A SYSTEMATIC EVALUATION OF ORCONECTES CF. BARRENENSIS FROM THE RED RIVER SYSTEM OF TENNESSEE AND KENTUCKY

Erin T. Bloom

A systematic evaluation of *Orconectes cf. barrenensis* from the Red River system of Tennessee and Kentucky

A Thesis

Presented to

The College of Graduate Studies

Austin Peay State University

In Partial Fulfillment

Of the Requirements for the Degree

Masters of Science

Erin T. Bloom

May, 2015

May, 2015

To the College of Graduate Studies:

We are submitting a thesis written by Erin T. Bloom entitled "A systematic evaluation of *Orconectes cf. barrenensis* from the Red River system of Tennessee and Kentucky." We have examined the final copy of this thesis for form and content. We recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science in Biology.

Research/Committee Advisor/Chair

Committee Member

Committee Member

Accepted for the Graduate and Research Council

Dean, College of Graduate Studies

Student of Permission to Use

In presenting this thesis in partial fulfillment of the requirements for the Masters of Science at Austin Peay State University, I agree that the library shall make it available to borrowers under the rules of the library. Brief quotations from this field study are allowable without special permission, provided that accurate acknowledgement of the source is made.

Permission for extensive quotation or reproduction of this field study may be granted by my major professor, or in her absence, by the Head of the Interlibrary Services when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Eric Bloom — 5/20/15

Signature — Date

For my grandfather, Tom Hudgins, who taught me at an early age that being stubborn isn't always a bad thing, and if people didn't like it, that they would just have to get over it. Thanks old man.

ACKNOWLEDGEMENTS

The completion of my thesis research and Master's degree would not have been possible without the help of many people. Dr. Rebecca Johansen, who served as my major advisor, provided essential guidance, resources, and support that lead to the completion of both. Her knowledge and passion for ichthyology and the field of biology is something that I admire and respect greatly. The contributions that she has made in completing this work were invaluable, and the guidance she has given me is so greatly appreciated; without it, this would not have been possible. I also thank her for always taking the time to listen and provide guidance for things that were not related to this project, but were just some of life's hardships.

I also thank my other two-committee members, Dr. Steve Hamilton and Dr. Dwayne Estes, for providing helpful criticisms and suggestions that facilitated the completion of this manuscript, and the time they took to read and provide valuable suggestions to improve the thesis.

Thank you to Dr. Mollie Cashner and her invaluable time and guidance in the lab. Without her guidance, I would still be sitting in front of the computer wondering why I was not seeing any bands on my gels. I appreciate the time and patience she gave to help in the completion of this work, and for the feedback and support she constantly contributed. She also taught me how to eat a "crawfish".

I also want to thank John Johansen for his guidance in identifying crayfish and for the hours of fieldwork that I suckered him into. John was invaluable in the completion of my fieldwork and maps included in this manuscript, and I am so appreciative of his time.

John was an appreciated and encouraging presence during this research, and I can't thank him enough for his time and support.

I would like to thank some of the other graduate students and labmates that helped in the completion of this project. Whether is was field work, lab work, or just lending an ear, Courtney Weyand, Brittany McCall, Kristen Anthony, Shawn Settle, and Zac Wolf served as essential supporters throughout this project. Without Brittany's help the morphological data collected for this project would not have been completed in such a timely manner. Courtney was my constant support system and was so encouraging. I am truly appreciative of all of the support and time she has given to this project and to me. I look forward to seeing Courtney and Brittany grow as professionals in their respective fields, and I hope I can lend my help and support as they have for me.

I also want to thank a variety of other individuals and institutions that aided in assistance with fieldwork, tissues, specimens, images, and locality information. Appreciation is extended to Bruce Bauer, David Withers, and Chris Taylor (and the Illinois Natural History Survey) for locality information and the loan of specimens, as well as Guenter Schuster and Carl Williams for images of the three focal taxa. I also want to extend an additional thank you to Guenter Schuster and Matt Thomas, for accompanying me into the field, even if I got a little lost. Thank you to Austin Peay's Center of Excellence for Field Biology and Austin Peay's Department of Biology, for funding and the use of vehicles.

Thank you to my parents, Shea and Eddie Chappell, for their endless support. They have always encouraged me to do things I am passionate about, to put my big-girl panties on when I feel like giving up, and to remind me that I tend to be my own worst

critic. Without their love and encouragement, this research and degree would not have been completed. Thank you to my brother, Garrett Chappell, for taking the brunt of their "love", when I couldn't come home as often as I would have liked.

And lastly, I would like to thank my boyfriend, Andy Dean. His support and patience with this degree, this project, and me are so appreciated. He has provided constant love and support (which included a lot of coffee and carbs), and he has been at my side through this entire process. From reading those early drafts of my thesis proposal, to supporting me when I left my teaching position, all the way to those last few days before my oral thesis defense, he has always been my biggest cheerleader, and a simple "thank you" will never be enough.

ABSTRACT

ERIN T. BLOOM A systematic evaluation of *Orconectes cf. barrenensis* from the Red River system of Tennessee and Kentucky (under the direction of REBECCA BLANTON JOHANSEN)

Orconectes barrenensis is endemic to the Green River system of Kentucky and Tennessee. The closely related species, *Orconectes mirus*, is restricted to Tennessee River tributaries in Tennessee and Alabama. Neither has been reported from the Red River (Cumberland River drainage) of Kentucky and Tennessee. However, a morphologically similar crayfish, referred to as *O. cf. barrenensis,* has been reported from this system. Whether the latter represents a disjunct population of *O. barrenensis* or *O. mirus,* or alternatively, represents a distinct undescribed species is not known. Furthermore, the hypothesis of a close phylogenetic relationship among these taxa inferred from their morphological similarities has not been tested.

The objectives of this work were to use molecular and morphological data to provide resolution for the phylogenetic relationships and taxonomic status of *O. cf. barrenensis*. Previously published primers were used to amplify and sequence two mitochondrial (COI and 16s) and two nuclear (28s and GAPDH) genes for the three focal taxa. Sequences of these genes for other *Orconectes* species were obtained from GenBank. Individual genes and a concatenated data set including all genes were used to generate hypotheses of phylogenetic relationships with Bayesian inference methods. To examine morphological variation between the three focal taxa, measurements and

meristics were taken for a standard suite of characters and were analyzed using univariate and multivariate tests.

Phylogenetic results from the most robust analysis that included all genes supported a close relationship among *O. cf. barrenensis*, *O. barrenensis,* and *O. mirus*, which were recovered as a well-supported clade. Within this clade, *O. cf. barrenensis* was monophyletic and divergent from *O. barrenensis* and *O. mirus*. *Orconectes barrenensis* was monophyletic, but with low support. *Orconectes mirus* was not monophyletic, but comprised of two geographically definable clades. Phenotypically, *O. cf. barrenensis* was distinguished from *O. mirus* and *O. barrenensis* based on several characteristics. The combination of genetic divergence and morphological distinctiveness observed support recognizing *O. cf. barrenensis* as a distinct species endemic to the Red River system. This species is known from only four localities, suggesting it has a small range and requires possible conservation efforts.

TABLE OF CONTENTS

CHAPTER

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

Introduction

Crayfishes (Decapoda: Crustacea) are small invertebrates found worldwide, in both aquatic and semi-aquatic habitats. Currently, there are 640 described species distributed among three families (Taylor and Schuster, 2004; Buhay and Crandall, 2005). Astacidae, Cambaridae, and Parastacidae are diagnosed by differences in gonopod morphology. In the United States the highest diversity of crayfishes is found in the Southeast, represented by members of the family Cambaridae, which has its greatest diversity in this region (Crandall and Buhay, 2008). The high crayfish diversity of the Southeast has been attributed to the high degree of habitat heterogeneity, associated with the many physiographic regions in the southeastern United States (Isphording and Fitzpatrick, 1992; Taylor et al., 1996). In the Southeast, Alabama has the highest species diversity with 85 currently recognized species followed by Tennessee and Kentucky with 76 and 52 species, respectively (Taylor and Schuster, 2004; Williams et al., 2004; Schuster et al., 2008). The bulk of this diversity is found in the two most common cambarid genera, *Cambarus* and *Orconectes*. Other genera found in the region include *Procambarus*, *Cambarellus*, *Fallicambarus*, and *Barbicambarus*.

Compared to other groups of freshwater organisms, such as fishes and amphibians, crayfishes have been relatively understudied. For many currently described species, distributional information, life history, or other basic biological data are lacking. Additionally, current estimates of species-level diversity likely represent a gross underestimate of actual crayfish diversity (Sinclair et al., 2004). For example, Crandall and Buhay (2008) estimated around 5-10 new crayfish species are described each year

with others still awaiting description. Sinclair et al. (2004) estimated almost 40 new species of crayfish have been described in the US alone and over 80 have been described worldwide since 1989.

Crayfish species have been described primarily through use of morphological characteristics to diagnose species (Fitzpatrick, 1967; Taylor, 1997; Taylor, 2000; Sinclair et al., 2004; Taylor and Knouft, 2006; Brienholt et al., 2012). These same characters also are used to reflect phylogenetic relationships of crayfishes. For example, reproductive structures of Form I males are used to distinguish species, but also to place species in subgenera and genera. For example, Form I males of the genus *Orconectes* are distinguished by gonopods with two long thin terminal processes, that usually terminate in a straight or gently curved form (Bouchard, 1972; Taylor and Schuster, 2004). The gonopods of *Cambarus* species have two terminal processes, but are usually thicker, rounded, and somewhat sickle shaped. *Procambarus* gonopods have more than two terminal elements, which are usually very short (Taylor and Schuster, 2004).

Morphology primarily has formed the basis for the current understanding of relationships and diversity of crayfishes in general, and specifically for the genus *Orconectes*. The current classification of species in subgenera of *Orconectes* is based almost exclusively on the gonopods of Form I males (Fitzpatrick, 1987; Bouchard and Bouchard, 1995; Taylor and Knouft, 2006). Although, morphological characters have formed the basis for crayfish taxonomy and classification, reliance on these characters alone may result in erroneous estimates of relationships or diversity as suggested by more recent studies that have utilized other data sources such as DNA. For example, some species show a high degree of phenotypic conservatism in certain morphological

characters, while others show high intraspecific diversity in those same phenotypic traits (Harris and Crandall, 2000; Sinclair et al., 2004). Others, like cave crayfishes, demonstrate convergence due to similar environments (Sinclair et al., 2004). Members of the genus *Orconectes* are good examples of these challenges, as they are occasionally considered the most difficult crayfish to identify with the use of traditional morphological characteristics. Members of *Orconectes* display homogeneity of morphology, particularly the gross morphology of the gonopods of the Form I males; early descriptions and diagnoses of these species are based on semi-quantitative comparisons of the gonopods (Fitzpatrick, 1967; Taylor and Knouft, 2006).

The use of genetic data to estimate crayfish relationships and diversity offers a viable alternative approach to test existing hypotheses stemming from morphological data alone and does so within a statistical framework (Sinclair et al., 2004). Genetic data is commonly used to estimate and redefine phylogenies and classification schemes (Thoma et al., 2014). However, as reliance on gene-based phylogenetic systematics becomes more widespread in crayfish studies, the accuracy of these phylogenetic hypotheses becomes more important (Crandall and Fitzpatrick, 1996). To increase the likelihood that estimated phylogenies are robust and represent the most accurate evolutionary history of a group, multiple independent data types should be evaluated for congruence (Galtier et al., 2009; Toews and Brelsford, 2012).

Orconectes barrenensis (Barren River Crayfish) is a species of crayfish that is known from Kentucky and Tennessee, where it is endemic to the Green River drainage, including the Barren, Nolin, and Rough River systems. It is typically found under cobble or gravel substrates in riffles of clear, small to large streams (Taylor and Schuster, 2004).

Prior to description as *O. barrenensis* by Rhoades (1944), this species was considered a subspecies of *O. rusticus*, but was distinguishable from the latter by having minute or absent lateral spines, and having broader chelae. In this description, *O. barrenensis* was also noted as having a shorter areola and more widely gaping fingers than *O. placidus*, but less gaping than *O. forceps*. Rhoades (1944) also noted variation in these chela characters between populations of *O. barrenensis* found in the Barren River and the Green River (Rhoades, 1944).

Orconectes mirus, the Wonderful Crayfish (Ortmann, 1931), is known from Alabama and Tennessee where it is endemic to the Tennessee River drainage, including the Elk River, Hurricane Creek, Crow Creek and Paint Rock River systems. It is known also for its light brown color, with light to dark brown patches (Schuster et al., 2008). *Orconectes mirus* differs from *O. barrenensis* by having a longer and wider areola, and having narrower chelae. Prior to description by Ortmann (1931), *O. mirus* was considered a local race (within the Tennessee River system) of *O. rusticus*, but was distinguishable from the latter by having a broader areola than *O. rusticus*; *O. mirus* also lacked tubercles on the carapace and chela, unlike *O. rusticus* (Ortmann, 1931).

Both species have typical *Orconectes* gonopods comprised of two long, thin elements, with the central projection overhanging the mesial process (Taylor and Schuster, 2004). However, gonopods of *O. mirus* Form I males have a shoulder at the base of the mesial projection, which is absent on gonopods of *O. barrenensis* males (Figure 1; Rhoades 1944).

Neither of these species is known to occur in the Red River system or elsewhere in the Cumberland River Drainage. However, a 2008 survey of the aquatic fauna of Fort

Campbell Military Base in Tennessee identified a crayfish, from several tributaries to the Red River system, that was morphologically similar to, but also distinct from, *O. barrenensis* (BHE, 2008). Later survey efforts by others for this crayfish, found specimens in another Red River tributary (West Fork Red River) located in Clarksville, TN and not associated with Fort Campbell (pers. comm. B. Bauer, 2014).

Based on the morphological features shared with *O. barrenensis*, the crayfish found in the Red River system was referred to as *O. cf. barrenensis*. Subsequently, others noted that this crayfish was morphologically similar to *O. mirus* and may be more closely related to that species (pers. comm., G. Schuster, 2013). Whether the crayfish referred to as *O. cf. barrenensis* is a disjunct population of one of these two currently recognized species (*O. barrenensis* or *O. mirus*) or possibly represents a distinct crayfish species, has not been tested (Schuster 2008). Furthermore, whether the phenotypic similarities among these three species reflect recent common ancestry or convergence in morphological traits is not known.

Objectives:

The main goals of this study were to provide the first assessment of the taxonomic status and phylogenetic relationships of *Orconectes cf. barrenensis* from the Red River system. These objectives were accomplished using an integrative approach that utilized both molecular data and an assessment of morphological diagnosability. Another goal was to determine the range of *O. cf. barrenensis*. Through the course of surveying other possible populations of *O. cf. barrenensis*, we identified two localities, Sycamore Creek and West Fork Obey River (Cumberland River drainage), that contained individuals that were morphologically aligned with our original Red River populations of *O. cf.*

barrenensis, and thus their taxonomic status and phylogenetic relationships to our three focal taxa were evaluated also. The specific hypotheses tested were: (1) *Orconectes cf. barrenensis* from the Red River represents a diagnosable lineage warranting species-level recognition; (2) *Orconectes cf. barrenensis* is most closely related to *Orconectes barrenensis* from the Green River or to *Orconectes mirus* from the Tennessee River as implied by morphology; and (3) the newly identified and morphologically similar crayfish populations from other Cumberland River tributaries represent other populations of *O. cf. barrenensis* known previously from only the Red River system (Cumberland River drainage).

CHAPTER II

Materials and Methods

Localities and Collection Methods:

Samples of the focal taxa, *O. cf. barrenensis*, *O. barrenensis* and *O. mirus* were collected using standard seining and dip netting methods (Bouchard, 1972; Taylor and Schuster, 2004) or borrowed from institutions (see Appendix I and II for localities and accession information). For individuals collected personally, specimens were placed in 70% ethanol for later study in the lab, or 95% ethanol for DNA preservation.

Specimens of *O. cf. barrenensis* were collected from all previously reported localities within the Red River, including sites found on Fort Campbell Military Base. Specimens of *O. barrenensis* were examined from localities representing each of the major systems of the Green River drainage from which this species is known, including the Nolin, Rough, and Barren River systems. *Orconectes mirus* specimens and tissues were examined from multiple localities across the range of the species in the Tennessee River drainage. Specific locality information for all specimens used in the molecular data analysis is provided in Appendix I and shown in Figure 2. Locality information for specimens used in the morphological data analysis is provided in Appendix II and shown in Figure 3.

Further sampling attempts were made in the Red River at non-historical localities for *O. cf. barrenensis*. However, no individuals of *O. cf. barrenensis* were collected. Those attempts were made at the three following localities: Red River on TN Hwy 236 (Tiny Town Road) at the Blueway in Montgomery Co., TN; Spring Creek on KY Hwy

294/Graysville Road, close to KY/TN state line in Todd Co., KY; and Bluehole Creek at the intersection of Stoke Rd. and Maton Rd. in Todd Co., KY.

Molecular Data Collection:

DNA Extraction - DNA was extracted from abdominal tissue using a Qiagen DNEasy Blood and Tissue kit and following the manufacturer's instructions except that a smaller volume of elution buffer (80 µl) was used to obtain a higher concentration of DNA and only one elution step was performed.

PCR and Sanger Sequencing - Four genes were targeted, amplified and sequenced, including the mitochondrial markers cytochrome oxidase subunit I (COI) and 16s (intron), and two nuclear markers 28s (intron) and GAPDH (exon). The same individuals were used to generate sequences for each gene. Mitochondrial genes, particularly COI are commonly used to assess divergence among closely related species (Crandall and Fitzpatrick, 1996; Hebert et al., 2003; Song et al., 2008). To assess relationships among deeper nodes, 28s, which is one of the most variable nuclear genes among Crustacea species (Brienholt et al, 2012) and GAPDH were also examined. The 16s intron is also commonly used in phylogenetic analyses, specifically in examining relationships among Crustacea (Schuburt et al., 2000).

Polymerase Chain Reaction (PCR) was performed using a 25 µl total volume solution including 2 µl of template DNA, 0.5 µl dNTPs, 2.5 µl 10X buffer, 0.75 µl $MgCl_2$, 0.25 µl Taq polymerase, 1 µl of each primer, and 17 µl of PCR water to amplify target loci.

Previously published primers, LCO1490 (5'-ggtcaacaaatcataaagatattgg-3') and HC02198 (5'-taaacttcagggtgaccaaaaaatca-3'), were used to amplify the COI gene (~700

bp) (Folmer et al., 1994). Thermocycler conditions included 35 cycles of the following: 30 s at 94°C, 30 s at 53°C and 90 s at 72°C, which occurs after an initial denaturation step of 3 minutes at 94°C. These steps were then followed by a final extension of 5 minutes at 72°C (Folmer et al. 1994; Taylor and Hardman, 2002).

The large non-coding subunit ribosomal RNA gene, 16s (~500 bp), was amplified with 16S-L2 (5'-tgcctgtttatcaaaaacat'3') and 16S-1472 (5'-agatagaaaccaacctgg-3') (Crandall and Fitzpatrick, 1996; Schubart et al., 2000; Mathews et al., 2008). Thermocycler conditions included 35 cycles of the following: 60 s at 94°C, 60 s at 48°C and 60 s at 72°C, which occurred after an initial denaturation step of 2 minutes at 94°C. These steps were then followed by a final extension of 10 minutes at 72°C (Crandall and Fitzpatrick, 1996; Mathews et al., 2008).

The nuclear 28s (~900 bp) gene, was amplified using primers rd3a (5'-agtacgtgaaaccgttcagg-3') and rD5b (5'-ccacagcgccagttctgcttac-3') (Breinholt et al., 2012). Thermocycler conditions included 40 cycles of the following: 60 s at 94°C, 60 s at 46°C and 60 s at 72°C, which occurred after an initial denaturation step of 3 minutes at 94°C. These steps were then followed by a final extension of 10 minutes at 72°C (Whiting et al., 1997).

GAPDH (~700 bp), a coding nuclear gene, was amplified using primers G3PCq157F (5'-tgacccttcattgctcttgacta-3') and G3PCq981R (5'-attacacgggtagaatagccaaactc-3') (Buhay et al. 2007; Mathews et al., 2008). The PCR conditions included 40 cycles of the following: 30 s at 95°C, 30 s at 60°C and 60 s at 72°C, which occurred after an initial denaturation step of 2 minutes at 95°C. These steps were then followed by a final extension of 10 minutes at 72°C (Mathews et al., 2008).

Products from all PCR reactions were visualized on an ethiduim bromide stained 0.8% agarose gel, run at 85 volts. Each well of the gel contained 5.0 µl of the PCR product and 1.5 µl of loading dye. A DNA ladder was also loaded for comparison of band fragment length and intensity. A negative control was also included.

Amplified products were sent to the University of Florida's Sanger Sequencing facility at the Interdisciplinary Center for Biotechnology Research. The same primers used in PCR were used to sequence each gene examined.

Molecular Data Analyses:

The resulting sequences were edited and aligned using CodonCode Aligner 3.6.1. The resulting alignments were used in multiple analyses to assess genetic diversity and phylogenetic relationships among populations and taxa.

Pseudogenes and Paternal Leakage - When examining genes from the mitochondrial genome of invertebrates, especially crayfishes, pseudogenes and paternal leakage are potential issues that must be considered and evaluated prior to further analyses using these loci (Song et al., 2008; Buhay, 2009). Pseudogenes occur when parts of the mitochondrial genome embed within the nuclear genome, which can then be passed on to future generations. Because the nuclear copies stem from the genes targeted in the mitochondrial genome, primers designed to amplify these mitochondrial genes may also amplify the nuclear copy. Because selective pressures applied to the true coding mitochondrial genome would hold no constraint on the nuclear genome, mutations typically arise in the nuclear copies that can eventually lead to discrepancies between individuals from the same population and to possible erroneous estimates of phylogeny among taxa (Song et al., 2008; Buhay, 2009). Paternal leakage occurs when both the

female and male contribute mitochondrial DNA to offspring. Typically, only the female contributes to the mitochondrial genome of offspring, because sperm mitochondria disappear during embryogenesis and typically do not contribute to the mitochondrial genome of the embryo (Budowle et al., 2003; Piganeau et al., 2004; Fontaine et al., 2007). When leakage occurs (i.e. there is a paternal contribution), recombination may occur, leading to production of different alignment regions among members of a species or population that appear to have confounding evolutionary histories (Posada and Crandall, 1998). Traditional phylogenetic analyses used are under the assumption that no recombination is occurring within these mitochondrial gene regions (Posada and Crandall, 1998; Piganeau et al., 2004).

Both pseudogenes and paternal leakage can cause erroneous results in phylogenetic estimations (Fontaine et al., 2007; Song et al., 2008; Buhay, 2009). With this in mind, methods presented in Song et al. (2008) were followed to evaluate the presence of such confounding data in the mitochondrial datasets generated herein (Thoma et al., 2014). Methods that identify potential problematic sequences, including assessing divergence levels among individuals and populations, conducting BLAST searches of individual sequences, and translating sequences to identify indels and stop codons inserted in mitochondrial gene reading frames were used (Song et al. 2008). Any sequences that showed signs of nuclear influences such as misplaced stop codons, unexpectedly high levels of sequence divergence, particularly among individuals of a population, or which were not recovered as the appropriate gene and taxon group in BLAST searches were discarded from any subsequent analyses.

Estimating Sequence Divergence - Average sequence divergence was estimated within

and between species, and within and between clades for each gene using DnaSP (Hebert et al., 2003; Librado and Rozas, 2009). Average sequence divergence among groups, particularly for COI, has been used as a criterion to identify species-level differences between crayfishes and coincides with the classification of currently recognized species (Grandjean et al., 2002). However, these data alone should not be used for taxonomic decisions.

Phylogenetic Analyses - Phylogenetic relationships were generated using Bayesian inference methods for the focal taxa, *O. cf. barrenensis*, *O. mirus,* and *O. barrenensis* (Ronquist and Huelsenbeck, 2003). Although, *O. cf. barrenensis* is hypothesized, based on morphology, to be a close relative to *O. barrenensis* from the Green River system or *O. mirus* from the Tennessee River system, it was possible that the shared characters reflect convergence and not a recent common evolutionary history. Thus, other representative *Orconectes* species were included in the phylogenetic comparisons. Inclusion of other species, and representatives of the genus *Cambarus* used as the outgroup, ensured that the true sister species to *O. cf. barrenensis* was identified and not overlooked due to exclusion from the analysis. Overall, increased taxonomic sampling is also known to provide more accurate estimations of relationships (Crandall and Fitzpatrick, 1996; Guindon and Gascuel, 2003; Ronquist and Huelsenbeck, 2003). Sequences for other *Orconectes* species were downloaded from GenBank. Table 1 provides a summary of all species examined for each gene data set that was obtained from GenBank.

All gene alignments were converted to NEXUS files to use in the appropriate programs. For the 28s and 16s alignments, gaps were treated as missing characters (Crandall and Fitzpatrick, 1996). There were no gaps in the COI or GAPDH alignment,

since they are coding regions (Mathews et al., 2008). COI was the only alignment where the first, second, and third codon position were identified (Maddison et al., 1997).

Bayesian analyses (BA) were conducted to generate phylogenetic hypotheses for each dataset independently and for a combined data set that included COI, 28s, 16s and GAPDH. In the independent analysis, the COI gene was partitioned by codon. The non-coding genes, 16s and 28s were analyzed as a single data partition. The coding gene GAPDH was also analyzed as a single data partition, due to the lack of variation between individuals within the dataset. In the combined analysis, partitions were set for the three-codon positions of the COI gene, as well as each individual marker, resulting in five partitions.

PAUP* 4.0, in conjunction with ModelTest, was used to evaluate 56 progressively complex models of sequence evolution (Swofford, 1993; Posada and Crandall, 1998). The best-fit model for each gene or data partition (summarized in Table 2) was selected using the Akaike information criterion (Posada and Crandall, 1998).

The selected models (Table 2) were implemented as parameters in phylogenetic estimation using the program MrBayes 3.1 (Posada and Crandall, 1998; Ronquist and Huelsenbeck, 2003). Two independent runs, consisting of 12 million generations were conducted for each dataset. Burn-in was determined by graphically examining the Maximum Likelihood scores at the sampled generations to find where the values converged. All trees recorded prior to burn-in were then discarded. The posterior probability at which a clade occurred in the remaining trees was used as an indication of node support. Inference about the history of the group in question was based on the posterior probability; those with the highest posterior probability were considered the

most likely estimates of relationships among taxa (Huelsenbeck et al., 2001).

Tests of Alternative Hypotheses - With the use of Mesquite 3.02 (Maddison and Maddison, 2015) and constraint tree searches, viable alternative hypotheses of relationships among the focal taxa that were not recovered in all estimated phylogenies were examined for support. Previously proposed hypotheses of monophyly and relationships among the focal taxa were examined with each data set and can be found in Table 3 (Crandall and Fitzpatrick, 1996; Taylor and Knouft, 2006; Buhay, 2009). Support for alternative hypotheses were assessed using a Bayesian-based approach. Constraint trees that represented alternative hypotheses were generated using Mesquite 3.02. The posterior distributions resulting from the Bayesian analyses of each locus data set were used to test each alternative hypothesis. Only the post-burnin trees of each Bayesian Analysis were examined. The program PAUP* 4.0 was used to filter the posterior distributions of the post-burnin trees to only keep those that met the criteria of the loaded constraint tree. The number of trees found supporting that constraint divided by the total number of trees examined was equivalent to the posterior probability of support for the constrained set of relationships (Farrell and Sequeira, 2004; Weisrock et al., 2006). If those alternative hypotheses were recovered in more than 5% of the post-burnin Bayesian trees from the posterior distributions, then the hypothesis could not be statistically rejected as an alternative hypothesis by the data (Weisrock et al., 2006).

Morphological Data Collection:

Morphological characters were examined from all major river systems from 20-30 Form I males and from 20-30 females and Form II males for each focal taxon (see Figure 3). Standard measurements (Table 4) were made following Rhoades (1944), Tierney

(1982), Taylor (1997, 2000), Cooper (2000), Schuster (2008), and Thoma (2014) and were taken with digital calipers to the nearest 0.1 mm (Table 4). Measurements were standardized by total length to account for variation in the size of individuals measured. An assessment of the presence/absence of a shoulder on Form I male gonopods and the presence/absence of cervical spines was also conducted (Rhoades, 1944; Ortmann, 1931).

Morphological Data Analyses:

The mean, range, and standard deviation for each size-standardized variable were calculated using JMP 10 (Tierney, 1982) and generated for each purported taxon, river system, drainage and clade.

Since most currently recognized species are distinguished by a combination of phenotypic variables, a multivariate Principle Components Analysis (PCA) also was conducted using SYSTAT 8 (Austin and Knott, 1996; Taylor, 1997; Taylor, 2000). A PCA of standardized measurements was used to examine variation in characteristics among purported taxa, river systems, river drainages, and clades (identified using mitochondrial and nuclear data herein). Principal components with eigenvalues > 1.0 and variables with component loadings > 0.5 (absolute value) were considered to have contributed most to any separation among groups, visualized using scatterplots of resulting factor scores (Table 5). These variables were also considered as potentially diagnostic or to be taxonomically informative (Austin and Knott, 1996; Taylor, 1997; Taylor, 2000).

CHAPTER III

Results

Sequence Divergence:

Overall sequence divergence levels within and between species for all datasets are found in Table 6. Average COI sequence divergence within *O. cf. barrenensis* was less than 1%, and average sequence divergence between *O. cf. barrenensis* and *O. barrenensis* and between *O. cf. barrenensis* and *O. mirus* was approximately 6%. This level is comparable to, or greater than, that observed between currently recognized closely related species. For example, the average sequence divergence was 6% between *O. barrenensis* and *O. mirus*, just over 2% between *C. distans* and *C. obeyensis*, and just under 5% between *O. rusticus* and *O. putnami*.

Overall sequence divergence of 16s within *O. cf. barrenensis* was less than 1%. Between *O. cf. barrenensis* and *O. barrenensis* there was around 3% sequence divergence, with similar levels seen between *O. cf. barrenensis* and *O. mirus*. Again, these levels are comparable to other recognized species, such as that found between *O. barrenensis* and *O. mirus*, which also was around 3%.

Sequence divergence levels found using our 28s and GAPDH datasets were comparable within and between species. In all cases divergence levels within and among focal species were less than 1%.

Following recommendations of Song et al. (2008) to assess potential nuclear influences in the mitochondrial data collected, issues were identified with the COI genes of one population of *O. barrenensis*, from the Nolin River. Individuals of this population had more than 12% sequence divergence. Levels of sequence divergence this high are

more typical of levels observed among genera of crayfish, and not expected among individuals of the same species from the same location (Sinclair et al., 2013). This finding was a strong indication that either pseudogenes were amplified, or that paternal leakage may have occurred in some individuals of the population. Although *O. barrenensis* COI sequences were included in the BLAST searches of these two individuals, one was recovered most similar to *O. compressus* and the other most similar to *O. forceps*; neither was similar to *O. barrenensis*. This was also a strong indication that there were problems with these sequences. Therefore these sequences were removed from subsequent analysis (Song et al., 2008). No other populations or individuals had characteristics of nuclear influenced mitochondrial genomes. Furthermore, no problems were noted within the 16s dataset.

Phylogenetic Analyses:

COI Gene Tree - The Bayesian Analysis (BA) of the COI dataset recovered a well-supported clade, which included all individuals of *O. barrenensis*, *O. mirus* and *O. cf. barrenensis* from the Red River system (Figure 4; Clade A). Within this clade *O. cf. barrenensis* was recovered as a well-supported clade with subclades (Figure 4; Clade B) corresponding to the two tributary systems of the Red River where the species has been found. Despite their morphological similarity to *O. cf. barrenensis* from the Red River system, the newly identified populations from other Cumberland River systems (Sycamore Creek and West Fork Obey River), were not recovered in this focal taxon clade, but were more closely related to *O. forceps* (Figure 4; Clade D).

The close relationship among *O. barrenensis*, *O. mirus*, and Red River *O. cf. barrenensis* inferred from their overall morphological similarities was well supported.

However, relationships among these taxa were less resolved. For example, *O. cf. barrenensis* from the Red River was recovered as sister to a clade containing *O. barrenensis* and a subset of *O. mirus,* but with less than 0.85 posterior probability support. Furthermore, neither of these latter species was monophyletic. The two clades recovered for *O. mirus* corresponded to different rivers systems of the Tennessee River, but those from the Flint and Crow Rivers were more closely related to a clade of *O. barrenensis* from the Rough and Barren Rivers (Figure 4; Clade C) than to *O. mirus* from the Elk River.

Individuals of *O. barrenensis* from the Nolin River were omitted from the COI dataset, due to those sequences potentially containing pseudogenes, based on our initial sequence divergence estimates and BLAST searches (refer to earlier in the results). A BA of the COI dataset was run with Nolin River individuals at one point, and these problematic individuals were recovered as highly divergent from other individuals of *O. barrenensis*, with one individual recovered in a clade with *O. forceps*, and the other recovered in a clade with *O. compressus* (which they were also recovered as in their BLAST searches).

16S Gene Tree - The BA of the 16s dataset recovered a well-supported clade for all *O. barrenensis*, *O. mirus,* and *O. cf. barrenensis* from the Red River (Figure 5; Clade A). As in the COI gene tree, the other *O. cf. barrenensis* from the Cumberland River populations (represented by those from the West Fork Obey River in this dataset) were not closely related to *O. cf. barrenensis* from the Red River, but recovered as sister to *O. forceps* (Figure 5; Clade B). *Orconectes cf. barrenensis* from the Red River was recovered as a clade (Figure 5; Clade C) sister to *O. mirus* from the Flint River and Crow Creek. This

clade was sister to a clade containing *O. barrenensis* and Elk River *O. mirus*, but as in the COI gene tree, neither *O. mirus* nor *O. barrenensis* was monophyletic. Relationships recovered among these taxa overall had low support. Individuals of *O. barrenensis* from the Nolin River were included in this dataset, as there were no indications of these sequences containing pseudogenes that were detected in the COI sequences.

28s Gene Tree - The BA of the nuclear 28s gene recovered *O. barrenensis*, *O. mirus*, and *O. cf. barrenensis* from the Red River in a well-supported clade, but relationships among these taxa were unresolved (Figure 6; Clade A). The Cumberland River individuals were not included in this analysis; attempts to amplify those individuals for this locus were unsuccessful.

GAPDH Gene Tree - The BA of the GAPDH dataset supported the monophyly of the three focal taxa. *O. mirus*, *O. barrenensis*, and *O. cf. barrenensis* from the Red River system, however relationships among these taxa were unresolved (Figure 7; Clade A), except that one individual of *O. mirus* and one of *O. cf. barrenensis* (Red River system) were recovered in a clade (Figure 7; Clade B). Populations from the West Fork Obey River and Sycamore Creek (Cumberland River system) also labeled as *O. cf. barrenensis* based on morphology, were not recovered as part of this clade but recovered in a separate divergent clade (Figure 7; Clade C). Due to limited taxon sampling, whether GAPDH supports a close relationship of Clade C crayfishes to *O. forceps* as observed with mitochondrial genes is not known.

Combined Gene Tree - The BA of the combined data set that included all genes, recovered a well-supported monophyletic clade for *O. barrenensis*, *O. cf. barrenensis* from the Red River system, and *O. mirus* (Figure 8; Clade A), supporting a close

relationship among these taxa as implied by their overall morphological similarity. However, relationships among these taxa were unresolved. Within the focal group clade, *O. cf. barrenensis* from the Red River system was recovered as a well-supported clade (Figure 8; Clade B). As observed in the COI and 16s gene tree, there was structure within this clade, which was representative of the different tributaries sampled.

The newly identified and morphologically similar populations from the Cumberland River were recovered as highly divergent from the Red River *O. cf. barrenensis,* and as in other analyses, were recovered in a clade that included *O. forceps* (Figure 8; Clade C). *Orconectes barrenensis* was recovered as monophyletic, but with low support. There was substantial geographic structure within this clade, representative of the different tributaries within the Green River system (Figure 8; Clade D). *Orconectes mirus* was not monophyletic. Those from Flint River and Crow Creek were recovered as sister to *O. barrenensis*, rather than to those from the Elk River system.

Hypothesis Tests - Under the Bayesian criterion, the alternative hypotheses and their statistical support are summarized in Table 3. Several of the alternative hypotheses tested were statistically significant, and could not be rejected as valid alternatives to relationships among the focal taxa.

Hypotheses tested using the COI dataset that could not be statistically rejected included the monophyly of *O. barrenensis* and a polytomy of *O. barrenensis* and *O. mirus* that was sister to *O. cf. barrenensis* from the Red River. Hypotheses tested using the 16s dataset supported the same two hypothesis, along with *O. mirus* being monophyletic as being equally likely explanations of the data.

Morphological Analyses:

Univariate comparisons for all measurements are provided for focal taxa (*O. mirus*, *O. barrenensis*, and *O. cf. barrenensis* from the Red River) in Table 4. Results of the multivariate Principal Components Analyses (PCA) of females and Form II males were not informative due to small sample sizes. This is partly due to the fact that focus was placed on examination of Form I male characteristics as these are typically used in species diagnoses. Results of the PCA of Form I males shown by scatterplots of factor scores demonstrated minimal to moderate overlap in variation (represented by polygons that bound all individuals of a given taxon) between *O. cf. barrenensis* and *O. mirus* (Figure 9). Minimal overlap was observed, however, between *O. barrenensis* and *O. cf. barrenensis*, with most individuals diverging along both PC2 and PC3. Variables that loaded most heavily (absolute value > 0.5) on these axes and which contributed most to separation include the width of the areola and length of the abdomen, rostrum, gonopod, and central and mesial projections. *Orconectes cf. barrenensis* had a shorter gonopod, central projection, and mesial projection and had a wider areola, longer abdomen, and shorter rostrum, compared to *O. barrenensis*. Despite the moderate overlap, most individuals of *O. cf. barrenensis* can be distinguished from *O. mirus* by a combination of those variables that loaded most heavily on PC3, which included the width of the areola and length of the abdomen and rostrum, with *O. cf. barrenensis* having a wider areola, longer abdomen, and shorter rostrum, compared to *O. mirus*.

Assessments regarding the presence/absence of a shoulder at the base of the central projection, and the presence/absence of cervical spines were also conducted. *Orconectes cf. barrenensis* can be further distinguished from *O. barrenensis* by having a

shoulder at the base of the central projection (Figure 10), which is a character that it shares with *O. mirus*. The presence/absence of the cervical spines were variable across our three focal taxa (Figure 11).

CHAPTER IV

Discussion

Gene-based estimates of phylogeny for the crayfish from the Red River system, *O. cf. barrenensis*, supported the existing hypothesis, stemming from their overall morphological similarity, of a close relationship of this taxon to either *O. barrenensis* (Green River system) or *O. mirus* (Tennessee River system). In all datasets examined *Orconectes cf. barrenensis* was consistently recovered as a clade closely related to *O. barrenensis* and *O. mirus.* Recovery of this relationship across multiple loci provides relatively strong support that the estimated relationship accurately reflects evolutionary history of the focal group. Numerous studies have demonstrated that the use of multiple loci, as opposed to single locus gene trees, provide more robust estimates of phylogenetic relationships among species (Crandall and Fitzpatrick, 1996; Maddison et al., 1997; Song et al., 2008).

Although, all data suggest *O. cf. barrenensis* is closely related to *O. mirus* and *O. barrenensis*, relationships among these three taxa varied among loci examined. One mitochondrial gene supported a sister relationship with *O. mirus,* while the other supported a clade containing *O. mirus* and *O. barrenensis* as the sister to *O. cf. barrenensis*. The latter could not be statistically rejected by either mitochondrial data set and overall was a better-supported hypothesis of the sister relationship for *O. cf. barrenensis.* This observation also suggests that *O. barrenensis* and *O. mirus* are more closely related to each other than either is to *O. cf. barrenensis* from the Red River.

Unfortunately, nuclear genes offered little to no resolution among these three closely related species. Relationships among these taxa were also unresolved in the

combined gene phylogeny. Lack of resolution among the three focal taxa in the nuclear datasets was not surprising given the general slower rate of mutation accumulation in such markers compared to mitochondrial genes (Moore, 1995). Unfortunately, this has led to a strong reliance on mitochondrial DNA, particularly COI, to estimate phylogenetic relationships among crayfishes (since it evolves more rapidly and offers more resolution for recently diverged species; Moore, 1995; Ballard and Whitlock, 2004). However, phylogenies estimated from multiple genes, both nuclear and mitochondrial, are more likely to reflect true evolutionary relationships (Maddison et al., 1997) and despite not providing resolution among focal taxa, the nuclear genes further support that *O. cf. barrenensis*, *O. barrenensis* and *O. mirus* are close relatives.

Although mitochondrial genes typically offer more resolution of terminal clades, sole reliance on mitochondrial DNA alone can lead to erroneous estimates of phylogeny. This is particularly true for macroinvertebrates that have documented potential for paternal leakage (Fontaine et al., 2007; Wolff et al., 2013) in the mitochondrial genome or the potential presence of mitochondrial pseudogenes (Song et al., 2008). Regarding the latter, Buhay (2009) expressed concern with mitochondrial sequences used in other studies of crayfish phylogeny (e.g., Taylor and Knouft, 2006), and highlighted sequences that contained potential psuedogenes. The author recommended following methods of Song et al. (2008) to assess the potential for nuclear influences on mitochondrial DNA sequences prior to use in phylogenetic estimation. Therefore the methods of Song et al. (2008) were followed in this study. Through these steps, COI genes from one population of *O. barrenensis* were suspect (Nolin River) based on the high degree of divergence observed among individuals from the same locality. These individuals were also

recovered in very different parts of the COI tree. While these sequences were removed from subsequent analyses and not used in phylogenetic inference of the focal taxon, this finding provides additional support for common nuclear influences on mitochondrial genes, which if ignored can lead to erroneous conclusions. For example, our findings from of the Nolin River would suggest two morphologically similar, but highly divergent genetically, forms of *O. barrenensis* in that system (neither of which was closely related to *O. barrenensis* from other parts of the Green River system).

Recovered phylogenetic relationships also support the previous hypothesis that *O. cf. barrenensis* from the Red River is a distinct species. This hypothesis was proposed based on noted morphological differences between it and *O. barrenensis* at the time of discovery (BHE, 2008). In all mitochondrial and the combined gene trees, *O. cf. barrenensis* was recovered as a single genetically divergent monophyletic clade. Observed mitochondrial sequence divergence also supports *O. cf. barrenensis* as a genetically distinct lineage. The relatively high divergence levels observed are indicators of long-standing isolation among the focal taxa. Average sequence divergence within *O. cf. barrenensis* from the Red River was less than 1%, but was approximately 6% between *O. cf. barrenensis* and *O. mirus* or *O. barrenensis*. This level is comparable to or higher than that observed between other closely related and currently recognized crayfish species. For example, average sequence divergence between *O. mirus* and *O. barrenensis* was 6% and that observed between *O. rhoadesi* and *O. alabamensis* (recovered as sister species using COI) was around 7%. This same pattern was observed for 16S, and these levels were similar to Sinclair et al. (2003), who reported expected divergence levels within species, among species, among genera, and among superfamilies for five different

gene regions, including COI (5.9% among species) and 16s (5.7% among species). Thoma et al. (2014) used average sequence divergence (COI average sequence divergence was around 6% among species, along with morphology and other molecular data) as an aid for species-level identification of *Cambarus callainus*.

The distinctiveness of *O. cf. barrenensis* is further supported by observed variation in phenotype among the three focal taxa. Examination of a suite of standard morphological characteristics suggests *O. cf. barrenensis* is potentially diagnosable based on a combination of characteristics, including areola width, length of abdomen and rostrum, gonopod size and presence of a shoulder at the base of the central projection. Species descriptions and other studies have examined and used a similar suite of morphological characteristics to demonstrate diagnosability (e.g., Austin and Knott, 1996; Taylor, 1997; Taylor, 2000). The congruence of molecular and morphological data, which supports *O. cf. barrenensis* as being both morphologically diagnosable and phylogenetically distinct, increases the robustness of the conclusion that *O. cf. barrenensis* from the Red River is a distinct lineage of crayfish that warrants recognition as a separate species. Such criteria have been used commonly to delineate and describe crayfish species (e.g., Taylor; 1997; Taylor, 2000; Thoma et al., 2014).

In addition to conclusions related to *O. cf. barrenensis* from the Red River system, additional findings were noteworthy. For example, results highlight that the monophyly of the other two focal species, *O. barrenensis* and *O. mirus*, warrants further evaluation. *Orconectes barrenensis* was recovered as monophyletic in the combined gene tree, but with low support. In the individual datasets, *O. barrenensis* was not monophyletic. Also, there was a substantial amount of geographic structure in the mitochondrial gene trees

within *O. barrenensis* corresponding to different tributaries of the Green River system, such as between the Barren River and the Rough River. Similarly, *O. mirus* was not monophyletic based on any gene, a result that has been observed previously (Crandall and Fitzpatrick, 1996). Two clades corresponding to different sets of tributaries in the Tennessee River system was observed in the COI, 16s, and combined dataset. However, the monophyly of these two species could not be statistically rejected as viable alternative hypotheses given the data examined. The lack of monophyly and noted geographic variation suggests these taxa require further study to evaluate the potential for unrecognized diversity in each.

Morphological variation within *O. barrenensis* and *O. mirus* was also examined. Although variation was noted within each, sample sizes were small, and thus, inferences were limited. One notable observation was that the presence of the cervical spines was variable across all three focal taxa. This was unexpected given that the original description and taxonomic keys currently used to identify *O. barrenensis* and *O. mirus* state that there are no cervical spines present on either species of crayfish (Orttman, 1931; Rhoades, 1944; Taylor and Schuster, 2004). Findings here suggest that cervical spines may be less reliable in diagnosing these species, or in taxonomic studies in general, than previously proposed. The unreliability of the use of cervical spines in diagnosing species also has been demonstrated with species of *Cambarus* (Taylor, 1997).

Another interesting finding was that despite having a strong morphological similarity to *O. cf. barrenensis* from the Red River, including lacking cervical spines and having generally the same coloration, the newly discovered crayfish from Sycamore Creek and West Fork Obey River (also referred to as *O. cf. barrenensis*) were highly

divergent genetically and closely related to *O. forceps*. Inconsistencies in gene-based estimates of lineage diversity and phylogeny and those inferred with traditional approaches of morphological diagnosability or shared morphological traits are common. These discrepancies may occur due to convergent evolution of morphological or genetic characters that are being used to estimate crayfish diversity and relationships (Fitzpatrick and Payne, 1968; Crandall and Fitzpatrick, 1996; Taylor and Knouft, 2006) or to other phenomena, such as mitochondrial introgression (Perry et al., 2002; Perry et al., 2011), or paternal leakage, that can lead to erroneous results in one or more of these datasets. The Cumberland River individuals, that were assumed to be related to or be newly discovered populations of *O. cf. barrenensis* from the Red River were in fact highly divergent from the Red River *O. cf. barrenensis*, and were recovered in a clade with *O. forceps*. This relationship was consistent across multiple gene trees. Because this observation was also observed in one of the nuclear gene trees, this implies that the recovered close relationship between populations from the Cumberland River tributaries and *O. forceps* is in fact not an artifact of mitochondrial introgression or paternal leakage. Alternatively, it seems more plausible that the morphological traits shared with *O. cf. barrenensis* from the Red River system stem from convergence associated with environmental factors or possibly reflect retained ancestral traits, rather than recent common ancestry of these crayfishes. This finding provides further evidence that morphological characters commonly used to ally crayfish species into subgenera or to diagnose taxa may be unreliable in some cases. Others have noted inconsistencies across morphology-based and gene-based estimates of diversity and phylogenetic relationships in crayfishes. For example, Crandall and Fitzpatrick (1996) demonstrated lack of congruence between the

existing morphology-based classification by Fitzpatrick (1987) and their estimates from a 16s gene phylogeny, which may have occurred due to convergence of morphological or genetic characters. There have been studies demonstrating hybridization or introgression within species of *Orconectes* (Perry et al., 2002; Perry et al., 2011), which also support inconsistencies between morphology- and gene-based estimates of phylogenetic relationships.

Discerning the underlying cause(s) leading to the observed pattern of shared morphological features between the genetically divergent Cumberland River *O. cf. barrenensis* and Red River system *O. cf. barrenensis* are beyond the focus of this study. However, the observed level of genetic divergence and phylogenetic relationships suggest there may be additional unrecognized crayfish diversity in the Cumberland River system; an observation previously noted by Bouchard (1972), but one that will require further evaluation.

While results strongly support that *O. cf. barrenensis* from the Red River represents a distinct species, additional morphological data are needed to fully diagnose it from its closest relatives. Studies completed by Taylor (1997, 2000), Cooper (2000), and Thoma et al. (2014) demonstrate the need for larger sample sizes of each sex, and the need to analyze a broad spectrum of morphological characteristics to diagnose and describe new species. While this study examined numerous characteristics, additional specimens of each sex and form type are needed. The distribution of *O. cf. barrenensis* within the Red River also requires further study. This has been completed in part, but attempts to identify other populations of *O. cf. barrenensis* have been unsuccessful.

While this may suggest they have a very limited range, additional exploration for this species in other areas of the Red River are needed to define its range.

At the conclusion of this study, *O. cf. barrenensis* had only been collected from four localities, three of those being on Fort Campbell Military Base. The species was abundant at the three sites on the base. However multiple sampling efforts were made at the Billy Dunlop Park locality on the West Fork Red River before any *O. cf. barrenensis* were collected and only eight specimens were obtained. Previous collections of this site (in 2007) resulted in approximately 25 individuals (per. comm., Bruce Bauer, 2014). Recent upstream development activity, such as residential development, and increasingly intensive row-crop agriculture may have contributed to declines in *O. cf. barrenensis* at this locality. Activities, such as these, that increase sediment loads to streams have been shown to negatively impact occurrence and abundance of crayfishes and other benthic organisms (Westhoff et al. 2006).

Orconectes barrenensis and *O. mirus* are listed as Currently Stable species (Taylor et al., 1996), and both have been collected from multiple sites (>5) within their native ranges (Kentucky and Tennessee and Alabama and Tennessee, respectively). *Orconectes cf. barrenensis,* based on current data, appears to have a small restricted range and the one locality observed that was not on Fort Campbell Military Base, may be impacted by increased expansion and development in Clarksville, TN. Furthermore, the Red River system, overall, is highly impacted by anthropogenic activities, particularly agriculture, with multiple tributaries included on the 303(d) list as Category 5 streams (TDEC, 2014). Events such as habitat destruction, degradation and alteration, as well as chemical pollution and the introduction of nonindigenous species increase the chance for

species loss or imperilment (Taylor et al., 1996; Taylor and Schuster, 2004). This has been documented before, with *Orconectes shoupi*, a species of crayfish that is not only restricted to a small native range in a system that is threatened by development, but also vulnerable to competition with an introduced species, *O. placidus,* in the same streams and surrounding systems (Bizwell and Mattingly, 2010). The restricted distribution seen in *O. cf. barrenensis* may not only reflect biogeographic limitations to its range, but also reflect range limitations or reductions stemming from anthropogenic events. Upon description, given these factors, *O. cf. barrenensis* would warrant state or potential federal protection and conservation actions. Additional studies of the ecology and life history of this crayfish are needed to guide future conservation and ensure its long-term persistence.

Table 1. GenBank sequences obtained and included in genetic analyses. Sequences used are listed by genus and species, with the associated Accession Number.

Gene	Species	Accession Number
COI	*Cambarus distans*	JX514489
	Cambarus fasciatus	JX514495
	Cambarus obeyensis	JX514498
	Cambarus scotti	JX514500
	Orconectes ascares	AY701227
	Orconectes alabamensis	AY701215
	Orconectes australis	EF207162
	Orconectes barrenensis	AY701228
	Orconectes barri	EF207164
	Orconectes carolinensis	AY701229
	Orconectes chickasawensis	AY701216
	Orconectes compressus	AY701217
	Orconectes cooperi	AY701218
	Orconectes cristavarius	AY701230
	Orconectes deanae	AY701205
	Orconectes difficulus	AY701206
	Orconectes etnieri	AY701219
	Orconectes forceps	AY701231
	Orconectes harri	AY701189
	Orconectes harfieldi	AY701207
	Orconectes hobbsi	AY701211
	Orconectes holti	AY701225
	Orconectes hylas	AY701232
	Orconectes illinoiensis	AY701226
	Orconectes immuni	AY701220
	Orconectes imcomptus	EF207166
	Orconectes indianensis	AY701198
	Orconectes inermis	AY701201
	Orconectes jonesi	AY701221
	Orconectes juvenillis	AY701233
	Orconectes kentuckiensis	AY701196
	Orconectes limosus	AY701199
	Orconectes longidtus	AY701234
	Orconectes luteus	AY701235
	Orconectes maleate	AY701208
	Orconectes marcrus	AY701236
	Orconectes medius	AY701237
	Orconectes meeki	AY701212
	Orconectes meanae	AY701238
	Orconectes mirus	AY701239
	Orconectes mississippiensis	AY701222

Table 1. Continued.

Gene	Species	Accession Number
COI	*Orconectes nais*	AY701223
	Orconectes neglectus	AY701241
	Orconectes ozarkae	AY701242
	Orconectes packardi	EF207168
	Orconectes pagei	AY701202
	Orconectes palmeri	AY701214
	Orconectes pellucidus	AY701203
	Orconectes punctimanus	AY701244
	Orconectes putnami	AY701245
	Orconectes quandruncus	AY701246
	Orconectes rhoadesi	AY701224
	Orconectes ronaldi	AY701247
	Orconectes rusticus	AY701249
	Orconectes saxatalis	AY701250
	Orconectes sloanii	AY701197
	Orconectes spinous	AY701251
	Orconectes validus	AY721593
	Orconectes williami	AY701252
	Orconectes wrighti	AY701200
16s	*Cambarus maculatus*	JX127864
	Cambarus obeyensis	JX514538
	Cambarus robustus	JX514558
	Cambarus scotti	JX514559
	Cambarus striatus	JX127861
	Orconectes alabamensis	KF771142
	Orconectes etneiri	KF771162
	Orconectes hartfieldi	KF771155
	Orconectes indianensis	KF771140
	Orconectes jonesi	KF771172
	Orconectes kentuckiensis	KF771118
	Orconectes meeki	KF771119
	Orconectes mirus	KF771178
	Orconectes mississippiensis	KF771143
	Orconectes obscurus	KF771120
	Orconectes perfectus	KF771175
	Orconectes placidus	AY609338
	Orconectes rhoadesi	KF771176
	Orconectes rusticus	JQ397607
	Orconectes sanbornii	JQ397609
	Orconectes validus	KF771138
	Orconectes wrighti	KF771177

Table 1. Continued.

Gene	Species	Accession Number
16s	*Orconectes enchsonianus*	EU433918
	Orconectes forceps	EU433919
	Orconectes roanldi	JX127865
	Orconectes deanae	JX127859
28s	*Cambarus lanconensis*	JX514643
	Cambarus maculatus	JS127595
	Cambarus parrishi	JX514690
	Cambarus pristinus	JX514690
	Cambarus robustus	JX514687
	Cambarus scotti	JX514688
	Orconectes deanae	JX127590
	Orconectes neglectus	JX514693
	Orconectes ronaldi	JX514694
	Orconectes roanldi	JX127596
GAPDH	*Orconectes cf. virilis*	EU596360
	Orconectes rusticus	JQ397617
	Orconectes sanbornii	JQ397620

Table 2. Models and parameters used for each data partition and implemented in the Bayesian analyses.

	1st Codon Position	2nd Codon Position	3rd Codon Position	Single Data Set
COI	HKY+I+G	GTR+I+G	TrN+G	-
16s	-	-	-	TVM+I+G
28s	-	-	-	TrN
GAPDH	-	-	-	TPM2+I

Table 3. Results of constraint tree searches of the Bayesian post-burnin trees. Trees tested were constrained to represent alternative viable hypotheses of relationships among focal taxa. Hypotheses that were recovered in more than 5 % of the post-burnin trees were considered to be statistically significant (*) and could not be rejected as viable alternative hypotheses under a Bayesian criterion.

Hypothesis Tested	**Combined**	**COI**	**16s**	**28s**	**GAPDH**
Monophyletic *O. barrenensis*	0.00%	24.67%*	44.10%*	4.29%	2.07%
Monophyletic *O. mirus*	0.16%	0.60%	6.54%*	0.50%	0.02%
Monophyletic *O. barrenensis*, sister to *O. cf. barrenensis*	0.00%	0.96%	2.26%	0.00%	0.00%
Monophyletic *O. mirus*, sister to *O. cf. barrenensis*	0.00%	0.17%	1.89%	0.00%	0.00%
Polytomy *O. barrenensis* and *O. mirus*, sister to *O. cf. barrenensis*	0.00%	17.23%*	19.52%*	0.00%	0.00%

Table 4. Standardized measurements of Form I males, Form II males, and females of *O. cf. barrenensis* from the Red River, *O. barrenensis,* and *O. mirus* that were made following Cooper (2006), Rhoades (1944), Schuster (2008), Taylor (1997, 2000), Thoma (2014) and Tierney (1982). Measurements were taken with digital calipers to the nearest 0.1 mm. Measurements were standardized by total length to account for variation in size of individuals measured and are reported as thousandth of total length

Measurements/Counts	***O. barrenensis***		***O. cf. barrenensis***		***O. mirus***	
Form I males (n = 78)	**Range**	**x ± SD**	**Range**	**x ± SD**	**Range**	**x ± SD**
Total Length	*29.36 – 45.58*	*37.13 ± 5.33*	*31.68 – 57.85*	*43.75 ± 6.96*	*34.04 – 65.83*	*46.05 ± 7.98*
Length of Right Chela	372 – 549	435 ± 52	313 – 512	426 ± 44	473 – 556	449 ± 63
Width of Right Chela	146 – 259	197 ± 29	134– 230	195 ± 25	82 – 244	199 ± 32
Length of Inner Margin Palm	103 – 168	131 ± 19	94 – 176	145 ± 22	64 – 181	146 ± 26
Length of Dactyl	207 – 356	266 ± 45	182 – 284	246 ± 21	159 – 341	262 ± 40
Length of Carapace	455 – 518	479 ± 18	408 – 498	476 ± 17	452 – 513	484 ± 16
Width of Carapace	218 – 264	239 ± 12	230 – 260	240 ± 6	203 – 260	233 ± 14
Length of Areola	124 – 184	159 ± 15	137 – 165	152 ± 7	140 – 188	159 ± 11
Width of Areola	14 – 39	28 ± 7	25 – 58	37 ± 9	19 – 43	34 ± 6
Length of Rostrum	85 – 166	139 ± 17	101 – 151	127 ± 10	109 – 169	142 ± 14
Width of Rostrum	57 – 82	72 ± 6	59 – 82	73 ± 6	58 – 81	72 ± 5
Length of Abdomen	509 – 555	533 ± 12	498 – 598	542 ± 24	495 – 563	520 ± 16
Width of Abdomen	194 – 237	212 ± 11	202 – 231	211 ± 6	186 – 224	206 ± 8
Length of Gonopod	123 – 158	136 ± 11	108 – 141	128 ± 10	117 – 139	127 ± 6

Table 4. Continued.

Measurements/Counts	*O. barrenensis*		*O. cf. barrenensis*		*O. mirus*	
Form I males (n = 78)	**Range**	**x ± SD**	**Range**	**x ± SD**	**Range**	**x ± SD**
Length of Central Projection	65 – 101	78 ± 11	53 – 85	72 ± 8	59 – 79	72 ± 5
Length of Mesial Projection	56 – 99	70 ± 10	39 - 75	60 ± 8	50 – 95	65 ± 8
Form II males (n = 62)						
Total Length	*30.72 – 53.51*	*45.02 ± 5.93*	*27.21 – 57.75*	*38.95 ± 5.63*	*31.57 – 56.43*	*42.51 ± 8.69*
Length of Right Chela	244 – 501	382 ± 55	195 – 421	317 ± 57	234 – 458	345 ± 99
Width of Right Chela	87 – 212	169 ± 28	65 – 179	135 ± 27	89 – 195	137 ± 43
Length of Inner Margin Palm	72 – 144	119 ± 18	68 – 169	110 ± 24	81 – 152	106 ± 28
Length of Dactyl	162 – 329	233 ± 37	114 – 253	182 ± 28	141 – 303	214 ± 67
Length of Carapace	473 – 515	491 ± 10	412 – 523	478 ± 18	451 – 523	483 ± 26
Width of Carapace	214 – 242	228 ± 7	201 – 260	226 ± 13	201 – 233	216 ± 10
Length of Areola	148 – 186	161 ± 9	134 – 200	150 ± 14	133 – 177	149 ± 17
Width of Areola	24 – 47	34 ± 5	13 – 59	35 ± 8	18 – 43	28 ± 8
Length of Rostrum	114 – 156	137 ± 9	67 – 156	128 ± 6	139 – 197	162 ± 21
Width of Rostrum	63 – 80	71 ± 4	55 – 88	72 ± 6	66 – 79	72 ± 5
Length of Abdomen	515 – 562	532 ± 13	509 – 570	541 ± 13	500 – 543	522 ± 17
Width of Abdomen	192 – 221	206 ± 7	161 – 231	207 ± 12	184 – 213	194 ± 111

Table 4. Continued.

Measurements/Counts	***O. barrenensis***		***O. cf. barrenensis***		***O. mirus***	
Females (n = 97)	**Range**	**x ± SD**	**Range**	**x ± SD**	**Range**	**x ± SD**
Total Length	*27. 49 – 54.13*	*40.32 ± 7.51*	*27.60 – 49.99*	*36.78 ± 6.28*	*35.05 – 65.56*	*44.45 ± 6.65*
Length of Right Chela	229 – 372	307 ± 34	209 – 331	271 ± 24	162 – 553	337 ± 63
Width of Right Chela	77 – 160	131 ± 19	67 – 146	117 ± 16	49 – 174	145 ± 25
Length of Inner Margin Palm	229 – 372	97 ± 16	62 – 135	94 ± 13	54 – 134	106 ± 17
Length of Dactyl	138 – 240	184 ± 21	116 – 184	154 ± 15	94 – 231	195 ± 26
Length of Carapace	455 – 508	481 ± 11	452 – 491	471 ± 8	431 – 500	473 ± 15
Width of Carapace	208 – 255	227 ± 9	196 – 231	220 ± 8	190 – 240	222 ± 11
Length of Areola	141 – 171	155 ± 6	132 – 159	146 ± 7	137 – 176	154 ± 9
Width of Areola	21 – 55	33 ± 6	16 – 24	41 ± 3	16 – 42	31 ± 7
Length of Rostrum	118 – 177	142 ± 13	114 – 166	71 ± 7	115 – 177	141 ± 11
Width of Rostrum	63 – 86	74 ± 5	56 – 93	71 ± 7	59 – 75	67 ± 4
Length of Abdomen	518 – 572	537 ± 13	209 – 605	512 ± 17	504 – 572	534 ± 15
Width of Abdomen	193 – 241	217 ± 11	184 – 241	215 ± 12	207 – 237	224 ± 9

Table 5. Principal component loadings for the fifteen morphological variables examined from Form I males of *O. cf. barrenensis*, *O. barrenensis*, and *O. mirus*. Those with loadings with absolute values > 0.50 were considered as variables that contributed most to separation among species as depicted in Figure 9.

	Principal Components	
Variable	**2**	**3**
Right Chela length	-0.117	0.067
Inner Palm length	-0.080	-0.130
Dactyl length	-0.207	0.176
Width of right chela	-0.081	-0.012
Carapace length	0.008	0.399
Carapace width	0.054	-0.125
Areola length	-0.154	0.059
Areola width	0.016	-0.628
Rostrum length	-0.140	0.615
Width of rostrum base	0.222	-0.376
Abdominal length	0.324	-0.682
Abdominal width	0.344	-0.222
Length of gonopod	0.915	0.035
Length of central projection	0.904	0.280
Length of mesial projection	0.824	0.294
% total of variance explained	18.255	12.169

Table 6. Average sequence divergence (%) among the three focal taxa. An "*" represents average sequence divergence found within that taxon. Note 16s average sequence divergence is not given for within *Orconectes cf. barrenensis* from Sycamore Creek and West Fork Obey River, because only one sequence was used. No *O. cf. barrenensis* from the Sycamore Creek and West Fork Obey River were amplified for the 28s gene region.

	Average Within Divergence			
Taxa	***COI***	***16s***	***28s***	***GAPDH***
O. cf. barrenensis from the Red R.	1.2	0.7	0.0	0.1
O. barrenensis	1.5	1.6	0.0	0.1
O. mirus	5.8	2.2	0.0	0.1
O. cf. barrenensis from Sycamore Ck. & West Fork Obey R.	2.1	-	-	0.1
	Average Between Divergence			
Taxa	***COI***	***16s***	***28s***	***GAPDH***
O. cf. barrenensis from Red R. vs. *O. barrenensis*	6.3	3.2	0.0	0.1
O. cf. barrenensis from Red R. vs. *O. mirus*	6.4	3.1	0.0	0.0
O. cf. barrenensis from Red R. vs. *O. cf. barrenensis* from Sycamore Ck. & West Fork Obey R.	11.8	5.0	-	0.4
O. barrenensis vs. *O. mirus*	6.4	3.0	0.0	0.1
O. barrenensis vs. *O. cf. barrenensis* from Sycamore Ck. & West Fork Obey R.	10.8	6.2	-	0.8
O. mirus vs. *O. cf. barrenensis* from Sycamore Ck. & West Fork Obey R.	10.0	6.1	-	0.8

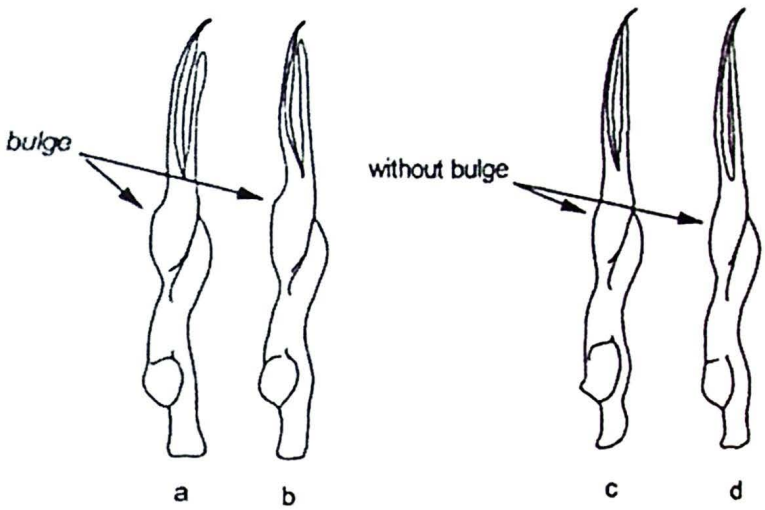

Figure 1. Gonopods of *O. mirus* Form I males have a shoulder (bulge) at the base of the central projection (a and b), which is absent on gonopods of *O. barrenensis* Form I males (c and d). Figure modified from Hobbs, 1976.

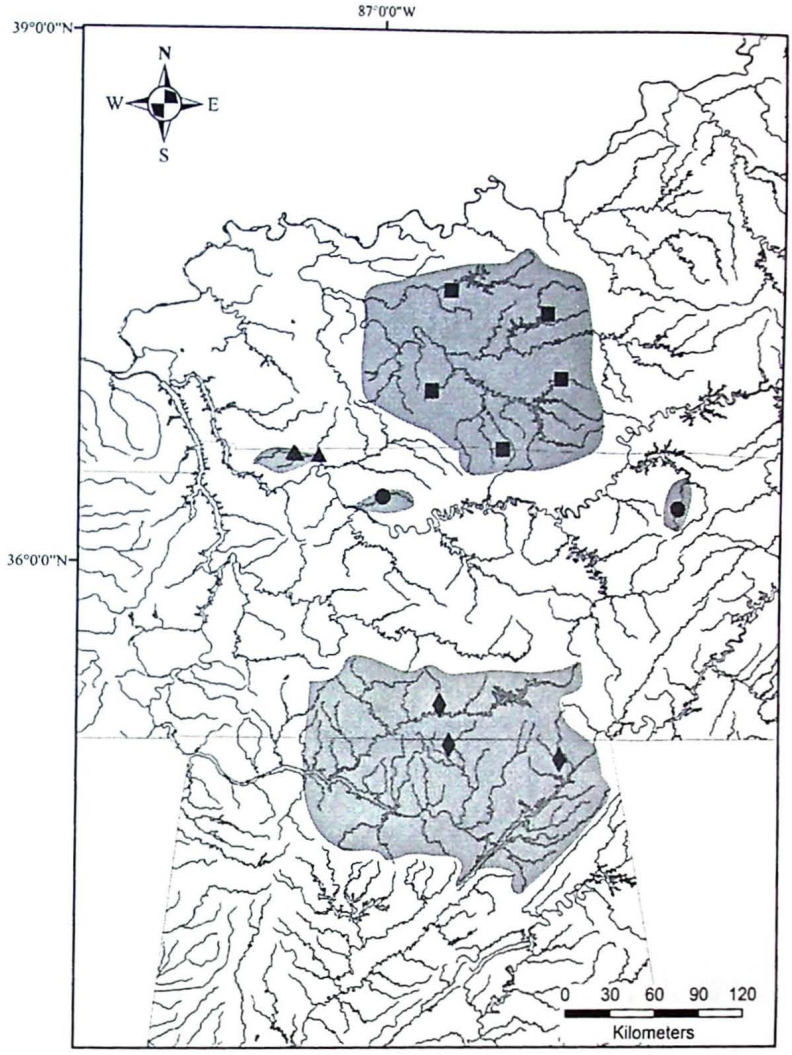

Figure 2. Localities sampled for individuals of the focal taxa used for genetic comparison. Localities include personally collected individuals and borrowed samples. Shaded areas encompass ranges of each taxon. Triangles represent sites sampled for *O. cf. barrenensis* from the Red River system. Squares represent sites sampled for *O. barrenensis* from the Green River system. Diamonds represent sites sampled for *O. mirus* from the Tennessee River system. Circles represent sites sampled for *O. cf. barrenensis* from Sycamore Creek and West Fork Obey River.

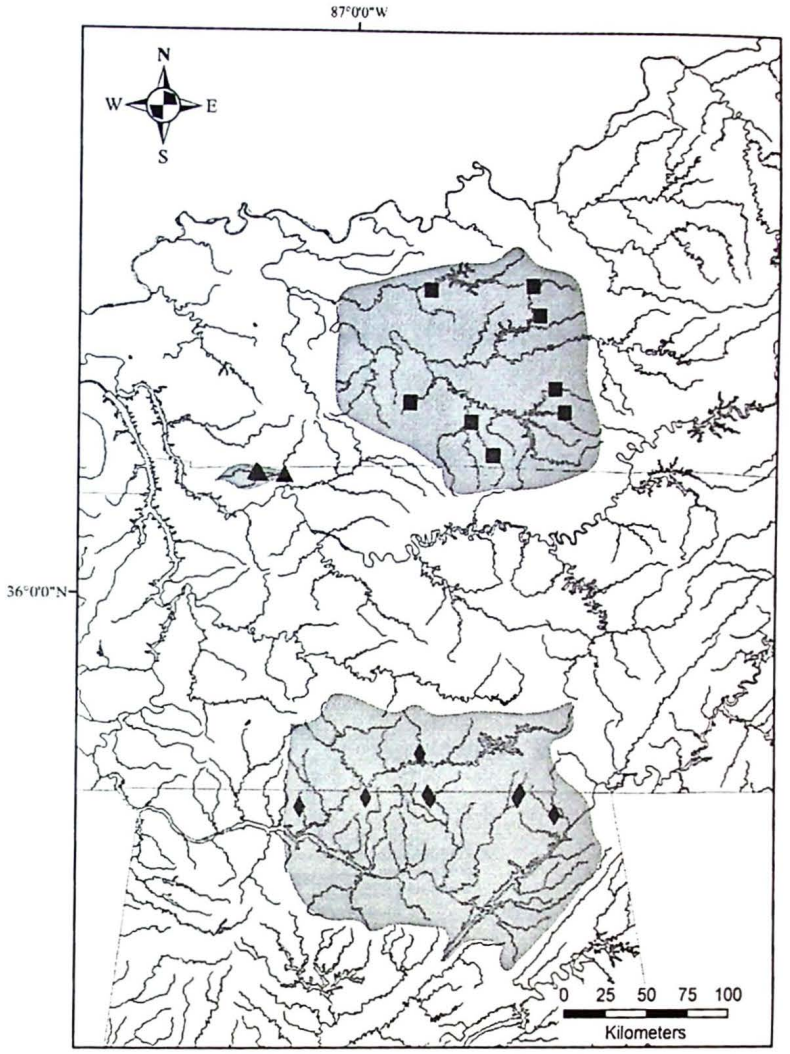

Figure 3. Localities sampled for individuals of the focal taxa (not including individuals of *O. cf. barrenensis* from Sycamore Creek and West Fork Obey River) used for morphological comparison. Localities represent those personally collected and museum specimens. Shaded areas encompass range of each taxon. Triangles represent sites sampled for *O. cf. barrenensis* from the Red River system. Squares represent sites sampled for *O. barrenensis* from the Green River system. Diamonds represent sites sampled for *O. mirus* from the Tennessee River system.

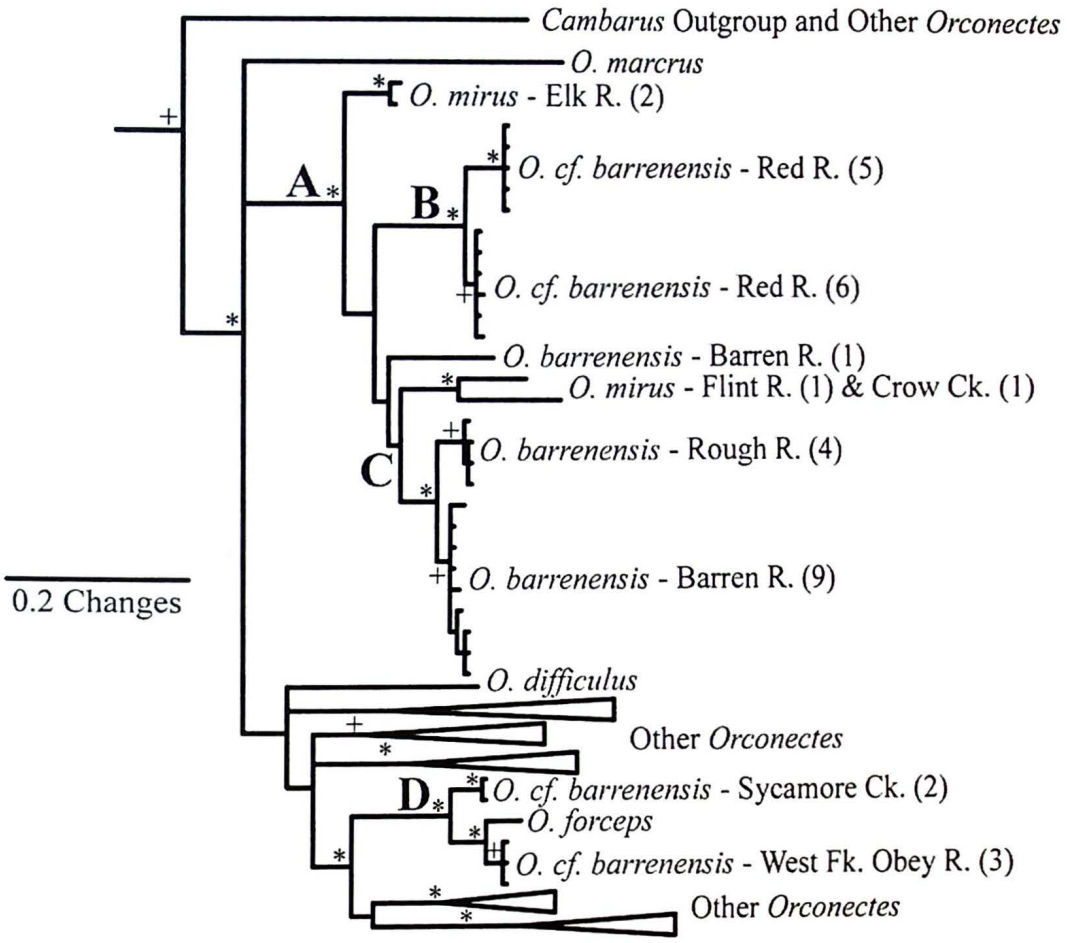

Figure 4. A 50% majority rule consensus tree resulting from the Bayesian analysis of the COI dataset. An "*" indicates 95% or greater posterior probability support for a clade and a "+" indicates clades with 85% - 95% posterior probability support. Letters highlight clades discussed in the results and discussion of the focal taxa. Numbers in parenthesis represent the number of individuals sequenced for a given population.

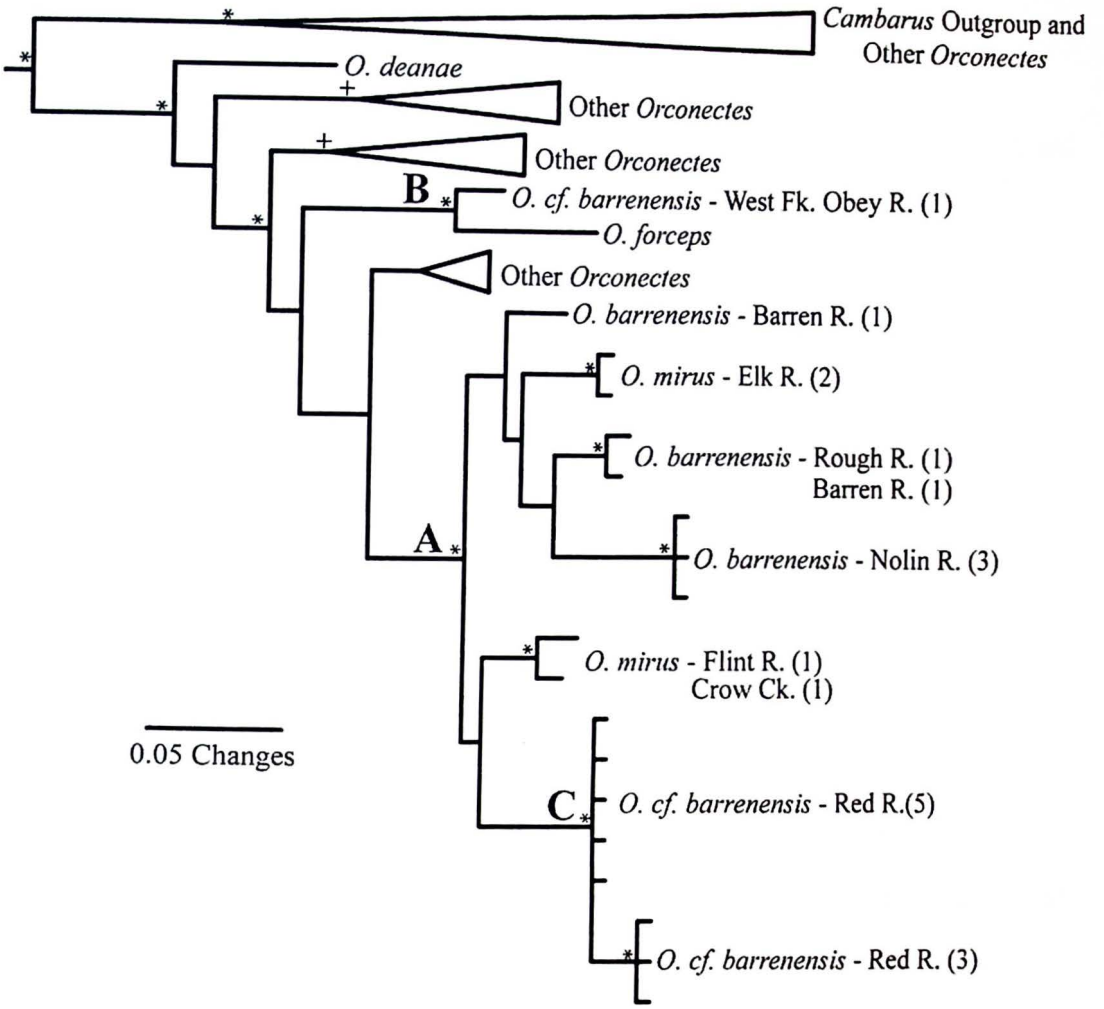

Figure 5. A 50% majority rule consensus tree resulting from the Bayesian analysis of the 16s dataset. An "*" indicates 95% or greater posterior probability support for a clade and a "+" indicates clades with 85% - 95% posterior probability support. Letters highlight clades discussed in the results and discussion of the focal taxa. Numbers in parenthesis represent the number of individuals sequenced for a given population.

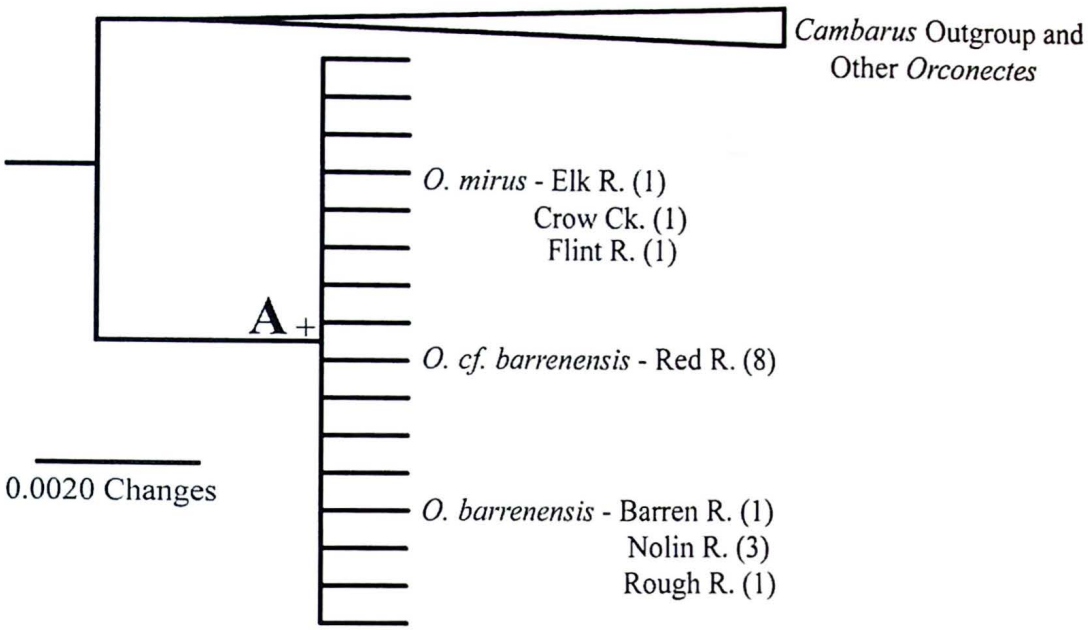

Figure 6. A 50% majority rule consensus tree resulting from the Bayesian analysis of the 28s dataset. An "*" indicates 95% or greater posterior probability support for a clade and a "+" indicates clades with 85% - 95% posterior probability support. Letters highlight clades discussed in the results and discussion of the focal taxa. Numbers in parenthesis represent the number of individuals sequenced for a given population.

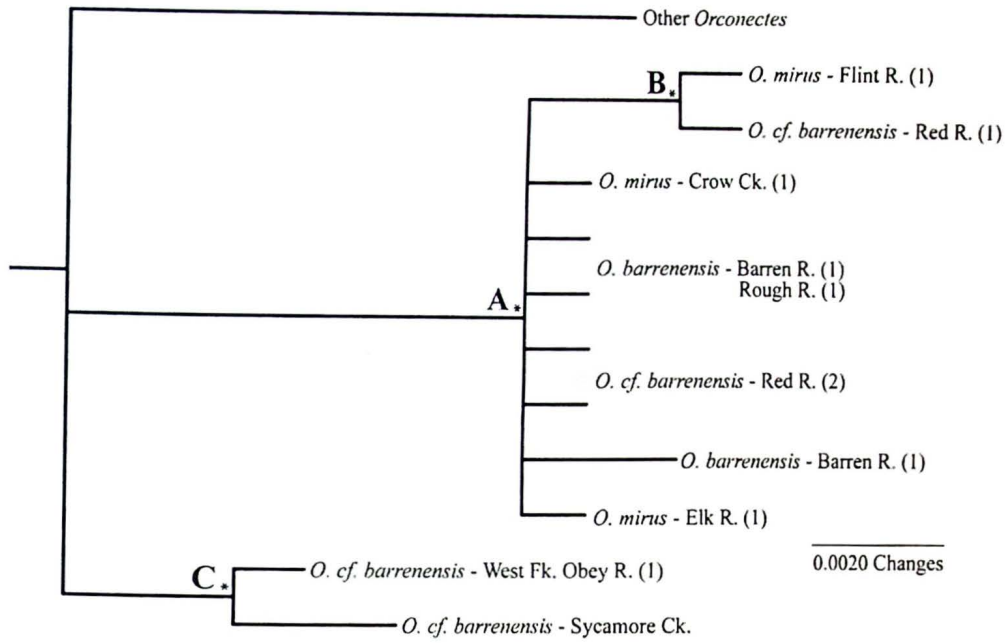

Figure 7. A 50% majority rule consensus tree resulting from the Bayesian analysis of the GAPDH dataset. An "*" indicates 95% or greater posterior probability support for a clade and a "+" indicates clades with 85% - 95% posterior probability support. Letters highlight clades discussed in the results and discussion of the focal taxa. Numbers in parenthesis represent the number of individuals sequenced for a given population.

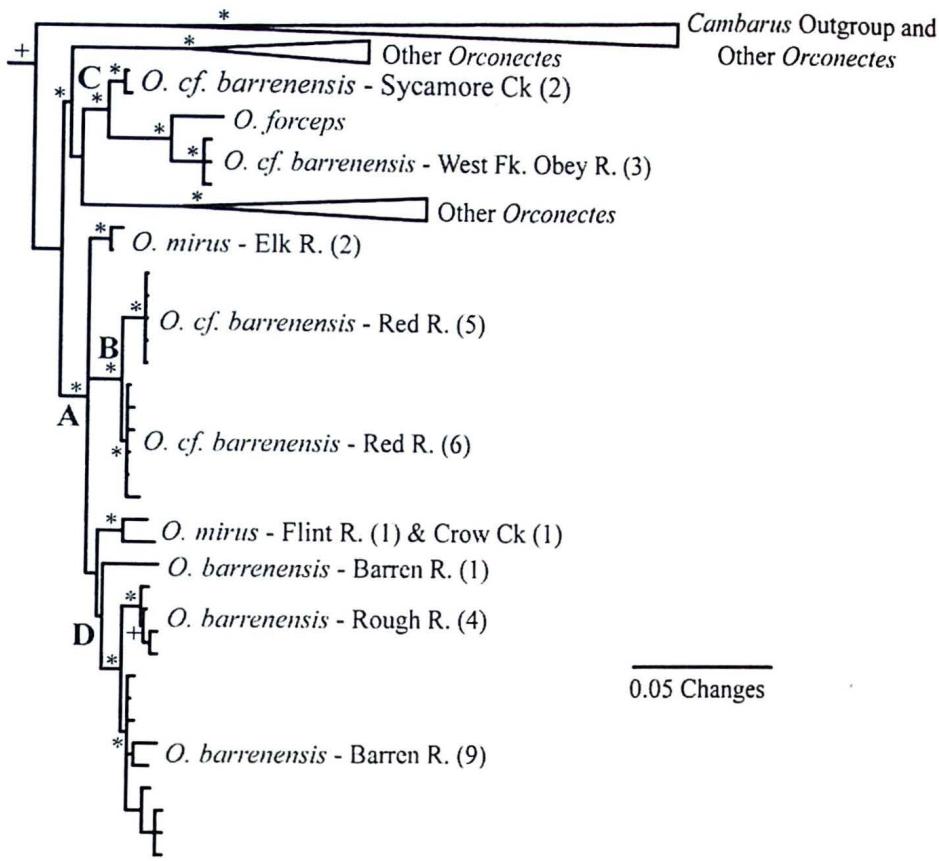

Figure 8. A 50% majority rule consensus tree resulting from the Bayesian analysis of the combined dataset. An "*" indicates 95% or greater posterior probability support for a clade and a "+" indicates clades with 85% - 95% posterior probability support. Letters highlight clades discussed in the results and discussion of the focal taxa. Numbers in parenthesis represent the number of individuals sequenced for a given population.

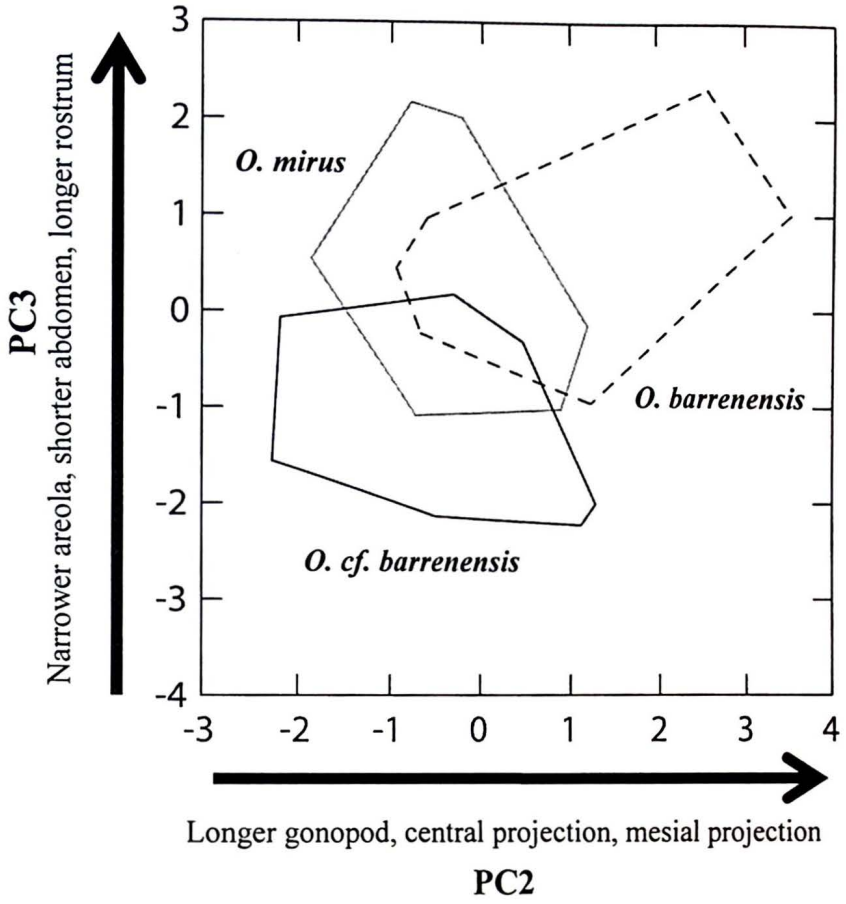

Figure 9. Results of the multivariate principal component analyses of Form I males for *O. barrenensis*, *O. mirus*, and *O. cf. barrenensis* from the Red River. Polygons bound all individuals examined for a given taxon. Variables that loaded most heavily on PC2 were length of gonopod, central projection and mesial projection; those variables that loaded most heavily on PC3 were width of areola and length of abdomen and rostrum. Component loadings for variables are provided in Table 7.

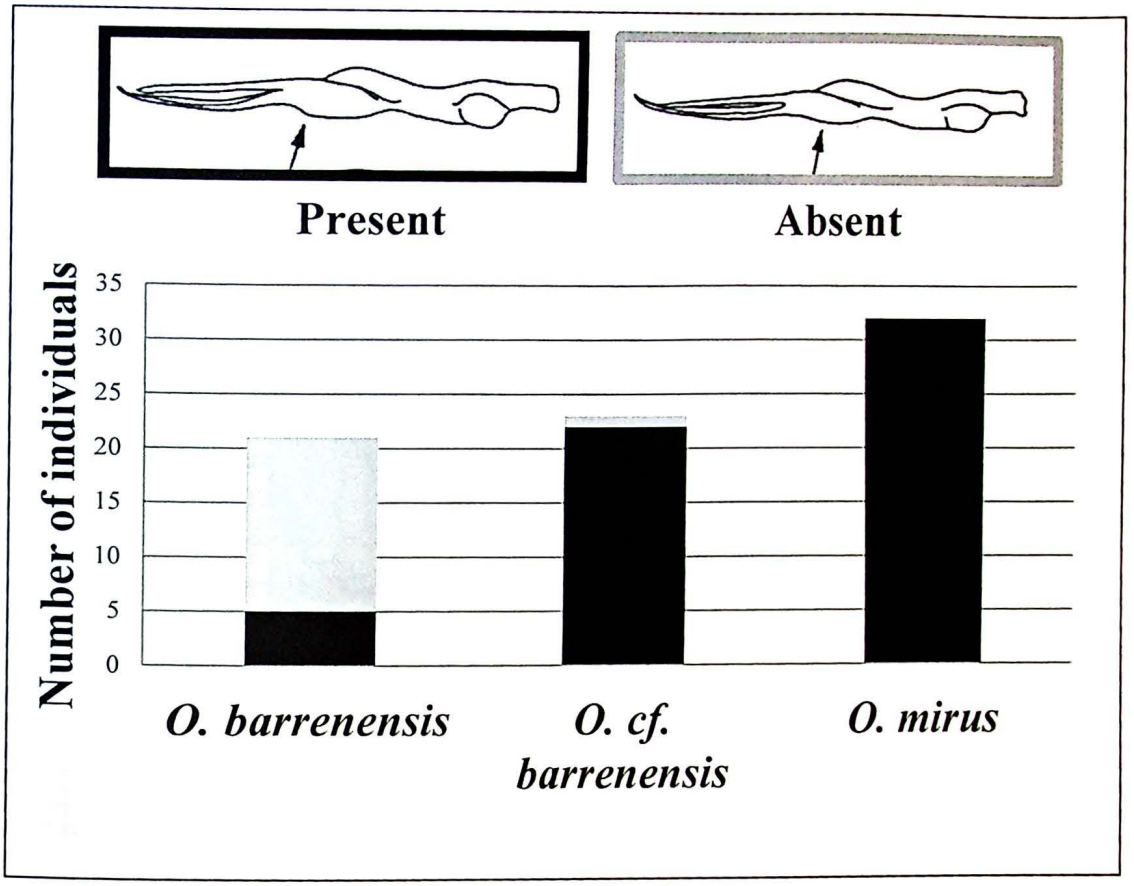

Figure 10. Quantitative assessment of the presence or absence of a shoulder at the base of the central projection of Form I male gonopods of *Orconectes. cf. barrenensis* from the Red River and from *O. barrenensis* and *O. mirus.*

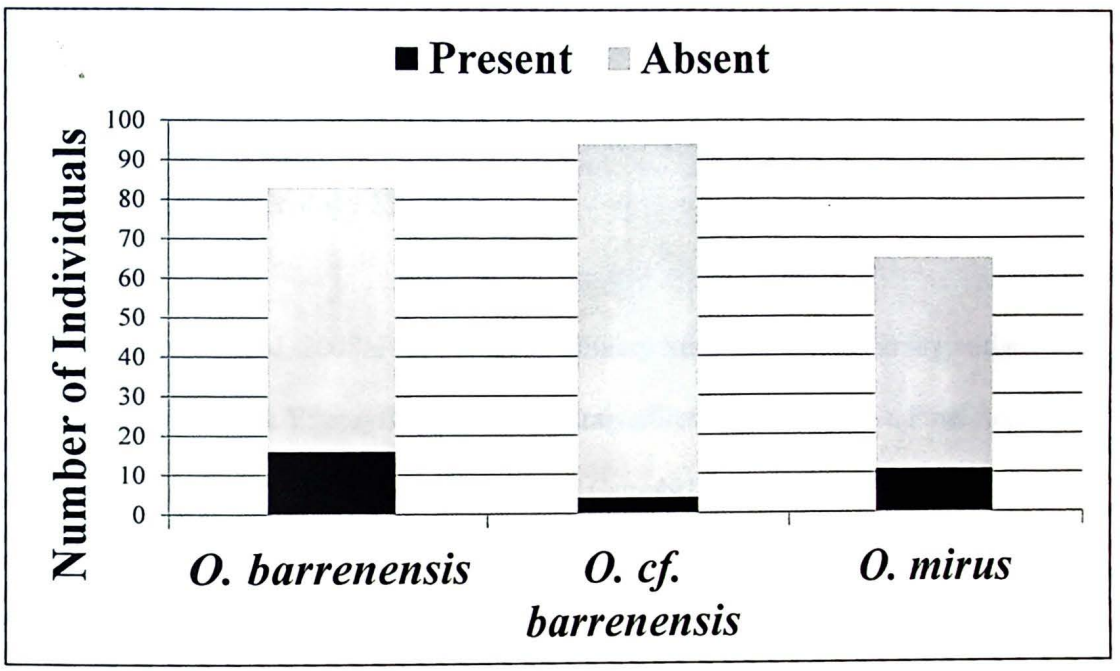

Figure 11. Quantitative assessment of the presence and absence of cervical spines in the three focal taxa.

LITERATURE CITED

Austin, C.M., and Knott, B. 1996. Systematics of the freshwater crayfish genus *Cherax* Erichson (Decapoda: Parastacidae) in South-western Australia: electrophoretic, morphological and habitat variation. Australian Journal of Zoology 44: 223-258.

Ballard, J.W., and Whitlock, M.C. 2004. The incomplete natural history of mitochondria. Molecular Ecology 13: 729-744.

BHE Environmental. 2008. Fort Campbell Military Reservation biodiversity initiative phases IV & V: crayfish, stoneflies, dragonflies, and damselflies. Final Report to US Army Corp of Engineers and Fort Campbell Conservation Branch, Environmental Division.

Bizwell, E.A., and Mattingly, H.T. 2010. Aggressing interactions of the endangered Nashville crayfish, *Orconectes shoupi*. Southeastern Naturalist 9(2): 359-372.

Bouchard, R.W. 1972, A contribution to the knowledge of Tennessee crayfish. Ph.D. dissertation, University of Tennessee. 113 pp.

Bouchard, R.W., and Bouchard, J.W. 1995. Two new species and subgenera (*Cambarus* and *Orconectes*) of Crayfishes (Decapoda: Cambaridae) from the Eastern United States. Notulae Naturae (471).

Brienholt, J.W., Porter, M.L., and Crandall, K.A. 2012. Testing phylogenetic hypotheses of the subgenera of the freshwater crayfish genus *Cambarus* (Decapoda: Cambaridae). PLoS ONE 7(9): 1-11.

Budowle, B., Allard, M., Wilson, M., and Chakraborty, R. 2003. Forensics and mitochondrial DNA: applications, debates, and foundations. Annual Review of Genomics and Human Genetics. 4: 119-141.

Buhay, J.E. 2009. "COI-Like" sequences are becoming problematic in molecular systematic and DNA barcoding studies. Journal of Crustacean Biology 29(1): 96-110.

Buhay, J.E., and Crandall, K.A. 2005. Subterranean phylogeography of freshwater crayfishes shows extensive gene flow and surprisingly large population sizes. Molecular Ecology 14(14): 4259-4273.

Buhay, J.E., Mone, G., Mann, N., and Crandall, K.A. 2007. Molecular taxonomy in the dark: evolutionary history, phylogeography, and diversity of cave crayfish in the subgenus *Aviticambarus*, genus *Cambarus*. Molecular Phylogenetics and Evolution 42: 435-448.

Cooper, J.E. 2000. *Cambarus* (Cambarus) *davidi*, a new species of crayfish (Decapoda: Cambaridae) from North Carolina. Proceedings of the Biological Society of Washington 113: 431-442.

Crandall, K., and Buhay, J. 2008. Global diversity of crayfish (Astacidae, Cambaridae, and Parastacidae – Decapoda) in freshwater. Hydrobiologia 595: 295-301.

Crandall, K., and Fitzpatrick, J. 1996. Crayfish molecular systematics: using a combination of procedures to estimate phylogeny. Systematic Biology 45(1): 1-26.

Farrell, B.D., and Sequeira, A.S. 2004. Evolutionary rates in the adaptive radiation of beetles on plants. Evolution 58(9): 1984-2001.

Fitzpatrick, J.F. 1967. The Propinquus group of the crawfish genus *Orconectes* (Decapoda: Astacidae). The Ohio Journal of Science 67(3): 129-172.

Fitzpatrick, J.F. 1987. The subgenera of the crawfish genus *Orconectes* (Decapoda: Cambaridae). Proceedings of the Biological Society of Washington 100(1): 44-74.

Fitzpatrick, J.G., and Payne, J.F. 1968. A new genus and species of crawfish from the southeastern United States (Decapoda, Astacidae). Proceedings of the Biological Society of Washington 8: 11-22.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3(5): 294-299.

Fontaine, K., Cooley, J., and Simon, C. 2007. Evidence for paternal leakage in hybrid periodical cicadas (Hemiptera: *Magicicada* spp.). PLoS One 2(9): 1-8.

Fricke, R. and Eschmeyer, W.N. 2010. A Guide to Fish Collections in the Catalog of Fishes Database. http://www.calacademy.org/scientists/projects/catalog-of-fishes. Accessed online 17 May 2015.

Galtier, N., Nabholz, B., Glemin, S., and Hurst, G.D.D. 2009. Mitochondrial DNA as a marker of molecular diversity: a reappraisal. Molecular Ecology 18(22): 4541-4550.

Grandjean, F., Harris, J., Souty-Grosset, C., and Crandall, K. 2002. Systematics of the European endangered crayfish species *Austropotamobius pallipes* (Decapoda: Astacidae). Journal of Crustacean Biology 22(3): 677-681.

Guindon, S., and Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696–704.

Harris, J.D., and Crandall, K.A. 2000. Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): implications for phylogenetic and microsatellite studies. Molecular Biology and Evolution 17(2): 284-291.

Hebert, P., Cywinkska, A., Ball, S., and deWaard, J. 2003. Biological identifications through DNA barcodes. Proceedings of The Royal Society 270: 313-321.

Hobbs, H.H. Jr. 1976. Crayfishes (Astacidae) of North and Middle America. Biota of freshwater ecosystems identification manual 9: 1-173.

Huelsenback, J.P., Ronquist, R.N., and Bollback, J.P. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294: 2310-2314.

Isphording, W.C., and Fitzpatrick, J.F. Geologic and evolutionary history of drainage systems in the southeaster United States. Biodiversity of the southeastern United States, aquatic communities. John Wiley and Sons, New York (1992): 19-56.

Leviton, A.E., Gibbs, R.H., Heal, E., and Dawson, C.E. 1985. Standards in herpetology and ichthyology: Part I. Standard symbolic codes for institutional resource collections in herpetology and ichthyology. Copeia 1985(3): 802-832.

Librado T., and Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25(11): 1451-1452.

Maddison, W.P., and Maddison, D.R. 2015. Mesquite: a modular system for evolutionary analysis. Version 3.03 http://mesquiteproject.org.

Maddison, D., Swofford, D., and Maddison, W. 1997. Nexus: an extensible file format for systematic information. Systematic Biology 46(4): 590-621.

Mathews, L.M., Adams, L., Anderson, E., Basile, M., Gottardi, E., and Buckholt, M.A. 2008. Genetic and morphological evidence for substantial hidden biodiversity in a freshwater crayfish species complex. Molecular Phylogenetics and Evolution 48(1): 126-135.

Moore, W.S. 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. Evolution 49(4): 718-726.

Ortmann, A.E. 1931. Crawfishes of the Southern Appalachians and the Cumberland Plateau. Annals Carnegie Museum 20(2): 61-160.

Perry, W.L., Lodge, D.M., and Feder, J.L. 2002. Importance of hybridization between indigenous and nonindigenous freshwater species: an overlooked threat to North American biodiversity. Systematic Biology 51(2): 255-275.

Perry, W.L., Feder J.L., and Lodge, D.M. 2011. Implications of hybridization between introduced and resident *Orconectes* crayfishes. Conservation Biology 15(6): 1656-1666.

Piganeau, G., Gardner, M., and Eyre-Walker, A. 2004. A broad survey of recombination in animal mitochondria. Molecular Biology 21(12): 2319-2325.

Posada, D., and Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14(9): 817-818.

Rhoades, R. 1944. The crayfishes of Kentucky, with notes on variation, distribution, and descriptions of new species and subspecies. American Midland Naturalist 31(1): 111-149.

Ronquist, F., and Huelsenbeck, J. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12).

Schubart, C.D., Neigel, J.E., and Felder, D.L. 2000. Use of the mitochondrial 16s rRNA gene for phylogenetic and population studies of Crustacea. Proceedings of the International Crustacean Congress. Vol. 2: 817-830.

Schuster, G. 2008. *Orconectes* (Trisellescens) *taylori*, a new species of crayfish from western Tennessee (Decapoda: Cambaridae). Proceedings of the Biological Society of Washington 121(1): 62-71.

Schuster, G., Taylor, C., and Johansen, J. 2008. An annotated checklist and preliminary designation of drainage distribution of the crayfishes of Alabama. Southeastern Naturalist 7(3): 493-504.

Sinclair, E., Fetzner, J., Buhay, J., and Crandall, K. 2004. Proposal to complete a phylogenetic taxonomy and systematic revision for freshwater crayfish (Astacidae). Freshwater Crayfish 14: 21-29.

Song, H., Buhay, J., Whiting, M., and Crandall, K. 2008. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proceedings of the National Academy of Science 105(36).

Swofford, D.L. 1993. PAUP: Phylogenetic analysis using Parsimony, Verson 3.1. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Taylor, C.A. 1997. Taxonomic status of members of the subgenus *Erebicambarus*, genus *Cambarus* (Decapoda: Cambaridae), east of the Mississippi River. Journal of Crustacean Biology 17(2): 351-360.

Taylor, C.A. 2000. Systematic studies of the *Orconectes juvenilis* complex (Decapoda: Cambaridae), with descriptions of two new species. Journal of Crustacean Biology 20(1): 132-152.

Taylor, C.A., and Hardman, M. 2002. Phylogenetics of the crayfish subgenus *Crockerinus*, genus *Orconectes* (Decapoda: Cambaridae), based on Cytochrome Oxidase I. Journal of Crustacean Biology 22(4): 874-881.

Taylor, C.A., and Knouft, J.H. 2006. Historical influences on genital morphology among sympatric species: gonopod evolution and reproductive isolation in the crayfish genus *Orconectes* (Cambaridae). Biological Journal of the Linnean Society 89(1): 1-12.

Taylor, C., and Schuster, G. 2004. The Crayfishes of Kentucky. Illinois Natural History Survey Special Publication No. 28. viii + 219 pp.

Taylor, C.A., Warren, M.L., Fitzpatrick, J.F., Hobbs, H.H., Jezerinac, R.F., Pflieger, W.L., and Robison, H.W. 1996. Conservation Status of Crayfishes of the United States and Canada. Fisheries 21(4): 25-38.

Thoma, R.F., Loughman, Z.L., and Fetzner, J.W. 2014. *Cambarus* (*Punticambarus*) *callainus*, a new species of crayfish (Decapoda: Cambaridae) from the Big Sandy River basin in Kentucky, Virginia, and West Virginia, USA. Zootaxa 3900 (4): 541-554.

Tennessee Department of Environment and Conservation. 2014. Year 2014 303(d) List Draft. http://www.tn.gov/environment/water/docs/wpc/2014-draft-303d-list.pdf. Accessed online 17 May 2015.

Tierney, A.J. 1982. Individual variation and taxonomic significance of the seminal receptacle of the crayfish, *Orconectes propinquus* (Girard) (Decapoda: Cambaridae). Crustacean 43(3): 284-296.

Toews, D., and Brelsford, A. 2012. The biogeography of mitochondrial and nuclear discordance in animals. Molecular Ecology 21(16): 3907-3930.

Weisrock, D.W., Shaffer, H.B., Storz, B.L., Storz, S.R., and Voss, S.R. 2006. Multiple nuclear gene sequences identify phylogenetic species boundaries in the rapidly radiating clade of Mexican ambystomatid salamanders. Molecular Ecology 15(9): 2489-2503.

Westhoff, J.T., Guyot, J.A., and DiStefano, R.J. 2006. Distribution of the imperiled Williams; Crayfish (*Orconectes williamsi*) in the White River Drainage of Missouri: associations with multi-scale environmental variables. The American Midland Naturalist 156 (2): 273-288.

Whiting, M.F., Carpenter, J.C., Wheeler, Q.D., and Wheeler, W.C. 1997. The Stresiptera problem: phylogeny of the holometabolous insect orders inferred from 18s and 28s ribosomal DNA sequences and morphology. Systematic Biology 46(1): 1-68.

Williams, C., Bivens, B., and Carter, B. 2004. Crayfish survey of Wilkerson Creek and two of its tributaries, Cumberland County Tennessee. Tennessee Wildlife Resources Agency.

Wolff, J.N., Nafisinia, M., Sutovsky, P., and Ballard, J.W. 2013. Paternal transmission of mitochondrial DNA as an integral part of mitochondrial inheritance in metapopulations of *Drosophila simulans*. Heredity 110: 57-62.

APPENDICES

APPENDIX I

Locality Information for Individuals Collected for Tissue Samples

Locality Information for Individuals Used In DNA Analyses

Individuals collected for extracting tissue samples or vouchered tissue samples (noted with an *) are found below and listed by state and county. Numbers following coordinate data are individual numbers, associating individual with the respective sequence that was amplified. Individuals collected personally are not formally accessioned but are currently housed at Austin Peay State University. Institutional abbreviations follow Leviton et al., 1985 and Fricke and Eschmeyer, 2010.

Alabama:

**O. mirus* - AL, Jackson Co.: Little Coon Creek, 1 mile W Rash Co Rd. 53; Lat: 34.87436 Long: -85.9101; EB001.01.

**O. mirus* - AL, Madison Co.: West Fork Flint River, 1 mile North Hazel Green, 0.25 miles upstream of 431/231; Lat: 34.958464 Long: -86.569478; EB003.01.

Kentucky:

O. barrenensis - KY, Allen Co.: Trammel Fork, at small bridge on Old State Rd; Site EB13-14; EB017.01, EB017.02, EB017.03, EB017.04, EB017.05.

O. barrenensis - KY, Barren Co.: Beaver Creek, at Glasgow-Munfordville Rd. bridge; Site EB13-07; Lat: 37.03811 Long: -85.919121; EB013.01, EB013.02, EB013.03, EB013.05.

O. rusticus - KY, Hardin Co.: Rough River, SR 920 crossing 1 mile N SR 86; Site EB13-05; EB011.01, EB011.02.

O. barrenensis - KY, Hart Co.: Roundstone Creek, at SR 1140 crossing; Site EB13-04; Lat: 37.40961 Long: 86.00249; EB010.01, EB010.02, EB010.03, EB010.04, EB010.05.

O. barrenensis - KY, Logan Co.: Gasper River, at the KY Rt. 73 bridge; Site EB13-13; Lat: 36.97114 Long: -86.70098: EB016.01, EB016.02, EB016.03, EB016.04, EB016.05.

O. barrenensis - KY, Ohio/Grayson Co.: Rough River, SR 54 crossing in Hites Falls; Site EB13-06; Lat: 37.54139 Long:-86.59554; EB012.01, EB012.02, EB012.03, EB012.05.

Tennessee:

O. cf. barrenensis (Cumberland R.) - TN, Cheatham Co.: Holt/Sycamore Creek, at Hwy 12 downstream of bridge; Site EB13-10; Lat: 36.36992 Long: -86.98711; EB015.01, EB015.02, EB015.03, EB015.04.

**O. mirus* - TN, Lincoln Co.: Cane Creek, 2.5 miles NW of Fayetteville, Boonshill; Lat: 35.18693 Long: -86.62732; EB002.01.

O. cf. barrenensis (Red R.) - TN, Montgomery Co.: Little West Fork of Red River, on Ft. Campbell at McNair Rd.; Site EB13-01; Lat: 36.61331 Long: -87.4971; EB006.01, EB006.02.

O. cf. barrenensis (Red R.) - TN, Montgomery Co.: Little West Fork of Red River, on Ft. Campbell at Pump Station Road and Boiling Springs; Site EB13-03; Lat: 36.62011 Long: -87.5063; EB008.01, EB008.02.

O. cf. barrenensis (Red R.) - TN, Montgomery Co.: Noah Springs Branch, on Ft. Campbell at Mabry Rd.; Site EB13-02; Lat: 36.62229 Long: -8751312; EB007.01, EB007.02.

O. cf. barrenensis (Red R.) - TN, Montgomery Co.: West Fork Red River, at Billy Dunlop Park on Boy Scout Rd.; Site EB13-15; Lat: 36.60891 Long: -87.36503; EB018.01, EB018.02, EB018.03, EB018.05, EB018.06.

O. cf. barrenensis (Cumberland R.) - TN, Overton Co.: West Fork Obey River, at Shiloh Church Rd. 14.5 km SE Liveingston, TN; Site JWJ13.001; Lat: 36.639712 Long: -86.264812; EB014.01, EB014.02.

**O. barrenensis* - TN, Sumner Co.: Unknown locality. EB004.01.

APPENDIX II

Locality Information for Individuals Used in Morphological Comparisons

Locality Information for Individuals Used in Morphological Comparisons

Individuals collected or borrowed (noted with an *) for morphological comparison are found below and are listed by state and county. Numbers preceding locality information are site numbers/INHS voucher numbers that correspond to sites in Figure 3. Individuals collected personally are not formally accessioned but are currently housed at Austin Peay State University. Institutional abbreviations follow Leviton et al., 1985 and Fricke and Eschmeyer, 2010.

Alabama:

O. mirus - *INHS 7291; AL, Jackson Co.: Hurricane Creek, 4 mi NNW Hytop, upstream Turkey Creek; Latitude: 34.984161, Longitude: -86.094057.

O. mirus - *INHS 11731; AL, Jackson Co.: Hurricane Creek, 2.05 km W SR 79 0.67 km S TN State Line; Latitude: 34.98349, Longitude: -86.09453.

O. mirus - *INHS 7293; AL, Jackson Co.: Hurricane Creek, 3.5 mi NNW Hytop; Latitude: 34.961966, Longitude: -86.108289.

O. mirus - *INHS 11366; AL, Jackson Co,: Little Coon Creek, 1 mile W Rash, Co. Rd. 53; Latitude: 34.87436, Longitude: -85.91041.

O. mirus - *INHS 11388; AL, Lauderdale Co.: Anderson Creek, 0.8 mi S Anderson Hwy, 207 crossing; Latitude: 34.916812, Longitude: -87.270628.

O. mirus - *INHS 11649; AL, Limestone Co.: Ragsdale Creek, 4 mi NE Elkmon, Shipley Hollow Rd and Ragsdale Creek; Latitude: 34.9649, Longitude: -86.9171.

O. mirus - *INHS 9031; AL, Lincoln Co.: Cane Creek, 3.5 mi NW Fayetteville, Boonshill R.; Latitude: 35.18693, Longitude: -86.62732.

O. mirus - *INHS 11720; AL, Madison Co.: Fowler Creek, 1 km NW Fisk, Elkwood Section Rd.; Latitude: 34.97653, Longitude: -86.58571.

O. mirus - *INHS 9012; AL, Madison Co.: West Fork Flint River, 1 mi N Hazel Green, 0.25 miles upstream Hwy 431/231; Latitude: 34.958464, Longitude: -86.569478.

O. mirus - *INHS 8806; AL, Marion Co.: Battle Creek near Martin Springs; Latitude: 35. 163264 Longitude: -85.790175.

Kentucky:

O. barrenensis - *INHS 8842; KY: Falling Timber Creek, 2.4 mi NNW Temple Hill, Hwy 63; Latitude: 36.918531, Longitude: -86.867809.

O. barrenensis - *INHS 4481; KY, Allen Co.: Trammel Creek, 5 mi SW Scottsville, US Rt. 31E; Latitude: 36.702238, Longitude: -86.24734.

O. barrenensis - *INHS 209; KY, Allen Co.: Trammel Fork, 6 mi S Scottsville; Latitude: 36.703329, Longitude: -86.250911.

O. barrenensis - EB13-07; KY, Barren Co.: Beaver Creek, at Old Glasgow, Mudfordville Rd. Bridge; Latitude: 37.03822, Longitude: -85.91912.

O. barrenensis - *INHS 8707; KY, Hardin Co.: Nolin River, 2.7 mi SSE Eastview Hwy. 84; Latitude: 37.561671, Longitude: -86.03708.

O. barrenensis - EB13-04; KY, Hart Co.: Roundstone Creek, at SR 1140 Crossing; Latitude: 37.40961, Longitude: -86.00249.

O. barrenensis - *INHS 5016; KY, Warren Co.: Gasper River, 10 mi W Bowling Green Hwy, 1083; Latitude: 36.99052, Longitude: -86.63140.

O. barrenensis - *INHS 4948; KY, Warren Co.: Trammel Fork, 1.5 mi W Aviation at Mt. Lebanon Rd.; Latitude: 36.87033, Longitude: -86.368456.

Tennessee:

O. cf. barrenensis - EB13-03; TN, Montgomery Co.: Little West Fork of Red River, on Ft. Campbell on Pump Station Rd. and Boiling Springs; Latitude: 36.62011, Longitude: -87.5063.

O. cf. barrenensis - EB13-01; TN, Montgomery Co: Little West Fork of Red River, on Ft. Campbell on McNair Rd.; Latitude: 36.61331, Longitude: -87.4971.

O. cf. barrenensis - EB13-02; TN, Montgomery Co.: Noah Springs Branch, on Ft. Campbell on Mabry Rd.; Latitude: 36.62229, Longitude: -87.51312.

O. cf. barrenensis - EB13-15; TN, Montgomery Co.: West Fork Red River, at Billy Dunlop Park on Boy Scout Rd.; Latitude: 36.60891, Longitude: -87.36503.

VITA

Erin Tyler Bloom was born in Franklin, TN in July 1988. She graduated high school in Dickson County, TN in 2006. She attended East Tennessee State University for one semester, before transferring to Austin Peay State University in January 2007. As an undergraduate she was awarded the Dr. Haskell C. Phillips and Estelle Judd Phillips Biology Scholarship in 2010. She joined Chi Omega Women's Fraternity in 2007, along with several honor societies, and graduated in December 2010 with a Bachelor of Science Degree in Biology, with a minor in Education.

Erin began teaching high school Biology in January 2010, and continued to teach, even after she began a Master of Science in Biology at Austin Peay State University in 2012. In May of 2014, she left the classroom and continued with her degree. During her time as a graduate student, she joined multiple student organizations, such as Southeastern Fishes Council, American Society of Ichthyologists and Herpetologists, and Austin Peay State University's American Fisheries Society. She has presented her research at several professional meetings, and also served as a co-author of other research, which included Southeastern Fisheries Council Annual Meeting in 2013 and 2014, Austin Peay State University's Graduate Student Extravaganza in 2013, 2014, and 2015, and the Southern Division of the American Fisheries Society in 2015. She was awarded a Graduate Student Research and Creative Activity Grant in 2013. At the Southeastern Fishes Council Annual Meeting in 2013, she was awarded third place for Best Student Poster Presentation. She completed her M.S. requirements and graduated with a 3.8 GPA Masters of Science in Biology in May of 2015.