in eastern Kentucky ranging from the northern edge of the Cumberland Plateau south to the state line (Hobbs et al., 1977). *O. sheltae* was only found at a single site, Shelta Cave in Huntsville, Alabama, which is located at the southern end of the Cumberland Plateau in northern Alabama, and Shelta Cave also houses populations of *O. a. australis* and *Cambarus* (*Aviticambarus*) *jonesi* (Cooper and Cooper, 1997). To the west of the Cumberland Plateau escarpment in northern Tennessee, in Ordovician limestone caves, *O. incomptus* was only recorded from four localities in two counties (Hobbs et al., 1977).

In a recent examination of the phylogeographic structure of the Cumberland Plateau assemblage of cave *Orconectes* (Buhay and Crandall, 2005), we found evidence for four lineages (excluding the extremely rare, possibly extirpated *O. sheltae*) using sequence data from the mitochondrial 16S gene of 461 individuals from 67 caves (Figures 1, 2). Rather than two subspecies of *O. australis*, each subspecies fell out
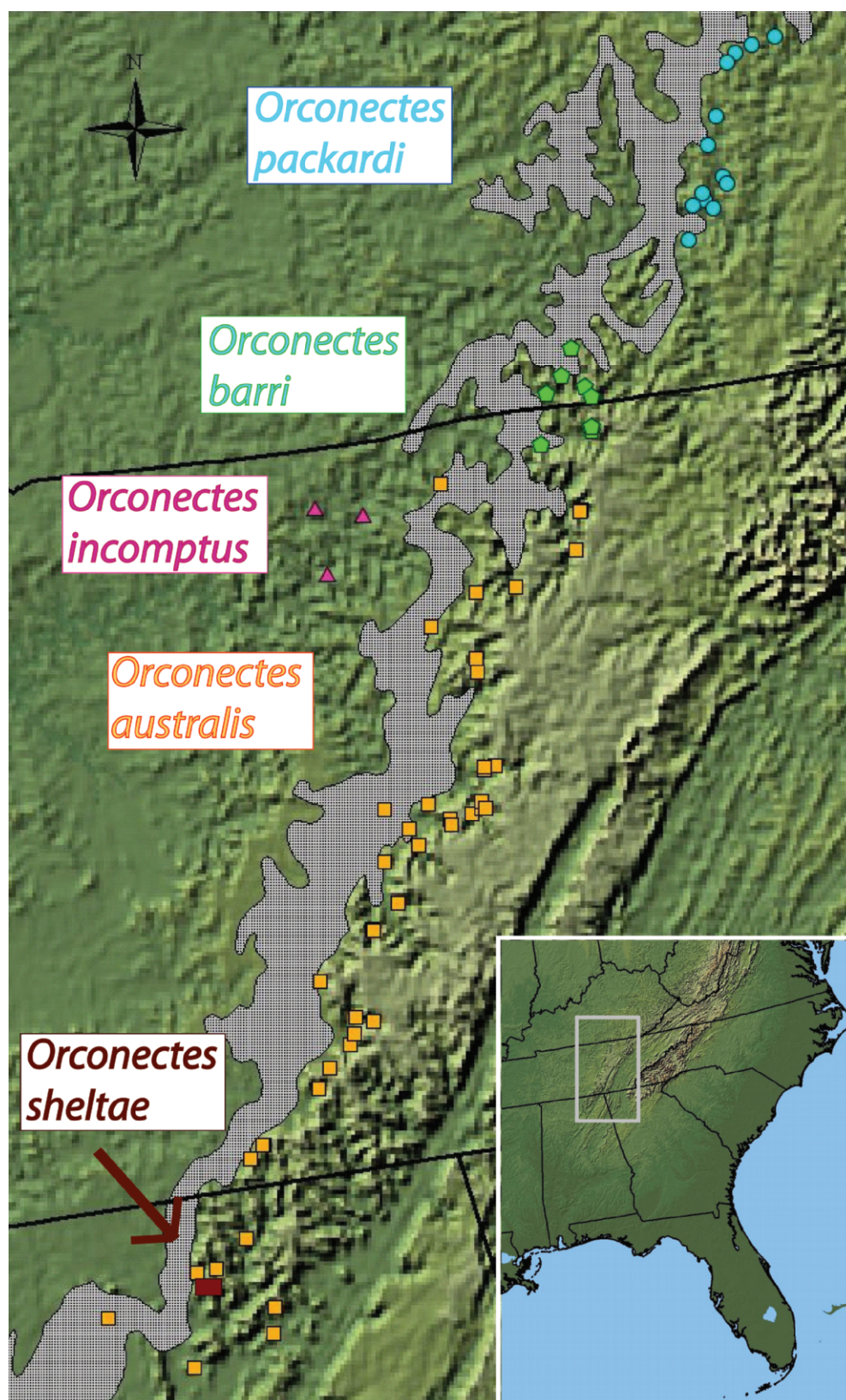
Fig. 1.   Distributions of *Orconectes* (*Orconectes*) *packardi* (blue circles), *O. incomptus* (pink triangles), *O. barri* (green hexagons), *O. australis* (orange squares), and *O. sheltae* (red rectangle) along the Cumberland Plateau of the southern Appalachians in the southeastern United States (adapted from Buhay and Crandall, 2005).
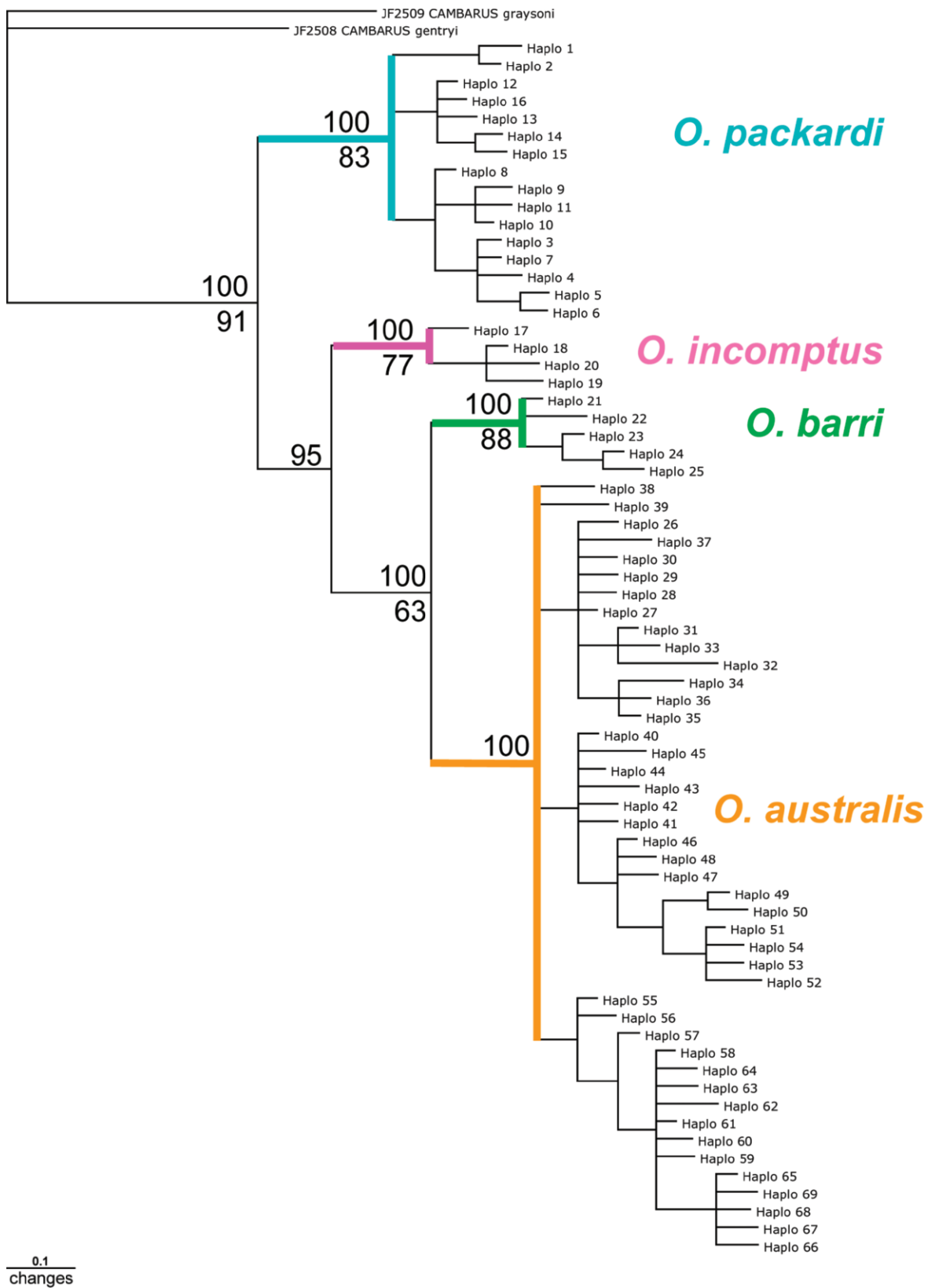
Fig. 2.   Phylogenetic relationships between cave *Orconectes* species based on 69 haplotypes of 16S mtDNA sequence data using a maximum likelihood approach in PAUP* (Swofford, 2001) and a Bayesian approach (Bayesian topology shown) (adapted from Buhay and Crandall, 2005). *Cambarus graysoni* and *Cambarus gentryi* were used as out-group taxa. Numbers below branches indicate bootstrap support and numbers above branches indicate posterior probabilities.

in strongly supported independent clades indicating that each is a separate taxon and furthermore, they were not sister taxa. In the area previously thought to be uninhabited by cave crayfishes (Hobbs and Barr, 1972), populations were located along the Kentucky—Tennessee border in Clinton County, Kentucky, and Pickett County, Tennessee. Hobbs and Barr (1972) suspected that northern populations of *O. australis australis* and southern populations of *O. a. packardi* come into contact with each other with "a limited amount of gene flow" creating "intergrades" at the state border. However, phylogeographic and phylogenetic analyses, revealed that these border populations represent a new species (Buhay and Crandall, 2005), which we formally describe in this paper as *Orconectes barri*.

Our study represents a novel approach to species diagnostics using a combination of phylogenetics, barcoding, and geography to diagnose species boundaries and infer new species. Recently, there has been a movement toward "integrative taxonomy" (Dayrat, 2005) which aims to delimit species boundaries using multiple resources, which may include geographic, behavioral, developmental, and/or genetic data, for species that are morphologically similar. We maintain that phylogenetic and/or morphological data are helpful in diagnosing species boundaries (Sites and Marshall, 2004), but some diagnoses (especially for morphologically cryptic or in our cave crayfish case, morphologically convergent) are more easily inferred using genetic data and can be misleading using morphological data (Wiens et al., 2003). Genetic data is particularly helpful for diagnosing species within a rigorous hypothesis-testing framework (Sites and Crandall, 1997; Templeton, 2001; Wiens and Penkrot, 2002). Thus we have attempted to include traditional elements in this species diagnosis including high resolution photographic images of gonopods (instead of drawings, to provide the actual structure instead of an interpretation of the structure) coupled with a phylogenetic analysis based on extensive geographic sampling as evidence of species boundaries via lack of gene flow and genetic diagnostic characters in the form of DNA barcodes (Hebert et al., 2003a, b).

The main goal of this study is to revise the taxonomy of this monophyletic group of cave crayfishes along the Cumberland Plateau in the southern Appalachians, including the description of a new species, *Orconectes barri*, and the elevation of the subspecies *O. a. packardi* to species level. This brings the number of stygobitic crayfish species and subspecies to 35 in the contiguous United States. We also provide genetic barcode information to aid in species diagnosis for newly discovered populations. Finally, we propose conservation status for each species to aid in future management decisions.

MATERIALS AND METHODS

Genetic Data Collection

Mostly tissue samples (claw or leg which can be regenerated) were removed from crayfish individuals, which were then placed back into the cave stream at the point of capture. In few cases, voucher specimens were taken to document new cave localities found after the most recent listing (Hobbs et al., 1977). Tissues and voucher specimens were immediately placed in 95% ethanol and given unique identification numbers (JC numbers for claw, leg, and tail/gill tissues from preserved whole specimens and Monte L. Bean Museum Crustacean Collection at Brigham Young University (BYUC) collection numbers for whole specimens). After tail or gill tissue

was removed from vouchers, the specimens were then placed in 70% ethanol for museum storage. Using a subset of the individuals used in the Buhay and Crandall (2005) study, sequence data from two additional mitochondrial genes (COI and 12S using primers listed in Buhay et al., 2007) were gathered, edited, and aligned by eye using BioEdit (Hall, 1999). The closest surface-dwelling ancestor to the group is possibly *Cambarus* (*Jugicambarus*) *gentryi* (Hobbs, 1970), *C.* (*Depressicambarus*) *graysoni* (Faxon, 1914), *C.* (*Glarecola*) *friaufi* (Hobbs, 1953), or *C.* (*Glarecola*) *brachydactylus* (Hobbs, 1953) based on preliminary analyses of nearly all species of *Orconectes* and *Cambarus* using multiple genes (Buhay and Crandall, unpublished data). For purposes of rooting the phylogenetic trees in the 12S and COI analyses, *C. friaufi* was used as the out-group. *Cambarus gentryi* and *C. graysoni* were used to root the 16S tree in the Buhay and Crandall (2005) study.

The gathering of 12S and COI data for this study was not meant for exhaustive phylogenetic analyses. Buhay and Crandall (2005) included phylogeographic and phylogenetic analyses using the 16S gene for 321 individuals of *Orconectes australis*, 93 individuals of *O. packardi*, 40 individuals of *O. barri*, and eight individuals of *O. incomptus*, because 16S is the most variable mitochondrial gene known for crayfish (Sinclair et al., 2003). The purpose of gathering 12S and COI data from a subset of the individuals sequenced for 16S in the Buhay and Crandall (2005) study was to show that these species can be easily distinguished from each other using different mitochondrial genes, of which COI is widely accepted as a barcode (see section below).

Although COI pseudogenes are especially problematic for the cave *Orconectes* (Buhay and Crandall, unpublished data), no indels or stop codons were found in the sequences. Phylogenetic analyses of the COI and 12S data were run in MrBayes 3.04b (Ronquist and Huelsenbeck, 2003) and PhyML (Guindon and Gascuel, 2003; http://atgc.lirmm.fr/phyml/). All parameters were estimated in MrBayes and PhyML except number of substitution types (nst) = 2 for COI and nst = 6 for 12S. The Bayesian analyses were run for two million generations over four chains (three heated, one cold) with 1/1000 trees sampled. The ML (maximum likelihood) estimates were run with random sequence addition and nodal confidence assessed via 500 bootstrap pseudoreplications. Three independent Bayesian and ML runs were performed to ensure convergence on similar results. Bootstrap support (BS) 70% and higher and Bayesian posterior probability (PP) 95% and higher are considered to be significant support for a clade (Felsenstein, 1985; Hillis and Bull, 1993; Huelsenbeck and Ronquist, 2001).

For purposes of delineating species' boundaries, we employed the lineage-based "Genealogical Concordance Species Concept" whereby a "genealogical species" is a group of organisms more closely related to each other ("exclusivity") than to organisms outside its group (Baum and Shaw, 1995).

Morphological Data Collection

Measurements of type specimens of the new species of *Orconectes* were taken using digital calipers (Fowler Sylvac, 6 inch, Model Number 54-100-444; Newton, Massachusetts) to the nearest hundredth for the 1) carapace: height, width, total length, and postorbital length; 2) rostrum: length and width; 3) areola: length and width; 4) pleon: length and width; 5) antennal scale: length and width; and 6) cheliped: length of lateral margin, length of mesial margin, width of palm, depth of palm, and length of dactyl. Digital photographs were taken using a Nikon D70 with AF Micro Nikkor 60 mm lens.

RESULTS

Phylogenetic Evidence for Species Diagnosis

The phylogenetic relationships of the Cumberlandian cave *Orconectes* assemblage were determined using mitochondrial sequence data from the 16S, COI, and 12S genes (485 base pairs, 705 bp, 383 bp, respectively, totaling 1573 bp) (Figs. 2, 3, 4). *Orconectes barri* was found to be a monophyletic group with strong bootstrap support and posterior probabilities, indicating no gene flow between this clade and the sister clade of *Orconectes incomptus*. In all analyses, *Orconectes australis packardi* is the most basal member of the faunal group and distinctly separate from *O. a. australis*. The phylogenetic and phylogeographic results statistically sup-
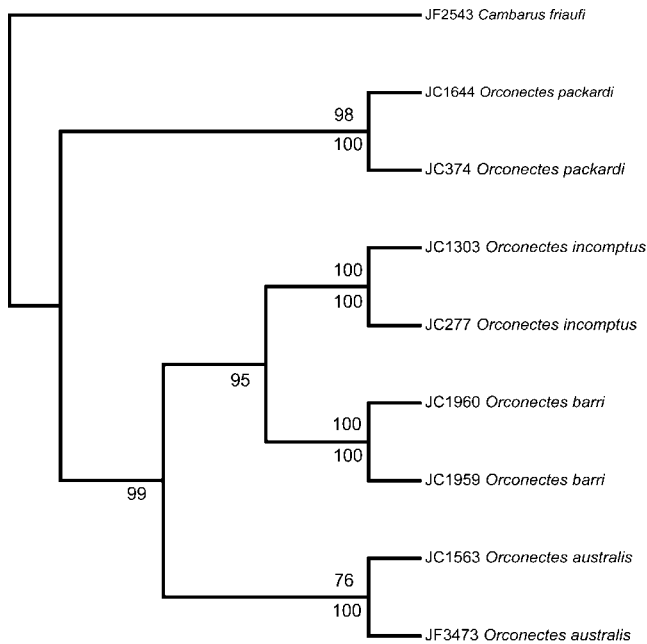
Fig. 3. Cladogram of the relationships between COI haplotypes for each of the species of Cumberlandian cave *Orconectes* (Maximum likelihood topology shown). Analyses done in PhyML are given above the nodes as bootstrap support (BS) percentages from 500 pseudoreplicates. Bayesian support values are given below the nodes as posterior probability (PP) percentages and are based on a consensus of 3000 trees (after 1000 were discarded as burnin). Support values are not shown for intra-specific groupings. Branch lengths not to scale.
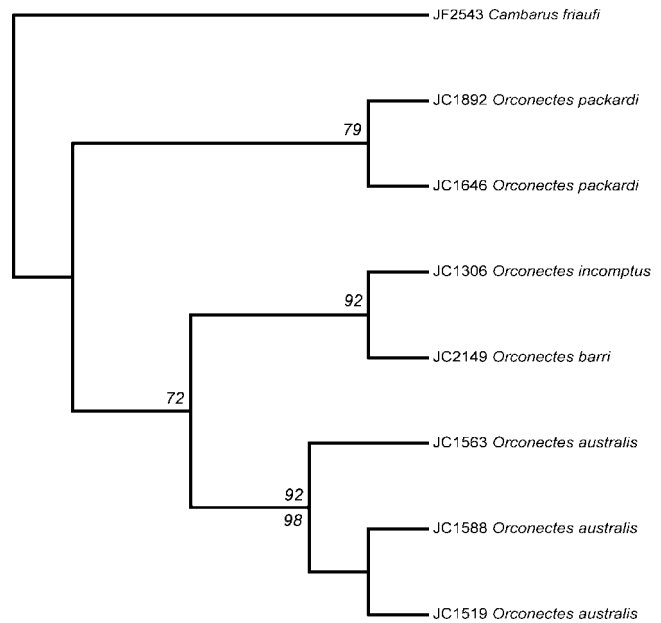

Fig. 4. Cladogram of the relationships between 12S haplotypes for each of the species of Cumberlandian cave *Orconectes* (Maximum likelihood topology shown). Analyses done in PhyML are given above the nodes as bootstrap support (BS) percentages from 500 pseudoreplicates. Bayesian support values are given below the nodes as posterior probability (PP) percentages and are based on a consensus of 3000 trees (after 1000 were discarded as burnin). Support values are not shown for intra-specific groupings. Branch lengths not to scale.

port the elevation of the subspecies *O. a. packardi* to species status, herein named *Orconectes packardi* (Rhoades, 1944), and the subspecies *O. a. australis* changed to recognize *O. australis* (Rhoades, 1941) as a distinct taxon. The 16S results (Buhay and Crandall, 2005) showed a phylogeny of (*O. a. packardi*+(*O. incomptus*+(*O. barri*+*O. a. australis*))) (Fig. 2). The COI phylogeny also showed significant support for four species, but the relationships were (*O. a. packardi* + ((*O. incomptus* + *O. barri*) + *O. a. australis*)) (Fig. 3). The 12S tree was less supported but mirrored the COI relationships (Fig. 4). Note that none of the phylogenetic analyses supported *O. a. packardi* and *O. a. australis* as sister taxa ($P < 0.001$; Shimodaira, 2002), therefore, they must be considered distinct species. This conclusion is further supported by the strong posterior probabilities and bootstrap values associated with each clade. Further genetic analyses is currently being conducted using nuclear markers to help resolve sister taxa relationships and gene flow routes (Buhay and Crandall, unpublished data). The taxonomic status of *O. sheltae* remains unknown until suitable genetic material can be isolated from preserved specimens or individuals are re-discovered in Shelta Cave. Resulting genetic sequences for this study were deposited into GenBank as accession numbers: EF207160-EF207168 for COI and EF207169-EF207176 for 12S.

SYSTEMATICS

Cambaridae Hobbs, 1942
Cambarinae Hobbs, 1942
Genus *Orconectes* Cope, 1872

Subgenus *Orconectes* Cope, 1872
Orconectes (Orconectes) barri n. sp.
Figs. 5, 6; Table 1

Diagnosis.—Albinistic; eyes reduced, without pigment, and recessed; rostrum with marginal spines or tubercles delimiting base of acumen, margins converging, and lacking median carina; postorbital ridges terminating cephalically in small spines; hepatic area with or without small tubercles and two or three minute cervical spines present; chelae inflated, with setae; mesial surface of palm of chelae with several irregular rows of tubercles; well-developed hooks on ischia of third pereiopods and small hooks on those of the fourth pereiopods. First pleopod of first-form male terminating in two elements (caudal process vestigial) (Fig. 5a, d), a short broad non-corneous mesial process and a corneous central projection. Annulus ventralis approximately two times broader than long.

Holotypic male, form I.—Body (Figures 5e, 6d) subovate, depressed. Pleon narrower than carapace width (6.11 mm and 6.56 mm, respectively). Areola broad (four to five times longer than wide) with approximately four punctations across the narrowest part. Length of areola approximately 40 percent of carapace length. Rostrum longer than broad, excavate, and with short acumen.

Carapace punctate dorsally and granulate laterally; hepatic area with few small tubercles. Pleon longer than carapace (17.78 mm and 15.48 mm, respectively). Cephalic section of telson with two strong spines in each caudolateral corner, mesial ones movable.
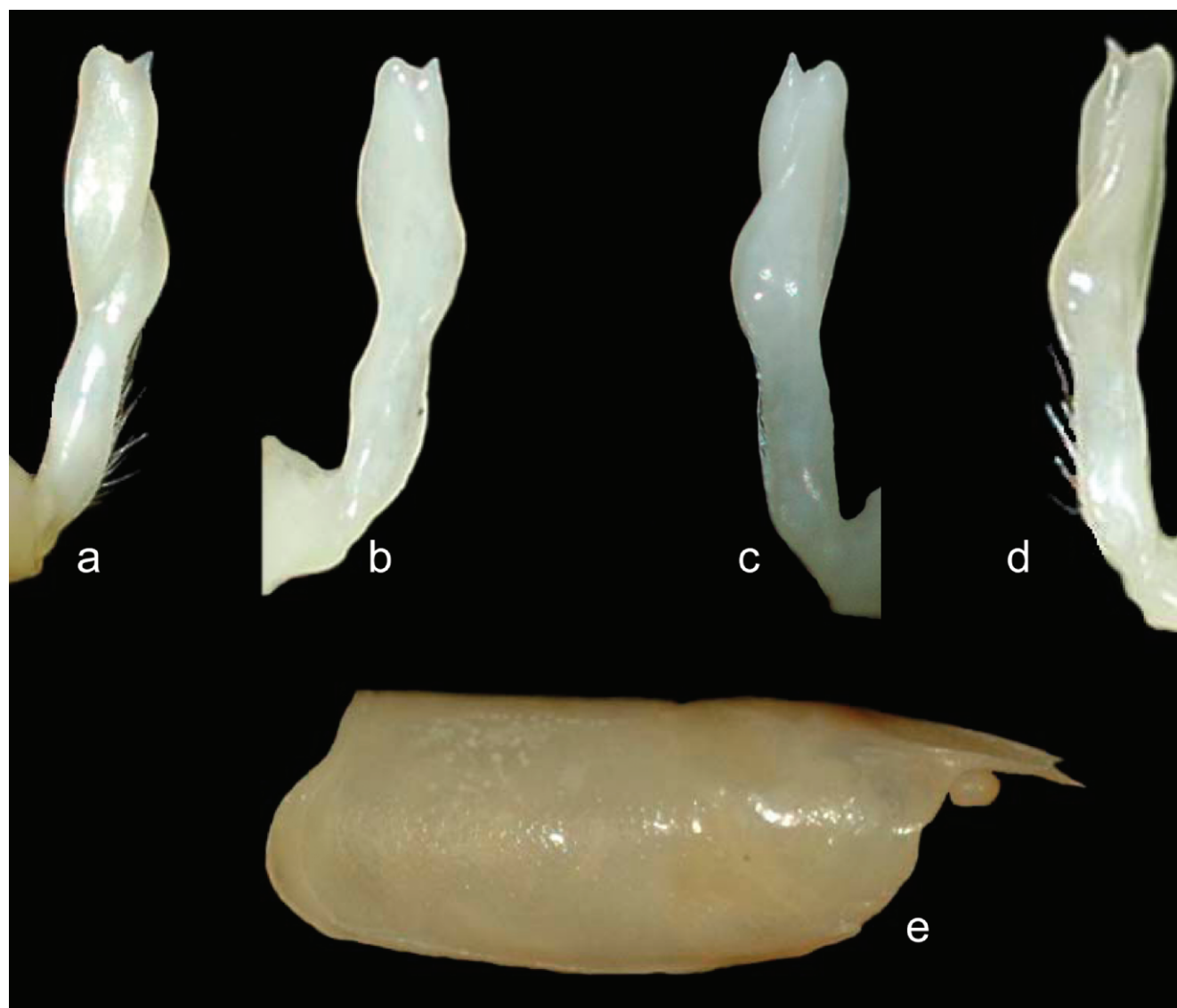
Fig. 5. *Orconectes* (*Orconectes*) *barri*, new species: a, mesial view of first pleopod of holotypic form I male; b, mesial view of first pleopod of morphotypic form II male; c, lateral view of first pleopod of morphotypic form II male; d, lateral view of first pleopod of holotypic form I male; e, lateral view of carapace of holotypic form I male.

Epistome broadly rounded cephalically. Eyes much reduced, hidden beneath rostrum in dorsal aspect. Antennules with prominent spine on ventral surface distal to midlength of basal podomere. Antennae extending caudally beyond telson. Antennal scale twice as long as wide; outer portion thickened and terminating in corneous spine.

Chelipeds somewhat inflated (Fig. 6a); mesial margin of palm about 1.6 times longer than palm width; dorsal surface of palm with tubercles mesially and both tubercles and punctations with fine setae; tubercles along mesial portion of palm, with about 13 in an irregular row. Fingers not gaping; carpus longer than broad; mesial surface of carpus with several prominent corneous spines; merus mostly tuberculate except ventrolaterally; tubercles along dorsal surface increase in size distally, but none corneous; ventrolateral and ventromesial margins with somewhat irregular row of 11 spike-like tubercles.

Ischia of third pereiopods with simple tapering hooks projecting proximally past distal margin of basis; ischia of fourth pereiopods with small, rudimentary hooks.

First pleopods symmetrical (Fig. 6c), barely reaching level of caudal margins of coxae of third pereiopods when pleon is flexed; shallowly situated in sternal groove; tip ending in two conspicuous parts as described in Diagnosis.

Allotypic female.—Differs from holotypic male in the following respects: minute hepatic spines, cervical tubercles smaller, setae on chelae longer, spines on merus longer and more prominent.

Annulus ventralis (Fig. 6b) subovate with high longitudinal median elevation devoid of shallow longitudinal trough; elevation highest along caudal half.

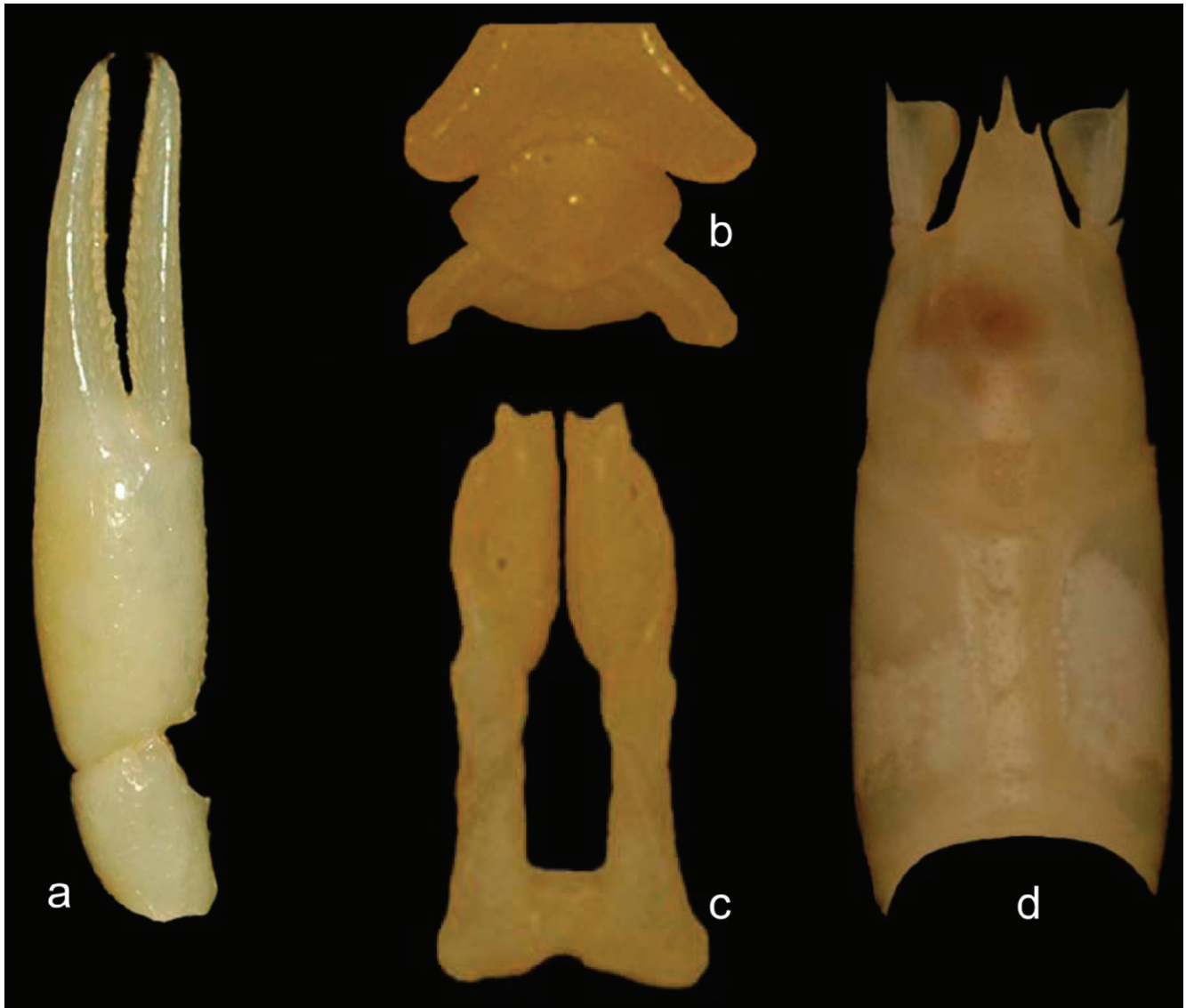Morphotypic male, form II.—Differs from holotypic male in the following respects: hepatic spines virtually absent,

Fig. 6. *Orconectes* (*Orconectes*) *barri*, new species: a, dorsal view of chela of holotypic form I male; b, annulus ventralis and portion of sternum of allotypic female; c, caudal view of first pleopods of holotypic form I male; d, dorsal view of carapace of holotypic form I male.

longer setae present on chela, tubercles on chela much reduced.

First pleopods (Fig. 5b, c) essentially similar to that of holotype but terminal elements more rounded and non-corneous.

Type locality.—Tonya's Cave, on Route 200, 2.5 km south of Powersburg, Wayne County, Kentucky. Situated just below the road, the cave entrance is an outflowing spring which drains into Carpenter Creek.

Common name.—Cumberland Plateau cave crayfish.

Disposition of Types.—The holotypic male form I (JC 2148), allotypic female (JC 2149), and morphotypic male form II (JC 2147) from Tonya's Cave, Wayne County, Kentucky, collected on 22 August 2004, are deposited in the United States Museum of Natural History (USNM), Smithsonian Institution, Washington, D.C, as USNM

1097953, USNM 1097954, and USNM 1097955, respectively. A female paratype (JC 2150; BYUC 04-60) is deposited at the Monte L. Bean Museum Crustacean Collection at Brigham Young University (BYUC), Provo, Utah. This female was also carrying sixteen eggs, each of which were removed and individually numbered (JC 2151-JC 2166).

Range.—*Orconectes barri* occurs in south central Wayne County, Kentucky near the town of Powersburg and in the watershed of Beaver Creek, which drains northward into Lake Cumberland, to northcentral Fentress County, Tennessee near the town of Pall Mall and in the watershed of Wolf River, which drains into Dale Hollow Lake of the Cumberland River basin.

The species identity has been confirmed with genetic analyses for nine caves in Wayne and Clinton Counties, Kentucky, and Pickett and Fentress Counties, Tennessee.

Table 1.   Measurements (mm) of *Orconectes barri*, new species.

Structure	Holotype JC2148 BYUC 04-62	Allotype JC2147 BYUC 04-63	Morphotype JC2149 BYUC 04-61
Carapace			
Height	5.27	5.36	4.73
Width	6.56	6.45	6.56
Total length	15.48	16.20	15.61
Postorbital length	12.97	13.36	12.92
Rostrum			
Width at base	2.10	2.03	2.01
Acumen length	1.16	1.22	1.06
Areola			
Length	6.22	5.96	5.78
Width	1.31	1.26	1.33
Abdomen			
Length	17.78	19.16	20.11
Width	6.11	5.64	6.04
Antennal scale			
Length	2.82	2.42	2.88
Width	1.47	1.46	1.52
Cheliped			
Length lateral margin chela	10.91	10.78	10.80
Length mesial margin palm	4.08	4.22	3.87
Width palm	2.48	2.44	2.39
Depth palm	1.71	1.85	1.80
Length dactyl	6.07	5.68	5.79

These cave sites of *Orconectes barri* include: KENTUCKY: Clinton County: 1) Buffalo Saltpeter Cave, tissue samples JC 1959-1961 collected on 20 August 2004; Wayne County: 2) Tonya's Cave (type locality), paratype female JC 2150 with eggs JC 2151-2166, morphotype male form II JC 2149, holotype male form I JC 2148, and female allotype JC 2147 (BYUC 04-60, 04-61, 04-62, 04-63, respectively) collected on 22 August 2004; 3) Redmond Creek Cave (formerly known as Kogers Cave), male form I JC 2113 and female JC 2114 (BYUC 04-68 and 04-69, respectively) collected on 19 August 2004, male form I (USNM 129284) collected by T. C. Barr and W. Andrews on 5 February 1967, male form I (USNM 145826) collected by W. Andrews and Terrence G. Marsh on 8 February 1969, male form II (USNM 145825) collected by W. Andrews and Terrence G. Marsh on 13 July 1968; 4) Grayson-Gunner Cave, tissue sample JC 1862 collected on 20 August 2004; 5) Stream Cave, tissue samples JC 1432-1435 collected on 10 February 2004; TENNESSEE: Pickett County: 6) Kathryn Cave, tissue samples JC 2031-2033 collected on 28 October 2004; 7) Clinton Cave, male form I JC 1639, female JC 1640, female JC 1641, male form II JC 1642 (BYUC 03-176) and tissue samples JC 1341-1342 collected on 20 September 2003; Fentress County: 8) Cornstarch Cave, male form II JC 1568 (BYUC 03-143) and tissue samples JC 342-349 collected on 7 August 2003; 9) Red Bud Cave, tissue sample JC 1516 collected on 2 September 2003.

Another seven caves were reported to have white crayfishes or have historical collections previously identified as either *Orconectes australis australis* or *O. a. packardi* which are herein renamed as *O. barri*. These caves with known populations of blind crayfishes that lie within the distribution of *O. barri* include: KENTUCKY: Wayne County (previously identified as *O. a. packardi*): 10) Sunnybrook Blowing Cave (also known as Blowing Cave), 1 male form I seen by Terrence G. Marsh and Andrew R. Boone on 21 August 1967, 2 females (USNM 145832) collected by R. A. Kuehne on 25 July 1965; 11) Horse Hollow Cave, 1 male form II and 7 females (USNM 129283) collected by Stewart B. Peck on 15 July 1964; 12) Johnson Fork Cave, 1 female (USNM 129285) collected by Thomas C. Barr on 10 July 1964; TENNESSEE: Fentress County (previously identified as *O. a. australis*): 13) Backstabber Pit, 3 individuals seen by Kristin Bobo in April 2004; 2) Sells Cave, 1 female (USNM 129255) collected by Thomas C. Barr and J. E. Crouch on 7 March 1959; 14) Wolf River Cave (also known as Blowing Cave), 1 female with eggs (USNM 129256), Thomas C. Barr and J. E. Crouch on 27 July 1954; 15) Shredded Goat Cave, 1 individual seen by John Swartz in April 2004; 16) York Cave, 1 individual seen by Joseph Douglas in January 2005. The species identity of these populations will be verified once samples and genetic barcodes can be obtained (see "DNA Barcoding" section, below).

Etymology.—Named in honor of Thomas C. Barr, Jr, author of "The Caves of Tennessee," Professor Emeritus, T. H. Morgan School of Biologic Sciences at the University of Kentucky. For five decades, Dr. Barr surveyed thousands of caves along the Cumberland Plateau due to his inordinate fondness for beetles, and even found time and patience to describe *Orconectes* (*Orconectes*) *incomptus* with Dr. Horton Hobbs, Jr. in 1972. The karst area around the Tennessee - Kentucky state line is a global hotspot of cave beetle biodiversity, and it is most appropriate to name this species after Dr. Barr, who climbed these hillsides inside and outside more than any other.

Crayfish associates.—*Cambarus* (*Erebicambarus*) *tenebrosus* (Hay, 1902) is also found in cave habitats containing *O. barri*.

<div align="center">DISCUSSION</div>

<div align="center">DNA Barcoding</div>

The National Center for Biotechnology Information (NCBI), which currently houses GenBank (an online repository for genetic data, http://www.ncbi.nlm.nih.gov/Genbank/), has recently incorporated a databank for genetic barcodes using the COI (cytochrome oxidase I) gene as the standard identification sequence for species (http://www.ncbi.nlm.nih.gov/BankIt/barcode/) that are then cross-referenced with the Barcoding of Life website (http://www.barcodinglife.com). This approach of DNA taxonomy is fast becoming an increasingly useful tool for species diagnosis, particularly in cases similar to this study, where cryptic species and their phylogenetic relationships are obscured by morphological convergence (Hebert et al., 2003a, b). Furthermore, scientists from across the globe are participating in this effort to document biodiversity in every form (Crandall and Buhay, 2004), including known described species as well as unknown or undescribed species by depositing sequences to assist the newly created Consortium for the Barcode of Life project (CBOL, http://barcoding.si.edu/). Genetic barcodes are not a means to replace traditional taxonomy,
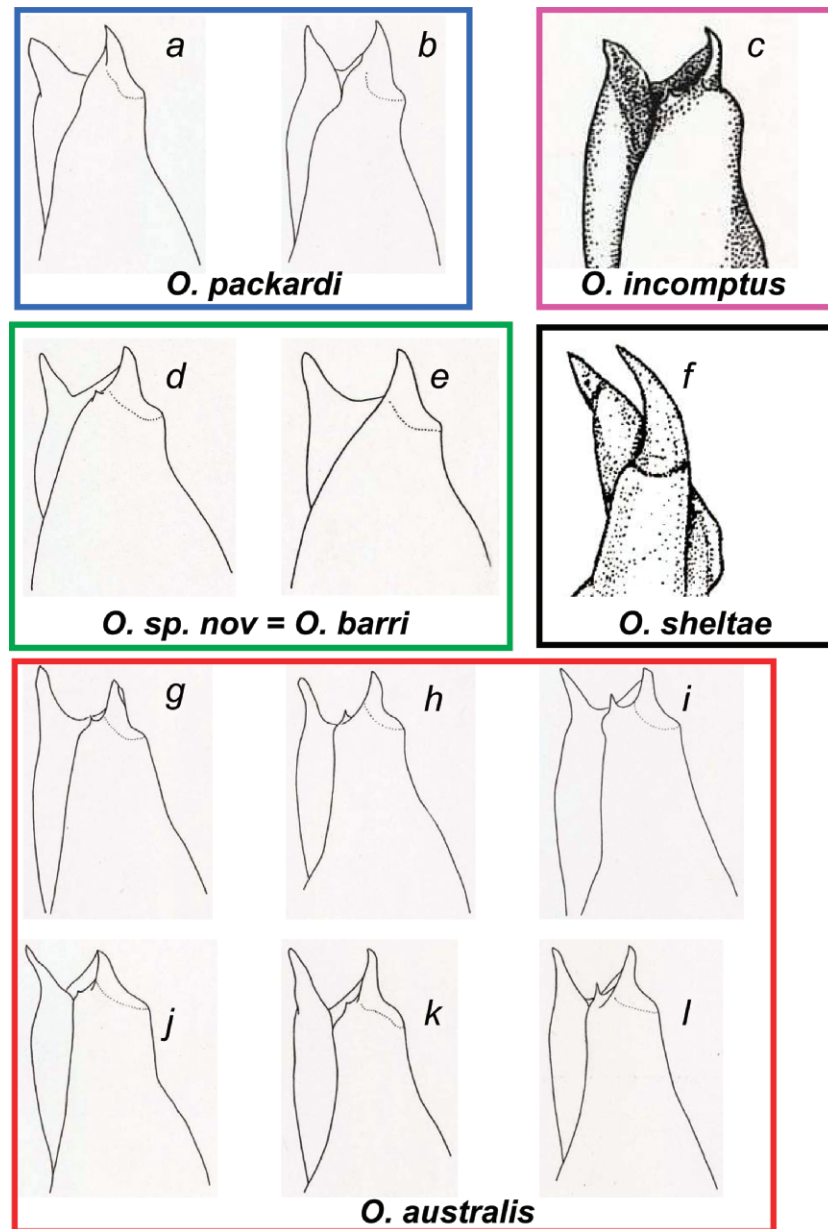
Fig. 7. Line drawings of form I male pleopods from each of the cave *Orconectes* species taken from Hobbs and Barr (1972) and Cooper and Cooper (1997) for morphological comparison. Pleopod drawings are representative of caves sampled for the Buhay and Crandall (2005) genetic study, including *Orconectes packardi*: a, Pine Hill Cave, Rockcastle Co., KY; b, Cumberland Crystal Cave (= Sloans Valley Cave, type locale), Pulaski Co., KY; *Orconectes incomptus*: c, Cherry Cave (type locale), Jackson Co., TN; *Orconectes barri*: d, Kogers Cave (= Redmond Creek Cave), Wayne Co., KY; e, Blowing Cave (same cove as Buffalo Saltpeter Cave), Wayne Co., KY; *Orconectes sheltae*: f, Shelta Cave (type locale), Madison Co., AL; *Orconectes australis*: g, Raven Bluff Cave, Overton Co., TN; h, Blind Fish Cave, Putnam Co., TN; i, Cumberland Caverns, Warren Co., TN; j, Big Mouth Cave, Grundy Co., TN; k, Limrock Blowing Cave, Jackson Co., AL, and l, Shelta Cave (type locale), Madison Co., AL. *O. sheltae* from Cooper and Cooper (1997) with permission of National Speleological Society.

but rather, are viewed as a new unique method to characterize and document life. We therefore have listed our COI sequences as barcodes (using keyword "BARCODE" in GenBank) for each of the four species, *Orconectes packardi*, *O. incomptus*, *O. barri*, and *O. australis* to aid in future diagnosis of samples from new cave localities.

Species Boundaries

In our case of cave crayfish, the use of a traditional morphological approach (Hobbs and Barr, 1972) for species delineation missed two cryptic species, and hence, identification "in the field" requires geographic location. The sole use of morphology of the pleopods for these species (Fig. 7) is inadequate for species diagnosis, both in the lab and in the field, particularly for *O. barri*, which was in the past considered "intergrades" of *O. packardi* and *O. australis* (Hobbs and Barr, 1972). Also, the traditional approach for diagnosing morphological differences using a "key" is therefore not appropriate for our cryptic cave crayfish species. Rather, genetic sequences (16S, COI, and/or 12S

data) and geography are the tools needed to determine the identity of newly discovered populations. Each of the cave *Orconectes* species has a clear and non-overlapping geographic boundary (Fig. 1) with exception of *O. sheltae* which co-occurs with both *O. australis* and *Cambarus jonesi* in Shelta Cave, Huntsville, Alabama (Cooper and Cooper, 1997).

Conservation Status

Using categories and criteria to evaluate species for endangerment (IUCN Red List version 3.1 criteria, www.redlist.org), we recommend conservation status for each species of cave *Orconectes* on the Cumberland Plateau based on the information currently available.

Orconectes sheltae should receive the highest protection, critically endangered (CR), as only 17 individuals were ever collected and another 97 individuals were examined and released during a study between 1968 and 1975 (Cooper, 1975; Cooper and Cooper, 1997) in Shelta Cave, Madison County, Alabama, which is the only known locale for the species despite search efforts by many biologists for the past three decades. *O. sheltae* 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 CR A2c, B1a, B1bi-v, B2a, C2i, D).

Orconectes incomptus is currently known from only twelve locations in three counties in Tennessee (Putnam County: Bartlett Cave; Smith County: Flat Rock Cave; and Jackson County: Cherry Cave (type locality), Carter Cave, Duds Cave, Elrod Cave, Flynn Creek Cave, Haile Cave, Hidden Cave, Jennings Rift Cave, Mahaney Cave, North Fork Cave). This species is found on both sides of the Cumberland River and was placed on the Red List in 1996 as vulnerable (VU) status. *O. incomptus* is state-listed as endangered (E) in Tennessee by the Department of Conservation (http://www.state.tn.us/environment/wpc/publications/E&Tlist.pdf). We agree that VU status should remain in effect, as the geographic extent of occurrence is less than 20,000 sq. km. with a severely fragmented range, area of occupancy less than 2000 sq. km, and inferred decline in the quality of the cave habitat (IUCN VU B1a, B1biii, B2biii).

Orconectes barri is currently known from sixteen cave localities (including seven caves to be verified using DNA barcodes) in four counties spanning Tennessee and Kentucky. Additionally, based on extremely low genetic diversity estimates (Buhay and Crandall, 2005), we suggest that this species be afforded vulnerable (VU) status. We feel that intensive field surveys may result in a few new localities of this species, but the area of occurrence is very rugged and remote, with some caves on inaccessible private property. This species meets criteria for vulnerability including geographic extent of occurrence less than 20,000 sq. km., fragmented range, inferred decline in the quality of cave habitat, area of occupancy less than 2000 sq. km, and a very small population size (IUCN VU B1a, B1biii, B2biii).

Orconectes packardi is currently known from two counties (Rockcastle and Pulaski Counties, Kentucky) and possibly in McCreary County, Kentucky (the historical sites were not accessible for our genetic study). This species is also found on both sides of the Cumberland River, and with intensive field surveys we are certain that many more cave localities will be found for this species. Major road construction is an imminent threat to many populations of this species, and although the criteria for vulnerable status are not met, we feel that its small, fragmented geographic range and moderate levels of genetic diversity (Buhay and Crandall, 2005) qualifies this species for "near threatened" (NT) status.

Orconectes australis is the most widespread of the Cumberlandian cave crayfish assemblage. This species is of "least concern" (LC) because of the large geographic range of extent and it is a common species through its distribution with high levels of genetic diversity (Buhay and Crandall, 2005).

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