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SYSTEMATICS AND BIOGEOGRAPHY OF *ORCONECTES*, SUBGENUS *TRISELLESCENS*, IN THE SOUTHEASTERN UNITED STATES, A TEST OF MORPHOLOGY-BASED CLASSIFICATION

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ABSTRACT

Diagnosable taxonomic units are fundamental to conservation biology and management of resources and the need for sound science in both fields is more pressing for aquatic ecosystems. Within freshwater crayfishes, the North American genus *Orconectes* is one of the most diverse in the World. Accurate assessments of species level relationships and species boundaries within the genus have historically been hampered by a low number of variable morphological characters and inadequate sampling from across the ranges of many taxa. We examine a diverse group of southeastern United States stream dwelling *Orconectes* in the subgenus *Trisellescens* using 16S, COI mtDNA, and morphology to resolve uncertainties in species boundaries. Our results suggest that strong divergences exist between taxa found above and below the Fall Line in parts of the southeastern United States and the taxonomy for taxa found in that region should remain unchanged. However, using both molecular and morphological datasets we are unable to determine species limits for some taxa found on and below the Fall Line. Analysis of DNA data suggests that historical and ongoing genetic events such as gene introgression may contribute to these uncertainties. For taxa found on and below the Fall Line, we suggest tentative, taxonomic assignments. Finally, we argue for increased sampling of independent molecular datasets and increased sample sizes for all cambarid crayfish biogeographic studies.

KEY WORDS: biogeography, crayfish, morphology, mtDNA, *Orconectes*, species boundaries

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INTRODUCTION

The conservation of biological diversity is one of the most important challenges facing humankind on earth. This challenge is exacerbated for freshwater ecosystems because these habitats may be the most threatened by human activity (Dudgeon et al., 2006). Meeting this conservation challenge requires accurate estimates of threats, distribution and abundance for as many taxa as possible. However, before these attributes can be assessed, taxa must be properly recognized, defined, and described. For purposes of biodiversity conservation and studies of evolutionary processes, the species is perhaps the most commonly used taxonomic unit.

The southeastern United States is recognized as a hotspot for temperate freshwater biodiversity, with exceptionally high species-level diversity in unionid mussels (Lydeard et al., 2004), fish (Warren et al., 2000) and crayfish (Taylor et al., 2007). Crayfish may be the least-studied group of these three groups as up-to-date state or drainage-level crayfish faunal assessments are lacking throughout the southeast. For example, Alabama may harbor the highest diversity of crayfish of any state or province in North America (Schuster et al., 2008), yet it lacks a peer-reviewed, statewide comprehensive survey for those taxa. To improve our understanding of Alabama crayfish, efforts by the authors began in 2005 to document range and abundance of crayfish within the state. These efforts have been complicated for some taxa by a lack of clear species limits and thorough examinations of morphological variation. The current study stems from the taxonomic confusion and need for broader faunal survey efforts for certain taxa.

The North American crayfish genus *Orconectes* (Cambaridae) is the third most diverse crayfish genus on Earth with over 90 taxa (Crandall and Buhay, 2008). The native ranges of its members extend from the Hudson Bay drainage of Canada to streams draining directly into the Gulf of Mexico. Fitzpatrick (1987) was the first to hypothesize relationships within the genus when he divided it into 10 subgenera based primarily on the morphology of the genitalia (= gonopods) of reproductively active (= form I) males. Besides further subdividing the more diverse subgenera into several "groups," interspecific relationships between members of the subgenera and groups were not addressed by Fitzpatrick (1987). More recently, the robustness of Fitzpatrick's classification for *Orconectes* has been challenged with phylogenies generated from allozyme (Fetzner, 1996) and mtDNA (Crandall and Fitzpatrick, 1996; Taylor and Knouft, 2006) data.

One of the more diverse subgenera (*sensu* Fitzpatrick) within *Orconectes* was *Gremicambarus* with 13 recognized

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species. With one exception, *O. compressus* (Faxon, 1884), Bouchard and Bouchard (1995) subsequently recognized Fitzpatrick's *O. alabamensis*-group as its own subgenus, naming it *Trisellescens*. Bouchard and Bouchard (1995) neglected to include *O. jonesi* Fitzpatrick, 1992a in their newly recognized *Trisellescens*, and with the description of *O.* (*T.*) *taylori* Schuster, 2008, the subgenus now numbers 11 species. *Trisellescens* occurs in the central United States from the upper Mississippi River drainage of Minnesota south to the Gulf Coastal drainages of the Alabama and Conecuh rivers. While the exact ranges of many described species in the subgenus are not clearly understood, the distribution of diversity within the subgenus is clearly uneven. Of the 11 members of the subgenus, all but *O. immunis* (Hagen, 1870) have ranges restricted to south of the Ohio River. Those 10 members are proposed to range from westward flowing headwater streams in the Yazoo (Mississippi) drainage in northcentral Mississippi east to Tennessee River tributaries in northeastern Alabama. The southern extent of their range is headwater streams of the Conecuh River drainage in Conecuh County, Alabama (Fig. 1). *Orconectes immunis* is one of the widest ranging crayfishes in the United States and occurs from northern Colorado to the Great Lakes drainage and Atlantic seaboard in Maine. Within the Mississippi River drainage, this species occurs from western Kentucky northward to Minnesota.

The taxonomy and distribution of members of *Trisellescens* found in the Mobile and Tennessee River basins of the southeastern United States has been problematic for many years, mainly due to a paucity of specimens (Cooper and Hobbs, 1980; Fitzpatrick, 1992). This lack of specimens facilitated an incomplete analysis of morphological variation and range and a lack of defining characteristics for many of the nominal species. In their description of three members of the subgenus, Cooper and Hobbs (1980) commented on their inability to identify specimens due to the lack of form I males. They also commented on the unreliability of "nonsecondary sexual characters" until limits of variation for those characters are determined. Fitzpatrick (1992) described *O.* (*T.*) *jonesi* from the Succarnoochee River drainage (Tombigbee basin) but likewise mentioned his inability to properly identify specimens from other regions of the Tombigbee Basin due to limited material. Because no additional work on the group has been conducted since their descriptions (Cooper and Hobbs, 1980; Fitzpatrick, 1992), recent investigations of *Orconectes* crayfish distribution and diversity in the region have been hampered by the inability to assign species level identifications to field collected specimens from large areas of the Tombigbee and parts of the Alabama and Tennessee River drainages.

Recent field efforts to better document the crayfish fauna of the state of Alabama has made a thorough examination of the problematic *Orconectes* (*Trisellescens*) more feasible. Crayfish collected by the authors in the Mississippi, Tennessee, and Mobile basins of Alabama and Mississippi have provided not only tissue samples for determining patterns of DNA variation, but also specimens for morphological analysis. The use of these two, independent datasets also allows for an assessment of the strength of phylogenetic signal of both, the merits of using traditional morphology-based classification for crayfishes in this group, and the formation of biogeographic hypotheses to explain distributions. Additional inherent benefits to a study of this type, is the determination of species and range boundaries. For this paper, our goals were to: 1) use DNA from two gene regions to construct a robust hypothesis of evolutionary relationship for nine of the eleven members (*O. taylori* and *O. immunis* not included) of *Trisellescens* in the Mississippi, Tennessee, and Mobile basins of Alabama and Mississippi; 2) test the strength of morphological characters traditionally used for species level identifications in the subgenus *Trisellescens* against the recovered phylogeny; and 3) assess the validity of species level recognition for *O. alabamensis* (Faxon, 1884), *O. chickasawae* Cooper and Hobbs, 1980, *O. etnieri* Bouchard and Bouchard, 1976, *O. jonesi*, *O. holti* Cooper and Hobbs, 1980, *O. mississippiensis* (Faxon, 1884), and *O. validus* (Faxon, 1914). Data will also help elucidate the ranges of valid species within the subgenus.

Material and Methods

Crayfish were collected from 55 sites across the Tennessee and Mobile River basins and from limited portions of the Mississippi, Pascagoula and Conecuh River basins between May 2001 and November of 2011 (Fig. 1). Additional specimens previously collected from the same drainages and housed in the Illinois Natural History Survey Crustacean and Tissue Collections (INHS) were also examined. When possible, type-localites for in-group taxa were sampled and topotypes of *O. cooperi* and *O. etnieri* are present in our molecular dataset. In addition, samples of *O. jonesi*, *O. chickasawae*, and *O. holti* collected within 16, 8 and 1.6 km, respectively, of their respective type localities are also present. Type localities of *O. validus* ("Huntsville, Madison Co., AL") and *O. mississippiensis* ("eastern Mississippi") were too vague to be accurately located and sampled. Specimens were collected in the field using either dip-nets, minnow seines, or backpack electrofishers and preserved in 70% ethanol. Tissue samples were taken from selected specimens by removing approximately 25-30 mg of muscle tissue from the pleon and placing it in 99% ethanol prior to, or shortly after, preserving the individual in 70% ethanol.

Total genomic DNA was isolated from 53 field preserved tissue samples (see the Appendix, which can be found in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/1937240x) using the QIAGEN DNeasy Blood and Tissue Kit™ per the manufacturer's instructions. Polymerase Chain Reaction (PCR) was used to amplify two mitochondrial genomic loci: 16S rDNA and partial COI. Amplification of the target regions used the primers FOLMER1 (5′-GGTCAACAAATCATAAAGATATTG-3′) and FOLMER2 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994) for COI and 16S-1472 (5′-AGATAGAAACCAACCTGG-3′) and 16S-16S17sub (5′-ATASRGTCTRACCTGCCC-3′) (Crandall and Fitzpatrick, 1996) for 16S. Reaction volumes followed either those of Taylor and Hardman (2002) or manufacturer's recommendations for the GE PuReTaq Ready-to-Go™ PCR beads using 1.0 μl F and R primers and 2.0 μl DNA template. Thermal cycling parameters followed Taylor and Hardman (2002). Amplified DNA was purified using the QIAGEN QIAquick PCR Purification Kit™ and sequenced with Perkin-Elmer BigDye V3.0 DNA Sequencing Kit according to the manufacturer's protocol with primers used in PCR. Sequenced product was visualized with either an ABI Prism 377 or ABI 3730XL automated DNA sequencer at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois Urbana-Champaign.

Complete double stranded sequences for the COI and 16S regions were collected for all individuals. Sequence chromatogram files were assembled, edited, and checked for stop codons using Sequencher 4.7 (GeneCodes) and aligned using MUSCLE (Edgar, 2004). Resulting alignment files were combined and analyzed for phylogenetic structure with PAUP* 4.0b10 (Swofford, 2002) and MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The possibility of saturation at the third codon position was examined by plotting 3rd position un-corrected pairwise sequence divergence values against total sequence divergence values in the Ape package of R (R Development Core Team). All composite sequence files were deposited in GenBank (see

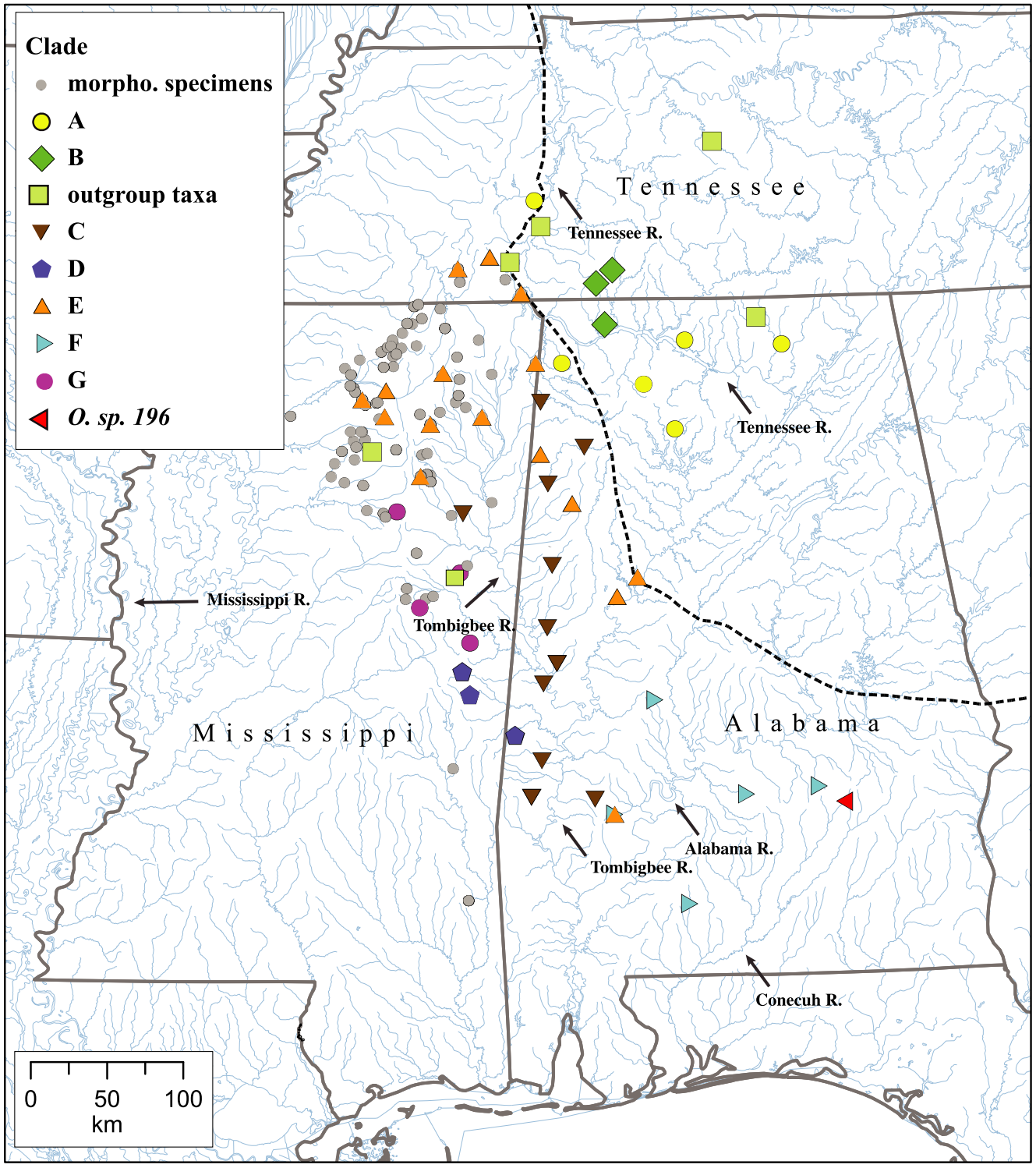

Fig. 1. Map of collecting locations of specimens used for the current study and clade assignments. Small gray dots indicate the location of specimens used for morphological analysis only. The dashed line indicates the physiographic feature known as the Fall Line. Some out-group taxa sampling locations occurred beyond the limits of this map and are not plotted.

the Appendix, which can be found in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/1937240x). To address the concerns of Song et al. (2008) that crayfish COI can contain nuclear-mitochondrial pseudogenes (numts), all COI sequences were carefully examined for the presence of indels, point mutations, and in-frame stop codons. Based on previous estimates of relationships (Crandall et al., 2000), *Procambarus acutus* (Girard, 1852) was used as the outgroup for *Orconectes* in phylogenetic analyses. Other species of *Orconectes* from other subgenera found in the eastern United States (*O.* (*Buannulifictus*) *meeki* (Faxon, 1898); *O.* (*Crockerinus*) *erichsonianus* (Faxon, 1898);

O. (*C.*) *obscurus* (Hagen, 1870); *O.* (*Hespericambarus*) *perfectus* Walls, 1972; *O.* (*H.*) *hartfieldi* Fitzpatrick, 1992b: *O.* (*Faxonius*) *indianensis* (Hay, 1896); *O.* (*F.*) *wright* Hobbs, 1948; *O.* (*Rhoadesius*) *kentuckiensis* Rhoades, 1944; *O.* (*Procericambarus*) *mirus* (Ortmann, 1931); and *O.* (*P.*) *neglectus* (Faxon, 1885)) were also added to the analyses to test the monophyly of the subgenus *Trisellescens* in that region. Given the monophyly of epigean members of *Orconectes* shown by Taylor and Knouft (2006), Breinholt et al. (2012), and Ainscough et al. (2013), members of other North American cambarid genera were not used as out-groups.

Phylogenetic analysis of the combined COI and 16S data set employed maximum parsimony and Bayesian optimality criteria. Parsimony analyses were conducted using the heuristic search option with tree bisection-reconnection branch swapping, steepest descent option, and 100 random addition sequences. Characters were treated as unordered, and all character transformations were weighted equally. Bootstrap analyses of the maximum parsimony tree was conducted with PAUP* using 1×10^5 pseudoreplicates using the fast-stepwise addition option to determine support (Felsenstein, 1985).

Prior to Bayesian analysis, the Akaike Information Criterion 1 test as implemented in ModelGenerator (v. 0.85, Keane et al., 2006) was used to determine an appropriate model of sequence evolution and to estimate its parameters for each of the two gene loci. Bayesian analyses were conducted on each gene locus and on a data matrix partitioned by locus (COI and 16S). For individual loci, searches were run with two simultaneous chains for 1×10^7 generations and sampling every 1000th generation. Burnin (Ronquist and Huelsenbeck, 2003) was determined by plotting likelihood scores for searches against generation number and looking for stationarity of scores in addition to looking for split frequencies below 0.01. For the partitioned dataset, 1×10^7 generations were run sampling every 1000th generation and burn-in was determined following the procedure used for single gene regions. Revmat, pinvar, statefreq, and shape were unlinked for partitioned dataset runs. Support for Bayesian analysis was assessed using posterior probability scores calculated as the frequency at which a particular node occurred in retained trees after each analysis reached stationarity.

For morphological analysis, the following morphometric measurements were recorded to the nearest 0.1 mm using dial calipers from fluid preserved specimens: post-orbital carapace length (POCL), areola length, areola width at its narrowest part, carapace width, carapace height, abdominal length, abdominal width, rostral length, rostral width, acumen length, antennal scale (AS) length, antennal scale (AS) width, telson length, pleopod length, central projection length, and mesial process length, and four chela measurements, including chela palm length, chela palm width, dactyl length, chela length along lateral margin. Total pleopod length (TPL) was measured from distal tip of central projection to base of knob at base of pleopod. Central projection length (CPL) was measured from distal tip of projection to anterior convergence point of "v" formed by the junction with the mesial process. Mesial process length (MPL) was measured from distal tip of process to anterior convergence point of "v" formed by the junction with the central projection. Rostral length was measured from the base of the acumen to the base of the right post-orbital spine. Rostral width was measured at the base of the post-orbital spines. Median carina, cervical spines and lateral rostral spines were scored as follows; 0 = absent, 1 = weakly present, or 2 = strongly present. Distinct excision in base of dactyl and excavation of rostrum was scored as present or absent. Tubercles along the mesial margin of the palm of the chelae were scored as 0 = low rounded or 1 = sharp and angled anteriorly. Number of punctations across narrowest part of areola, number of rows of tubercles on mesial margin of chela palm, number of palmer tubercles in most mesial row (= first row), number of tubercles in inner row (= second row), number of cervical spines, number of cephalic section telson spines, and number of sub-palmar tubercles also were recorded. All specimens from a single collecting event were assumed to be of the same taxon unless obvious differences in the primary characters traditionally used for taxonomic classification (pleopod shape, areola type, rostrum type, chelae shape) were noted. Sympatric collections of specimens with different morphotypes only occurred once and those specimens were recorded as different taxa.

We analyzed mensural morphological characters (but not qualitative or meristic characters) via sheared principal component analysis (PCA) using SAS (program developed by D. L. Swofford and modified by M. L. Warren, Jr.) (Bookstein et al., 1985; Warren, 1992). In sheared PCA, the effect of body size is concentrated in the first principal components axis, thereby removing body size effects from components II and III (Mayden, 2010). The sheared PCA procedure log transforms data. For specimens with closed areolas, areola width was zero. We added 0.01 to each 0 value to allow log transformation, but the resulting variation was so large that the procedure extracted the effect of areola width, rather than body size from components II and III. So, we removed areola width from the sheared PCA. Because many specimens had damaged, regenerated, or missing chelae, we had a larger data set of individuals without ($N = 237$) than with ($N = 177$) chela measurements, and we conducted sheared PCA on both data sets.

Non-continuous/ordinal data (meristic counts and qualitative assignments of development of characters) were not included in the SPCA, so we analyzed the ordinal data separately to assess whether any were informative for distinguishing among the clades identified in the genetic analyses. First, we conducted an agglomerative cluster analysis (PC-ORD 6.12) on all ordinal data for all measured specimens for which we had complete data ($N = 177$, excluded individuals lacking chelae). For the cluster analysis, we used relative Sorenson distance and a flexible beta group linkage method with beta $= -0.25$. We then used Kruskal-Wallis H-tests to test for significant differences among clades by each variable. Significant results were followed by post-hoc pairwise Mann-Whitney U-tests to determine which clades differed significantly from others (PASW Statistics 18.0).

RESULTS

Indels, point mutations, or in-frame stop codons were not present in any of our COI sequences. As such, we do not believe that numts are present in our COI dataset. Third codon position saturation was not present in our dataset as third position divergence values did not plateau when plotted against total sequence divergence. ModelGenerator selected the HKY + I + G model of evolution for 16S while the K81uf + I + G was selected for COI. The first 1×10^6 generations of both single and combined gene regions Bayesian analyses were discarded as the burn-in generations as log-likelihood values for these trees had not stabilized. Split frequencies between Bayesian runs were less than 0.01. With a few exceptions, Bayesian analyses of the separate gene loci recovered similar trees with the same major clades. The 16S tree (Fig. S1 in the Appendix, which can be found in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/1937240x) did not have strong support for clade A while the COI tree (Fig. S2 in the Appendix, which can be found in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/1937240x) did not recover *O. jonesi* 114 in clade D, *O. chickasawae* 117 and *O. etnieri* 345 in E, or *O. holti* 278 and 118 in clade F with strong support (Figs. S1 and S2). Sample *O. chickasawae* 271 was recovered in clade C with 16S and clade E with COI. Given the similarity in results, all proceeding results and discussion will focus on the Bayesian tree recovered from the combined analysis.

Bayesian analysis recovered a monophyletic *Trisellescens* in regards to those species represented in the dataset (Fig. 2). Within *Trisellescens*, two major groups (clade No. 1 and No. 2) and seven clades (clades A-G) were recovered with strong support (0.95 or higher). One group (clade No. 1) contains members found on or above the Fall Line and includes a monophyletic *O. validus* (clade A), a monophyletic *O. alabamensis* (clade B), *O. cooperi*, and *O. rhoadesi*. The other group (clade No. 2) contains the remaining members of *Trisellescens* found below the Fall Line, including one potentially undescribed species and five well supported clades (C, D, E, F, G). Of the terminals we tentatively identified to species using literature based morphological characters, only *O. holti* was recovered as monophyletic in clade No. 2. One specimen of *Trisellescens*

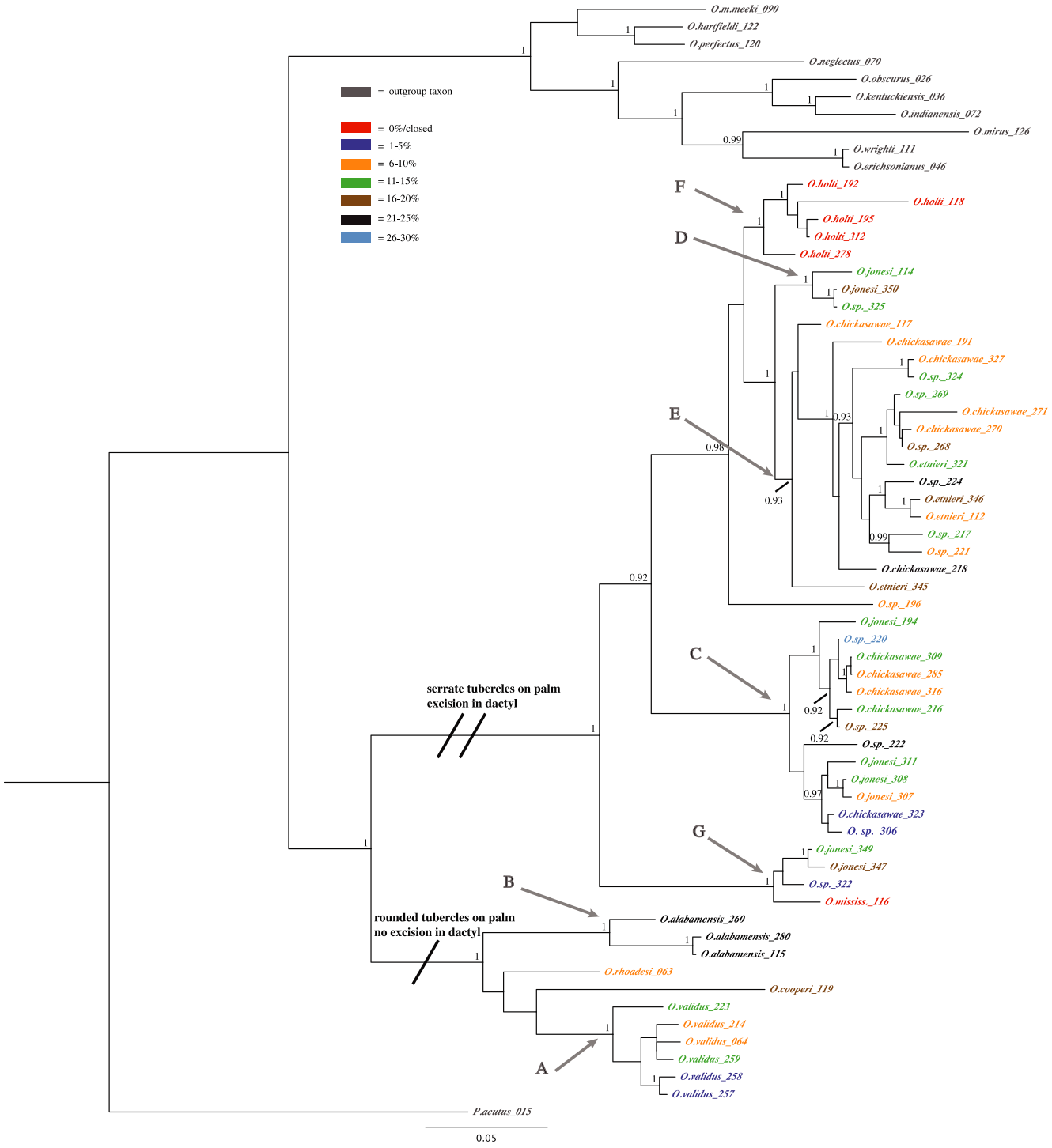

Fig. 2. Phylogram recovered from Bayesian analysis of combined partial COI and 16S sequence data. Colors indicate areola width as a percentage of carapace length: purple = 1-5%, orange = 6-10%, green = 11-15%, brown = 16-20%, black = 21-25%, blue = 26-30%. Red = closed or linear areola, gray = out-group taxa. Capital letters and No. 1 and No. 2 indicate clades as described in Results section.

(*O.* sp. 196) unidentifiable to species using morphology was recovered in clade No. 2 but was not recovered in one of the five clades (Fig. 2). This specimen was a form II male and, as such, was not used in the morphological analysis.

Parsimony analysis recovered 1248 most parsimonious trees of 1292 steps. The number of variable and parsimony informative sites for the COI region was 226 and 188 respectively, and 125 and 97, respectively, for 16S. A 50% majority rule consensus tree recovered both major groups (clade No. 1 and No. 2) as monophyletic with over 70% bootstrap support and all seven clades with identical composition with over 80% support with the exception of clade F, which re-

ceived 64% support (Fig. S3 in the Appendix, which can be found in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/1937240x). The consensus parsimony tree also differed by being unable to resolve relationships between clades D, E and F, and *O.* sp. 196, *O. chickasawae* 117 and *O. etnieri* 345. These latter two taxa were recovered as part of clade E in the combined Bayesian analysis (Fig. 2).

Morphological characters were measured and counted on 237 male form I specimens from the Tennessee, Tombigbee, Pascagoula and Mississippi river drainages. Included in these specimens were all form I specimens used for DNA analysis. Analysis of discrete morphological characters found variation in six characters; presence/absence of median carina, rostrum being excavated versus flat, type of tubercles on mesial margin of the palm, presence/absence of a distinct excision on the dactyl, number of palmer tubercles in most mesial row (= first row), and number of tubercles in inner row (= second row). The latter four chelae characters were only recorded on individuals with non-regenerated chelae (176 of the 237 specimens). The number of tubercles in both the first and second rows on the mesial margin of the palm were hyper-variable, even within individual collections of specimens. Across all specimens, tubercles in the first row ranged from 4 to 10 with mean values for all clades (clade 1, 2, A-G) recovered in Bayesian analysis ranging from 7.1 to 7.7. Across all specimens, tubercles in the second row ranged from 1 to 10 with mean values for all clades recovered in Bayesian analysis ranging from 4.5 to 6.2. Given the variation found in these two characters within and between collections and the lack of geographic or phylogenetic pattern, both were removed from further analyses. Strong median carinas were only found on individuals identified as *O. alabamensis*. Low, rounded tubercles along the mesial margin of the palm (= first row) and the absence of an excision on the dactyl were found on individuals occurring above the Fall Line and were identified as either *O. alabamensis*, *O. validus*, *O. rhoadesi*, or *O. cooperi*. The alternative character states for mesial margin tubercle shape and excision on the dactyl were found on all individuals occurring on or below the Fall Line. Flat rostrums were found on specimens identified as *O. cooperi* and *O. alabamensis* above the Fall Line and scattered across individuals found below the Fall Line in clades C and E. Geographically, specimens below the Fall Line with flat rostrums were found in the upper Tombigbee and lower Tennessee River drainages in northwestern Alabama, northeastern Mississippi, and southern Tennessee. Sample 196 of an unidentified *Orconectes* (*Trisellescens*) from the upper Conecuh River drainage also possessed a flat rostrum.

We present sheared PCA results only for the larger morphometric data set without chela measurements. In the sheared PCA on the data set including chela measurements, chela variables loaded strongly on PC-I, which removes effects of individual size, but not on sheared PC-II or PC-III (loadings < 0.21). In addition, measurements that loaded most strongly on sheared PC-II and III did not differ between analyses with and without chelae measurements.

The size axis (PC-I) accounted for 74% of the variation in the data, and sheared PCA axes II and III accounted for 11% and 6%, respectively. On PC-II, gonopod central projection and mesial process lengths were strongly contrasted with acumen length (Table 1). On PC-III, acumen length loaded strongly in the positive direction, followed by central and mesial projection lengths more weakly.

Table 1. Variance loadings for axes from Sheared PCA analysis of form I male *Orconectes* (*Trisellescens*) spp. Abbreviations explained in Materials and Methods.

Measurement	PCL (size)	Sheared PC-II	Sheared PC-III
POCL	0.304	0.048	−0.144
Carapace width	0.309	0.012	−0.148
Carapace height	0.266	0.178	−0.093
Areola length	0.319	0.067	−0.193
Rostral width	0.248	0.080	−0.055
Rostral length	0.243	0.077	−0.149
Acumen length	0.181	0.383	0.864
AS width	0.267	0.087	−0.064
AS length	0.221	0.174	0.076
Pleon length	0.210	0.141	0.040
Telson length	0.281	0.055	−0.081
Pleon width	0.267	0.039	−0.110
Pleopod TPL	0.237	−0.089	0.008
Pleopod CPL	0.237	−0.607	0.202
Pleopod MPL	0.244	−0.603	0.261

Based on plots of PC-II versus PC-III, clades A, B, D, F and G separated from one another with the exception of clade G being nested within clade F. Clades C and E form a large cloud with some degree of overlap with these other clades (Fig. 3).

Comparing genetic and morphometric results revealed several outliers in morphometric space. Specimen 116, identified in the field as *O. mississippiensis*, was a distinct outlier from the rest of clade G along both the areola width and PC-II axes (Fig. 3). The other four individuals

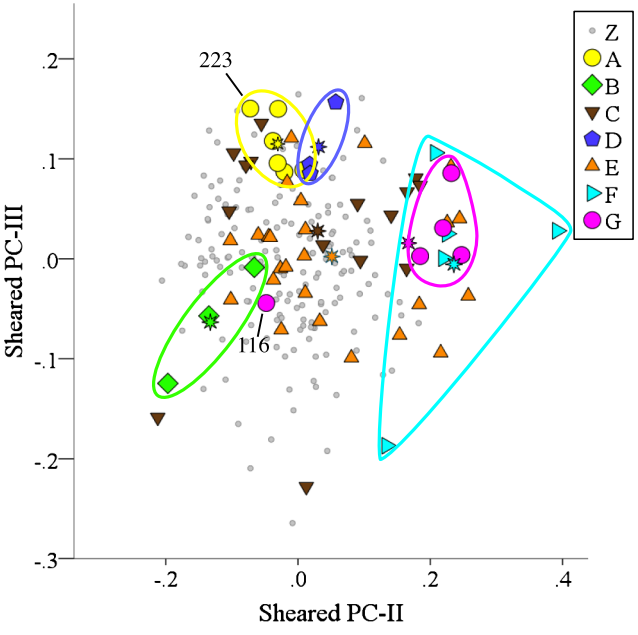

Fig. 3. Plot of sheared PCA axes II versus III for *Orconectes* (*Trisellescens*) spp. Letters in legend correspond to clades identified in Figs. 1 and 2. Z = location of specimens used for morphological analysis only.

measured from clade G were from collections 347 and 349 and were identified in the field as *O. jonesi*. Genetically, specimen 116 was somewhat distinct from the remainder of clade G, but was not distinct enough to meet our clade cutoff (Fig. 2). Specimen 223 appeared widely separated from the remainder of clade A along the areola width axis but grouped tightly with them along PC-II and -III. All members of the clade were identified in the field as *O. validus*.

In a dendrogram resulting from the cluster analysis of ordinal data, only clade F formed a monophyletic cluster. The clade F cluster was distinct from all other clades in lacking setae on the fingers and from all but clade A in having no punctations in the areola. This latter character is synonymous with having a closed areola.

Kruskal-Wallis H-tests resulted in no significant overall differences among clades for the following nine variables: number of lateral rostral spines, number of branchiostegal spines, location of widest point on antennal scale, number of rows of tubercles on mesial margin of the palm, number of tubercles in row 1, number of tubercles in row 2, number of small subpalmar tubercles, number of telson spines, and number of midcarpal tubercles or spines. Only the latter was invariate. In addition, the number of cervical spines differed significantly in the Kruskal-Wallis test, but not in any of the pairwise tests when using exact significance levels. The eight remaining variables differed significantly among clades overall and between at least one pair of clades (Table 2).

Clade F was the most easily distinguished clade, differing significantly from all others in having no setae on the fingers and from all but clade A in having no punctations in the areola, although the latter also occurred in outliers in clades C, E and G.

Clade B was the only clade with no postorbital spines, though there was no significant difference from clade D due to small sample sizes. Clade B also differed from all others in typically having no or only barely developed cervical spines.

Clade A differed from all other clades except B in having no excision in the dactyl (though one individual from clade F had no excision as well) and differed from B in many other characters, including having well-developed carina, postorbital, and cervical spines, 0-2 versus 3-4 punctations in the areola, and 1 versus 0 subpalmar tubercles.

No characters distinguished clearly among clades C, D, E and G.

DISCUSSION

General Matters

When initiated, the goal of our study was to use a molecular dataset to help resolve uncertainties in taxonomic status and distribution of members of a closely allied group of *Orconectes* crayfishes in the lower Tennessee and Tombigbee river drainages. Much of this uncertainty is rooted in the lack of cladistic analysis of morphological characters for the genus and, to a lesser extent, a lack of specimens from across purported ranges of the taxa in the group. Our field sampling (Fig. 1) has helped address the latter issue, however, these collections have not provided data to assist with the former. Historically, the recognition of species boundaries within *Orconectes* has used range and unique combinations of a handful of morphological characters, mainly shape of the form I male genitalia (gonopods), width of areola, shape of rostrum, and shape of mandible (Faxon, 1884; Fitzpatrick, 1987; Taylor, 2000). As the number of species in the genus has steadily grown to over 90 species and subspecies, additional morphological characters with discrete, easily diagnosed character states have not been reported in the literature. This strongly uneven ratio of characters to taxa has likely prevented the formulation of cladistic hypotheses of relationships. The same paucity of characters is also most likely responsible for the lack of morphology-based estimates of relationships in the other two large genera of crayfishes (*Procambarus*, *Cambarus*) found in Cambaridae.

Table 2. Results of Mann-Whitney pairwise comparisons of characters between clades (upper case letters). Clades with the same lower-case letter did not differ significantly from each other. Only ordinal characters with a significant result in Kruskal-Wallis comparisons of all clades are included. Numbers below lower-case letters are median (minimum-maximum). Differences were considered significant when exact, two-tailed p-values $\leqslant 0.05$.

Character	Clade						
	A ($N = 6$)	B ($N = 3$)	C ($N = 14$)	D ($N = 3$)	E ($N = 23$)	F ($N = 5$)	G ($N = 5$)
	a	a	b	ab	b	c	ab
Development of setae on fingers	2.0 (1-2)	2.0 (2-2)	1.0 (1-2)	2.0 (1-2)	1.0 (0-2)	0.0 (0-0)	2.0 (1-2)
	ab	c	c	c	c	a	bc
Number of punctations	0.0 (0-2)	4.0 (3-4)	3.0 (0-4)	4.0 (2-5)	2.0 (0-5)	0.0 (0-0)	3.0 (0-4)
	a	a	b	b	b	b	b
Extent of excision in dactyl	0.0 (0-0)	0.0 (0-0)	1.0 (1-1)	1.0 (1-1)	1.0 (1-1)	1.0 (0-1)	1.0 (1-1)
	a	b	a	a	a	a	a
Development of cervical spines	2.0 (2-2)	0.0 (0-1)	2.0 (2-2)	2.0 (2-2)	2.0 (2-3)	2.0 (2-2)	2.0 (2-2)
	a	b	a	ab	a	a	a
Postorbital spine development	2.0 (2-2)	0.0 (0-0)	2.0 (1-2)	2.0 (2-2)	2.0 (1-2)	2.0 (2-2)	2.0 (2-2)
	ab	a	b	b	b	b	b
Carina, present or absent	0.0 (0-1)	1.0 (1-1)	0.0 (0-0)	0.0 (0-0)	0.0 (0-1)	0.0 (0-0)	0.0 (0-0)
	a	b	c	abc	c	c	ab
Number of large subpalmar tubercles	1.0 (1-1)	0.0 (0-0)	2.0 (2-2)	1.0 (1-2)	2.0 (2-2)	2.0 (2-2)	1.0 (0-2)
	a	a	b	ab	a	ab	ab
Development of suborbital angle	1.0 (0-1)	0.0 (0-1)	1.5 (1-2)	1.0 (1-2)	1.0 (0-2)	1.0 (1-1)	1.0 (1-1)

Using partial COI and 16S mitochondrial genes, our resulting phylogeny yields conflicting information on the utility of morphological characters traditionally used for *Orconectes* classification. As such, our ability to recognize species boundaries for some members of the *Trisellescens* group is hampered. We believe that a combination of physiographic and genetic attributes may explain our results.

Our phylogeny recovers two strongly supported and divergent (12.0% average un-corrected pairwise divergence in COI, 8.0% for 16S) clades (Fig. 2) of *Trisellescens* members that are closely tied to physiography of the region. Clades A and B and our single samples of *Orconectes cooperi* and *O. rhoadesi* (clade No. 1) are only found at or above the Fall Line whereas all other specimens occur in a large clade found at or below the Fall Line (clade No. 2). The Fall Line through Alabama and other regions of the southeastern USA demarks the boundary between the Gulf Coastal Plain and provinces of the Appalachian Highlands Region. Stream reaches near the Fall Line are typically characterized by shoals and waterfalls of varying heights and the Fall Line serves as a barrier to the distribution of many other species of aquatic fauna, including fish (Swift et al., 1986; Boschung and Mayden, 2004) and mussels (Williams et al., 2008). The distribution of chelae characters (tubercle shape and presence/absence of dactyl incision) precisely tracks the above/below Fall Line split. In addition, the combination of unique character states in specimens found above the Fall Line allows for unambiguous species recognition: *O. alabamensis* possesses longer pleopod elements (elements longer than 30% of total pleopod length) (Fig. 4), well-developed postorbital and cervical spines, and a flat rostrum with a median carina; *O. validus* possesses the same type of pleopod elements with an excavated rostrum, poorly developed postorbital and cervical spines, and no median carina; *O. rhoadesi* possesses longer pleopod elements and weakly developed cervical spines; *O. cooperi* possesses longer pleopod elements, very short fingers on the chelae, chelae covered with short setae, and weakly developed cervical spines. The presence of unambiguous morphological character differences between clades 1 and 2 is also demonstrated in our molecular results. Un-corrected pairwise divergence values between members of both clades averaged 12.0% for COI and 8.0% for 16S. These values are only slightly lower than the recovered divergence values between *Orconectes* members and our *Procambarus* out-group taxon (13.0% and 9.0%) and equal to those reported for complete COI divergences between members of the same two genera by Taylor and Hardman (2002).

We believe that topography most likely explains the monophyly of species we found, using both morphological and molecular characters, in taxa found above the Fall Line. Streams in this region containing *Trisellescens* occur in the Highland Rim and Cumberland Plateau physiographic regions where elevations range from 150 to 460 m (Mettee et al., 1996). Thus streams generally have steeper gradients and form discrete sub-basins as stream valleys are narrow and can differ by up to 150 m in elevation from surrounding ridges (Mettee et al., 1996). A vicariance model of speciation could be invoked as it is likely that such steep topology leads to reduced gene flow and increased isolation in populations inhabiting these sub-basins over longer periods of time.

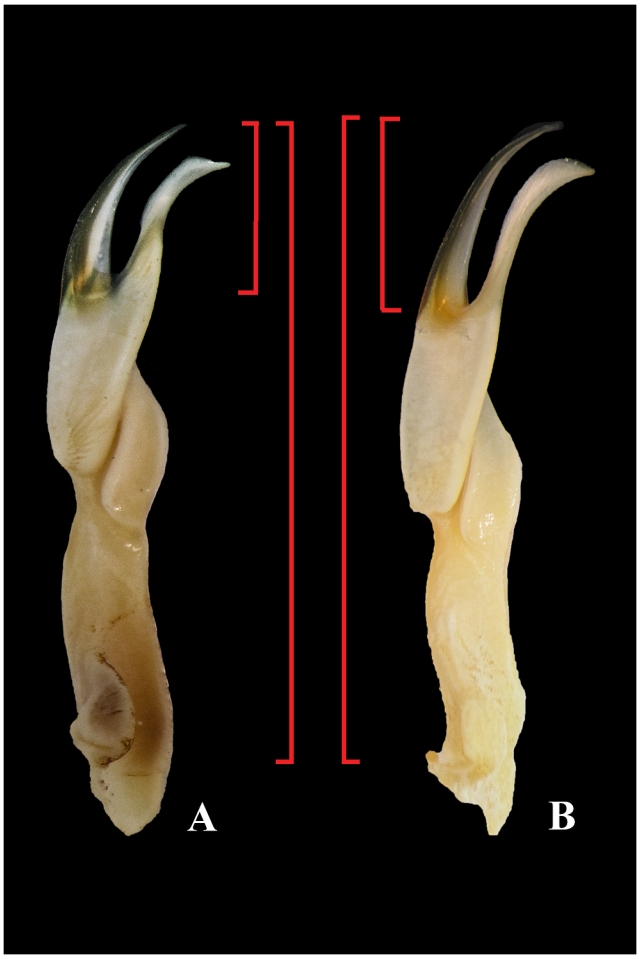

Fig. 4. Form I male pleopod types. A, *O. chickasawae* = "short pleopod elements"; B, *O. validus* = "long pleopod elements".

Our identification to species of some taxa found in clade No. 2 should be considered preliminary given the morphological characters recorded for specimens collected by us and the lack of clear distinguishing characters given in the species descriptions. This is particularly true for *O. chickasawae* and *O. jonesi*. Both species were only known to occur allopatrically in the Tombigbee River drainage and both possess the shorter pleopod (Fig. 4) form and have identical rostrums, leaving the width of the areola as the only character on which to base identifications. In their descriptions, *O. chickasawae* (Cooper and Hobbs, 1980) and *O. jonesi* (Fitzpatrick, 1992) are described as having highly variable areola widths. *Orconectes chickasawae*'s areola ranges from being closed to open with a length to width ratio of at least 6, whereas in *O. jonesi* the ratio ranges from 2 to 4. Both descriptions comment on the lack of additional specimens for morphological analysis. In many cases, we found specimens within the range of one of these species that had the areola width of the other species. *Orconectes holti* and *O. mississippiensis* identifications were based on the presence of a closed areola while *O. etnieri* were identified as such by the presence of longer pleopod elements and a flat rostrum. Specimens identified as *O.*

sp. had character combinations not matching those for the previously mentioned species in this group and the abundance of unidentified samples in our dataset attests to the taxonomic uncertainty in *Trisellescens* found below the Fall Line.

A discussion of our recovery and composition of clade No. 2, containing all samples coming from taxa found below the Fall Line, requires multiple hypotheses. Our DNA results illustrate: 1) the recovery of strongly supported and strongly divergent (8-11.0% for COI and 3.5-5.0% for 16S) clades (C-G) within clade No. 2; 2) a pattern of peripheral isolation along the southern edges of the lower Tombigbee and Alabama river drainages (Fig. 1); and 3) discordance between morphology and DNA (Fig. 2) in most cases.

Our clades D, F, and G (Fig. 1) all occur near the edges of the known distribution of *Trisellescens*. Both clades D and G occur along the southwestern edge of the subgenus' range in eastern flowing tributaries of the Tombigbee River. One sample in clade G did occur just across the drainage divide in a headwater tributary of the Yalobusha-Mississippi River drainage. The geographic distribution of clade F (lower Alabama River drainage and one headwater stream of the adjacent Conecuh drainage) represents the extreme southeastern limit of members of this subgenus. Clades C and E have wide distributions. Clade C occurs from the upper Tombigbee drainage to within 100 km of the confluence of the Tombigbee and Alabama rivers and clade E ranges from headwaters of the Hatchie (Tennessee R. dr.) and Tallahatchie (Mississippi R. dr.) drainages south within the Tombigbee drainage to within 100 km of the Tombigbee/Alabama confluence. Clades D, F and G appear to represent dispersing populations from the core range of *Trisellescens* below the Fall Line (lower Tombigbee River) and the isolation of individuals in these clades could be attributed to distance alone under a peripheral isolation model of peripatric speciation (Coyne and Orr, 2004). A similar scenario may also explain the phylogenetic and geographic position of sample No. 196. A single small form II male was collected from that location so we are unable to assign a species name to it using morphology. Given that this individual was very divergent (Fig. 2) from other members of clades D, E, and F and its peripheral (and easternmost) distribution for members of the subgenus, we believe this sample may represent a new taxon. Additional samples from this portion of the upper Conecuh River drainage will be necessary to determine its status. Our clades C and E encompass individuals occurring nearest the Fall Line in the upper and middle Tombigbee River drainage and adjacent headwater tributaries of the Tennessee and Mississippi (Hatchie and Tallahatchie) river drainages (Fig. 1).

The genus *Orconectes* likely arose from a *Procambarus*-like stock (Hobbs, 1988; Crandall et al., 2000) and Hobbs (1988) hypothesized that the genus evolved from ancestral stock on or near the Cumberland Plateau between extreme northeastern Alabama to southwestern Kentucky. From that region, members of the genus dispersed north and west (Hobbs, 1988). The lack of members of the subgenus and presence of only one member of the genus *Orconectes* in the upper Coosa and Tallapoosa river drainages in Alabama and Georgia supports that pattern in general. It suggests that members of the currently recognized *Trisellescens* have moved west off of the Fall Line and dispersed or are dispersing south, southeast and southwest along the Tombigbee River drainage. The distribution of *Trisellescens* in southern Tennessee, extreme northeastern Mississippi and northwestern Alabama indicates an ancestral connection between the lower Tennessee River and upper Tombigbee River drainages. Support for the connection can also be seen in several fish species (Metee et al., 1996; Boschung and Mayden, 2004). Likewise, the cross-basin distribution in the Tombigbee and Mississippi river drainages of *Trisellescens* is a pattern also seen in unionid mussels (Haag et al., 2002) and fish (Ross, 2002).

The distribution of morphological character states across our phylogeny supports species boundaries in some cases but not others. Our recovery of clades C, E, and G are examples of discordance. The composition of these clades contains multiple nominal species based on our preliminary identifications and thus multiple states of characters used for identification of species in *Trisellescens*. Historically, pleopod shape (length and degree of curvature of both terminal elements) has been the primary determinant for the inferred relationships of *Orconectes* crayfishes at the subgeneric level (Fitzpatrick, 1987; Hobbs, 1989; Bouchard and Bouchard, 1995) and for species recognition (Hobbs, 1989). Within *Trisellescens*, two different pleopod forms have been used for species delimitations, a short element form where the length of the terminal elements are less than 30% of the total length of the pleopod and a long element form where terminal elements are greater than 30% of pleopod length (Fig. 4). Both pleopod forms are recovered in clades C, E, and G (Fig. 5), suggesting that pleopod shape may be homoplastic and not an accurate measure of relationship between some *Trisellescens* species. The incongruence between morphology and molecular estimates of phylogeny has also been observed for the other *Orconectes* subgenera (Fetzner, 1996; Taylor and Knouft, 2006) and was recently demonstrated within the cambarid genus *Cambarus* where chelae morphology is more heavily used for phylogenetic estimates (Breinholt et al., 2012). Alternative hypotheses to our observed results are: 1) that ancestral genetic polymorphisms still exist within these clades occurring in the upper and middle Tombigbee River drainage, parts of the lower Tennessee, and adjacent tributaries of the Mississippi river drainages and incomplete lineage sorting is present; or 2) mitochondrial gene introgression/hybridization between sympatric species in this region has occurred or is occurring (Funk and Omland, 2003). Gene introgression has recently been suggested to commonly occur in other southeastern aquatic taxa (Bossu and Near, 2009; Heckman et al., 2009). Given the lower gradient and less dissected nature of the Tombigbee River drainage as compared to streams above the Fall Line, we believe that there is a higher likelihood of increased gene flow between populations. Thus geography and the lack of isolating mechanisms may be responsible for the clouded resolution for members of *Trisellescens* in this region. Without an additional independent nuclear genetic marker, it is impossible for us at this time to support or refute either of these two alternative hypotheses. During the course

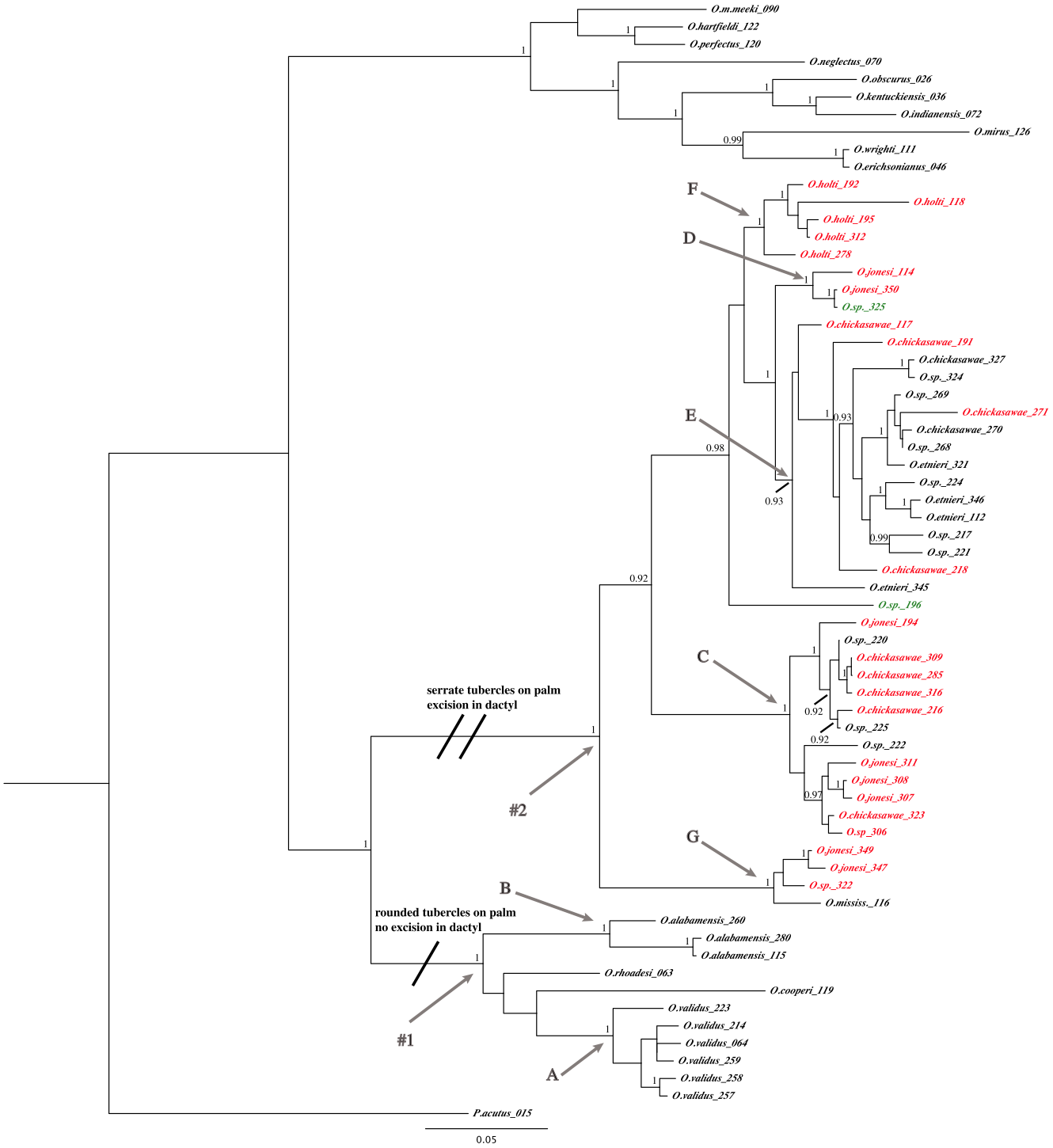

Fig. 5. Phylogram recovered from Bayesian analysis of combined partial COI and 16S sequence data. Red taxon name = short pleopod elements, black = long pleopod elements, green = unable to determine due to non-form 1 sample.

of our study, two nuclear DNA gene regions were sequenced in an attempt to create an additional dataset. However, both Histone H3 and EF1 Alpha failed to produce more than 2 polymorphic sites within *Trisellescens* taxa and thus did not provide suitable phylogenetic signal for further analysis.

Within clades D and F, a single pleopod character state is present (Fig. 5). All samples within both clades had short form I pleopod elements, although sample *O. jonesi* 325 is undetermined because only a single form II male was collected at the site. All members of clade F also possessed a closed areola.

Since it was used by Fitzpatrick (1992) and Cooper and Hobbs (1980) to distinguish *Trisellescens* species, the value of areola width as a taxonomically informative character

within the subgenus must be examined. When open, we found areola width to be highly variable within and between species and clades (Fig. 2) across the Tombigbee River and parts of the Tennessee and Mississippi River drainages with widths ranging from 1 to 29% of areola lengths (SD = 21%). For example, within samples we identified as *O. chickasawae* and *O. validus* based on range and pleopod shape, areola widths ranged from 3 to 24% and 1.8 to 15.0%, respectively, of areola length. A closed or linear areola was found in three locations on our tree, wholly within clade F, and in single samples in clades C and G. Others have hypothesized that the character may be influenced by environmental conditions (Jezerinac, 1985) and Taylor (1997) found the character to vary by latitude in one species of *Cambarus*. Our results agree with these two works and suggest that, when open, areola width is an unreliable character for assessing species boundaries and relationships.

Finally, our multivariate analysis of 20 morphological measurements using PCA and 13 discrete/ordinal characters using agglomerative cluster analysis and Kruskal-Wallis H-tests resulted in equally ambiguous results and did not provide strong support for clade level recognition of taxa. Extensive two-dimensional overlap is seen between almost all clades recovered in our DNA phylogeny (Fig. 3). Statistically significant ordinal character differences between clades are discussed below.

Taxonomic Implications

One of our goals for this study was to assess the validity of species status for the species of *Trisellescens* in our study. Our results would thus assist with species-level identifications of field-collected or museum specimens. By defining a species as a monoyphyletic clade with at least one invariable morphological character state other than areola width when open, we are able to meet that goal in some cases. However, the disagreement between morphology and molecules in some samples below the Fall Line does not allow us to form operational hypotheses of species for some taxa. The discovery and future analyses of informative molecular (nuclear) and morphological characters will likely provide a clearer perspective of species limits below the Fall Line. Until that time, we offer the following comments and propose the following species limits as a tentative taxonomic operating arrangement.

Orconectes validus and *O. alabamensis* were both easily diagnosed in the field due to the presence of unique morphological character combinations and were recovered as monophyletic in our analysis. Both taxa possess long pleopod elements (Fig. 4), non-serrate tubercles on the mesial margin of the palm. They differ in that *O. alabamensis* possess a median carina, while *O. validus* lacks one. Our analysis of ordinal data found that postorbital and cervical spine development, number of punctations in the areola, and number of subpalmar tubercles also differed between the two. Their taxonomic status and range remains unchanged.

While we only sequenced one individual of *O. cooperi*, the species has a very restricted range in the Flint River drainage on the Cumberland Plateau. The species also has a unique combination of morphological characters (long pleopod, short fingers on the chelae, and very smooth margins of the chelae palm) and was very divergent in terms of mtDNA from other samples found above the Fall Line. As such, we feel confident that the taxon is valid.

Orconectes rhoadesi is also represented in our dataset by a single individual. The species occurs in the Duck, and limited portions of the Cumberland and Tennessee river drainages in central Tennessee. A more thorough examination of morphological and molecular datasets will be needed to confirm its taxonomic status. For now, however, we find no reason to question its validity.

Tissue samples of the recently described *O. taylori* were not available for this study. The species occurs in the North Fork Obion River drainage in extreme northwestern Tennessee. Because it possesses a unique suite of characters (short pleopods, strong median carina, and low palmar tubercles), its taxonomic status should remain unchanged until an analysis of molecular markers from specimens can be integrated into a study such as this one.

Orconectes holti is supported as a valid taxon with both molecular and morphological data. The species has a unique character combination of short pleopod elements (Fig. 4), serrate tubercles along the palm, no long setae on the fingers of the chelae, and a closed areola. Its range remains unchanged from that cited by Hobbs (1989), that being in the lower Tombigbee and Alabama River drainages in southcentral Alabama.

Orconectes mississippiensis is diagnosed by having a closed areola and long pleopod elements (Cooper and Hobbs, 1982). We collected only one sample that matched that description (sample No. 116). Sample No. 116 is recovered as the basal-most member of clade G. Since other members of that clade contain both short and long pleopod elements (Fig. 5), in addition to both wide and closed areolas, we cannot assign diagnostic morphological criteria for this lineage. Further complicating the recognition of *O. mississippiensis* is ambiguity of its type-locality, given as "eastern Mississippi" by Faxon (1884). Until additional characters can be analyzed, we withhold judgment on the validity of *O. mississippiensis* as a name applied to *Trisellescens* populations currently known to occur in eastern flowing tributaries of the Tombigbee River drainage upstream of the Sucarnoochee River and in adjacent headwaters of the Yalobusha River drainage.

The remaining named species (*O. chickasawae*, *O. etnieri*, and *O. jonesi*) did not form monophyletic groups based on DNA sequences. In addition, the morphological character states of both pleopod shape and areola width were variable and multivariate character analysis was inconclusive for diagnosing clades. Our identification of specimens as *O. jonesi* was based on the presence of a wide areola (>25% of areola length) and short pleopod elements following Fitzpatrick's (1992) description. This results in a polyphyletic *O. jonesi* in our tree. For example, one specimen in clade D had an areola of less than 12% while numerous individuals in clade C and one in clade E possessed both short pleopod elements and wide areolas (Fig. 2). Specimens in clade D do appear to have a discrete peripheral range within *Trisellescens* and may represent *O. jonesi* if areola width is discounted as a reliable character. Since this clade occurs in the Sucarnoochee River drainage as listed in its description

by Fitzpatrick (1992), we suggest that the name be applied to individuals with short pleopods found in that drainage.

Assigning names to members of our clades C and E is not possible. Prior to analysis, two species names or *O.* sp. were assigned to the specimens collected in the upper and middle Tombigbee River drainage and adjacent headwater tributaries of the Tennessee and Mississippi (Hatchie and Tallahatchie) river drainages. *Orconectes* sp. was used for specimens with long pleopod elements and a narrow areola and *O. chickasawae* was used if in possession of the same areola but short pleopod elements. The name *O. etnieri* was applied to specimens collected in the Tennessee or Mississippi river drainages of western Tennessee with long pleopod elements since the literature restricted its range to those drainages (Bouchard and Bouchard, 1976; Hobbs, 1989). Besides the extreme upper Tombigbee and adjacent headwater tributaries of the Tennessee and Mississippi (Hatchie and Tallahatchie) river drainages, the clades C and E have sympatric ranges, and members of both clades possess both pleopod types and widely variable areola widths (closed, narrow to wide) (Fig. 2). If our molecular results are to be interpreted as the true evolutionary history, then no combination of morphological characters is available to delimit species within clades C and E. Clearly, imperfect taxonomy could be partially responsible for the discordance between the species tree and gene tree. Average uncorrected pairwise divergence rates within both clades (clade C = 2.6% COI, 0.9% 16S; clade E = 3.5% COI, 1.6% 16S) suggest phylogenetic structure within both and border on species level recognition for some within-clade lineages. Our field collection procedures usually consisted of vouchering all individuals encountered, with one or two individuals having muscle tissue preserved for DNA analysis. Sympatric collections of long vs. short pleopod types were not observed in any of our site collections; however, within-sample variation in areola width was frequently present. Our lack of sympatric collections of pleopod types at individual sampling locations adds support to the contention that some phylogenetic structure has begun to occur within clades C and E. Species-level diversity within *Orconectes* is generally lower in the Coastal Plain compared to the Central Highland regions and collections of sympatric species *Orconectes* in that former region is rare.

Until additional independent nuclear molecular markers that show adequate levels of phylogenetic signal between closely related taxa are identified and larger within-site collections of tissue are made, it is impossible for us to determine if current or historical hybridization, gene introgression, or incomplete lineage sorting may be occurring in these clades. Since both clades are strongly divergent from one another (average pairwise divergence = 9.7% COI, 4.3% 16S) two evolutionary lineages are present but until new informative molecular and/or morphological characters are found, we suggest that "*O. etnieri* species complex" be applied to species of *Trisellescens* in clades C and E as *O. etnieri* is the oldest available name.

CONCLUSIONS

We used both molecular and morphological characters to examine the evolutionary history of a group of similar crayfish found in a relatively small geographic region. While our ability to draw conclusions based on our analyses of morphology and molecules is hampered by imperfect taxonomy, our results illustrate the continued discordance between both types of data within North American crayfish. We believe it is unwise to wholly accept recovered phylogenies based on a single data type. The agreement between both types of data in specific geographic regions (above the Fall Line) suggests that morphology may not be too plastic to be of use. These data support the current taxonomy for *Orconectes* (*Trisellescens*) in that region of the southeastern United States. However, our recovery of deeply divergent groups without diagnostic morphological characters on and below the Fall Line, alternatively suggests that the morphological signal has been obscured by selective pressure or historical genetic events. Until new sources of phylogenetic signal are found, we reject the hypothesis of species status for one taxon below the Fall Line. Taken in total, our results indicate the importance of fine-scale examination of both morphology and molecules when examining the biogeographic and evolutionary history of crayfish species.

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APPENDIX

Table S1. Locality and depository information for all taxa sequenced. * = topotypes, INHS = Illinois Natural History Survey Crustacean Collection, uncat. = uncataloged, Cr. = creek, R. = river, Br. = branch, Fk. = fork, trib. = tributary, Co. = county.

Species	Sample locality (lat. (N), long. (W))	INHS catalog number	Genbank accession number
Orconectes (*Trisellescens*)			
O. validus 214	Borden Cr., Lawrence Co., AL; 34.4974, 87.3868	11169	Pending
O. validus 258	Dry Cr., Limestone Co., AL; 34.7517, 87.0861	11278	Pending
O. validus 257	trib. Rock Cr., Winston Co., AL; 34.2298, 87.1722	11325	Pending
O. validus 259	unnamed spring, Colbert Co., AL; 34.6307, 87.9672	11360	Pending
O. validus 064	Turkey Cr., Decatur Co., TN; 35.5923, 88.1449	8852	Pending
O. validus 223	Hurricane Cr., Madison Co., AL; 34.7122, 86.3946	11184	Pending
O. cooperi 119*	Briar Fk., Madison Co., AL; 34.8760, 86.5704	9010	Pending
O. rhoadesi 063	trib. Harpeth R., Williamson Co., TN; 35.9209, 86.8461	8784	Pending
O. alabamensis 115	Fantail Br., Wayne Co., TN; 35.0981, 87.7107	9007	Pending
O. alabamensis 280	Factory Cr., Wayne Co., TN; 35.1752, 87.5930	11330	Pending
O. alabamensis 260	Buffler Spring, Lauderdale Co., AL; 34.8559, 87.6549	11243	Pending
O. sp. 225	Buttahatchie R., Marion Co., AL; 34.1326, 87.8184	11182	Pending
O. sp. 220	Beaver Cr., Lamar Co., AL; 33.9196, 88.0786	11159	Pending
O. sp. 306	Factory Cr., Sumter Co., AL; 32.7383, 88.1325	11899	Pending
O. sp. 222	Gum Cr., Franklin Co., AL; 34.4023, 88.1211	11180	Pending
O. sp. 196	Olustee Cr., Baldwin Co., AL; 32.0032, 86.0606	11188	Pending
O. sp. 325	Pewticfaw Cr., Kemper Co., MS; 32.6802, 88.6493	Uncat.	Pending
O. sp. 324	Bay Springs Br., LaFayette Co., MS; 34.4281, 89.3942	Uncat.	Pending
O. sp. 221	Yellow Cr., Tuscaloosa Co., AL; 33.3603, 87.4607	11165	Pending
O. sp. 217	Hells Cr., Fayette Co., AL; 33.8028, 87.9104	11141	Pending
O. sp. 268	Bear Cr., Tishamingo Co., MS; 34.6343, 88.1543	11207	Pending
O. sp. 269	Sand Cr., Itawunba Co., MS; 34.3210, 88.5414	11204	Pending
O. sp. 224	Hamilton Mill Cr., Marion Co., AL; 34.0964, 88.1293	11172	Pending
O. sp. 322	Topashaw Cr., Chickasaw Co., MS; 33.7647, 89.1501	Uncat.	Pending
O. chickasawae 309	Tussle's Cr., Greene Co., AL; 32.8569, 88.0348	11913	Pending
O. chickasawae 285	Hughs Cr., Pickens Co., AL; 33.0704, 88.0990	11273	Pending
O. chickasawae 316	Tussle's Cr., Greene Co., AL; 32.8569, 88.0348	11913	Pending
O. chickasawae 216	Coalfire Cr., Pickens Co., AL; 33.4367, 88.0584	11171	Pending
O. chickasawae 323	trib. Chuquatonchee R., Monroe Co., MS; 33.7496, 88.6828	Uncat.	Pending
O. chickasawae 327	Duncan's Cr., Pontotoc Co., MS; 34.3277, 89.2347	Uncat.	Pending
O. chickasawae 271	Soctalaoma Cr., Chickasaw Co., MS; 33.9729, 88.9823	11205	Pending
O. chickasawae 270	Mubby Cr., Pontotoc Co., MS; 34.2816, 88.9095	11206	Pending
O. chickasawae 218	Mill Cr., Tuscaloosa Co., AL; 33.2481, 87.6061	11170	Pending
O. chickasawae 191	Beaver Cr., Wilcox Co., AL; 31.9619, 87.6532	11190	Pending
O. chickasawae 117	Little Tallahatchie R., Union Co., MS; 34.4818, 89.2239	9027	Pending
O. jonesi 194	Mill Cr., Marengo Co., AL; 32.0515, 87.7870	11156	Pending
O. jonesi 311	Kinterbush Cr., Choctaw Co., AL; 32.2844, 88.1505	11919	Pending
O. jonesi 307	Wahalak Cr., Choctaw Co., AL; 32.0695, 88.2277	11917	Pending
O. jonesi 308	Wahalak Cr., Choctaw Co., AL; 32.0695, 88.2277	11917	Pending
O. jonesi 350	Running Tiger Cr., Kemper Co., MS; 32.8173, 88.7001	Uncat.	Pending
O. jonesi 114	Alamuchee Cr., Sumter Co., AL; 32.4386, 88.3375	9050	Pending
O. jonesi 347	Mill Cr., Winston Co., MS; 33.1977, 88.9947	Uncat.	Pending
O. jonesi 349	Sand Cr., Noxubee Co., MS; 32.9852, 88.6449	Uncat.	Pending
O. etnieri 321	Hatchie R., Union Co., MS; 34.5812, 88.8158	Uncat.	Pending
O. etnieri 112	Snake Cr., McNairy Co., TN; 35.2644, 88.4732	9033	Pending
O. etnieri 346	Keith Br., McNairy Co., TN; 35.1984, 88.7022	Uncat.	Pending
O. etnieri 345*	Houston Br., Hardin Co., TN; 35.0486, 88.2515	Uncat.	Pending

Table S1. (Continued.)

Species	Sample locality (lat. (N), long. (W))	INHS catalog number	Genbank accession number
O. holti 118	Sand Cr., Perry Co., AL; 32.6336, 87.3569	9057	Pending
O. holti 312	Burnt Corn Cr., Conecuh Co., AL; 31.4255, 87.1477	11925	Pending
O. holti 195	Dry Cedar Cr., Lowndes Co., AL; 32.0637, 86.7363	11153	Pending
O. holti 192	Beaver Cr., Wilcox Co., AL; 31.9619, 87.6532	11190	Pending
O. holti 278	Ramer Cr., Montgomery Co., AL; 32.0993, 86.2301	11461	Pending
O. mississippiensis 116	Catalpa Cr., Oktibbeha Co., MS; 33.4001, 88.7102	9018	Pending
Orconectes (*Buannulifictus*)			
O. m. meeki 090	Big Piney Cr., Newton Co., AR; 35.7815, −93.2907	8899	Pending
Orconectes (*Crockerinus*)			
O. erichsonianus 046	Turnbo Cr., Decatur Co., TN: 35.4408, 88.1002	8356	Pending
O. obscurus 026	Nine Mile Cr., Onieda Co., NY; 43.2072, −75.1435	6739	Pending
Orconectes (*Hespericambarus*)			
O. hartfieldi 122	Cowpen Cr., Calhoon Co., MS; 34.1020, 89.3229	9048	Pending
O. perfectus 120	Catalpa Cr., Oktibbeha Co., MS; 33.4005, 88.7102	9019	Pending
Orconectes (*Faxonius*)			
O. indianensis 072	Clifty Cr., Johnson Co., IL; 37.5707, 88.7258	8783	Pending
O. wright 111	S. Fk. Beason Cr., Hardin Co., TN; 35.2314, 88.3245	9034	Pending
Orconectes (*Procericambarus*)			
O. mirus 126	Cane Cr., Lincoln Co., TN; 35.1869, 86.6273	9031	Pending
O. n. neglectus 070	Indian Cr., Stone Co., MO; 36.5057, −93.5268	8789	Pending
Orconectes (*Rhoadesius*)			
O. kentuckiensis 036	Sinking Cr., Christian Co., KY; 36.8816, 87.6093	7421	Pending
Procambarus (*Ortmannicus*)			
P. acutus 015	trib. Cypress Cr., Union Co., IL; 37.3616, 89.0687	6763	AF474366

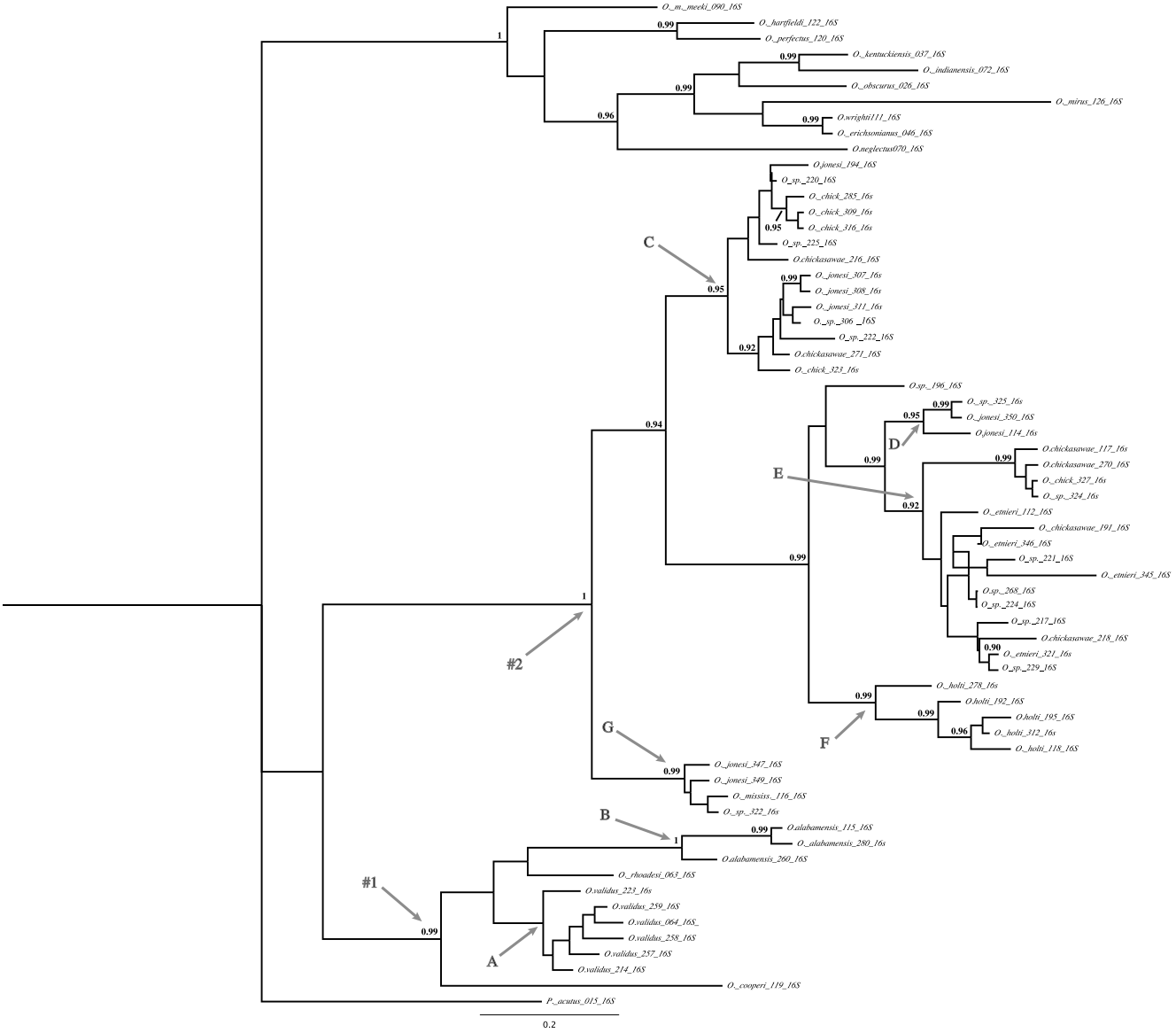

Fig. S1. Phylogram recovered from Bayesian analysis of 16S sequence data. Numbers indicate posterior probabilities. Clade labels as described in Fig. 1.

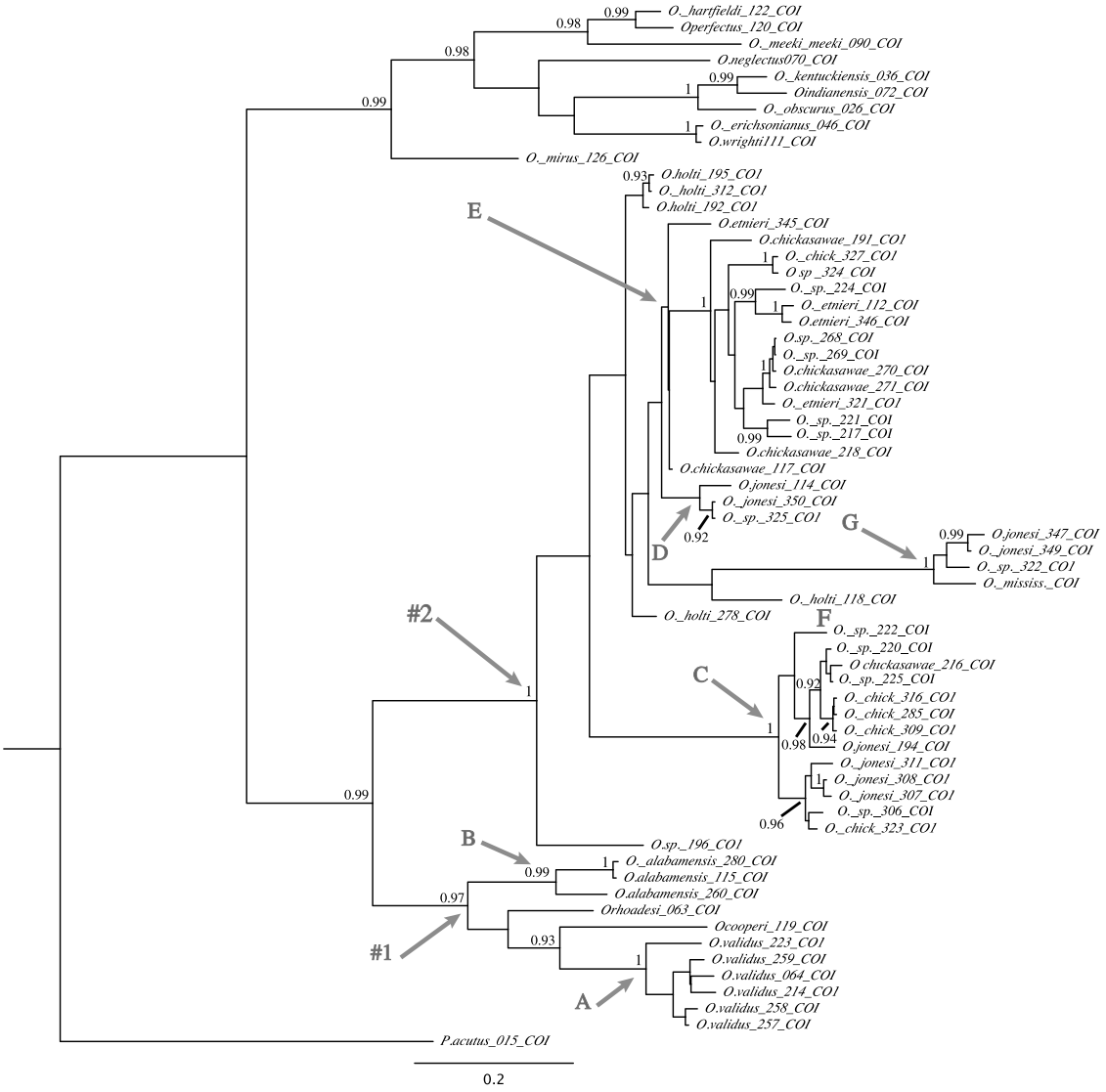

Fig. S2. Phylogram recovered from Bayesian analysis of partial COI sequence data. Numbers indicate posterior probabilities. Clade labels as described in Fig. 1.

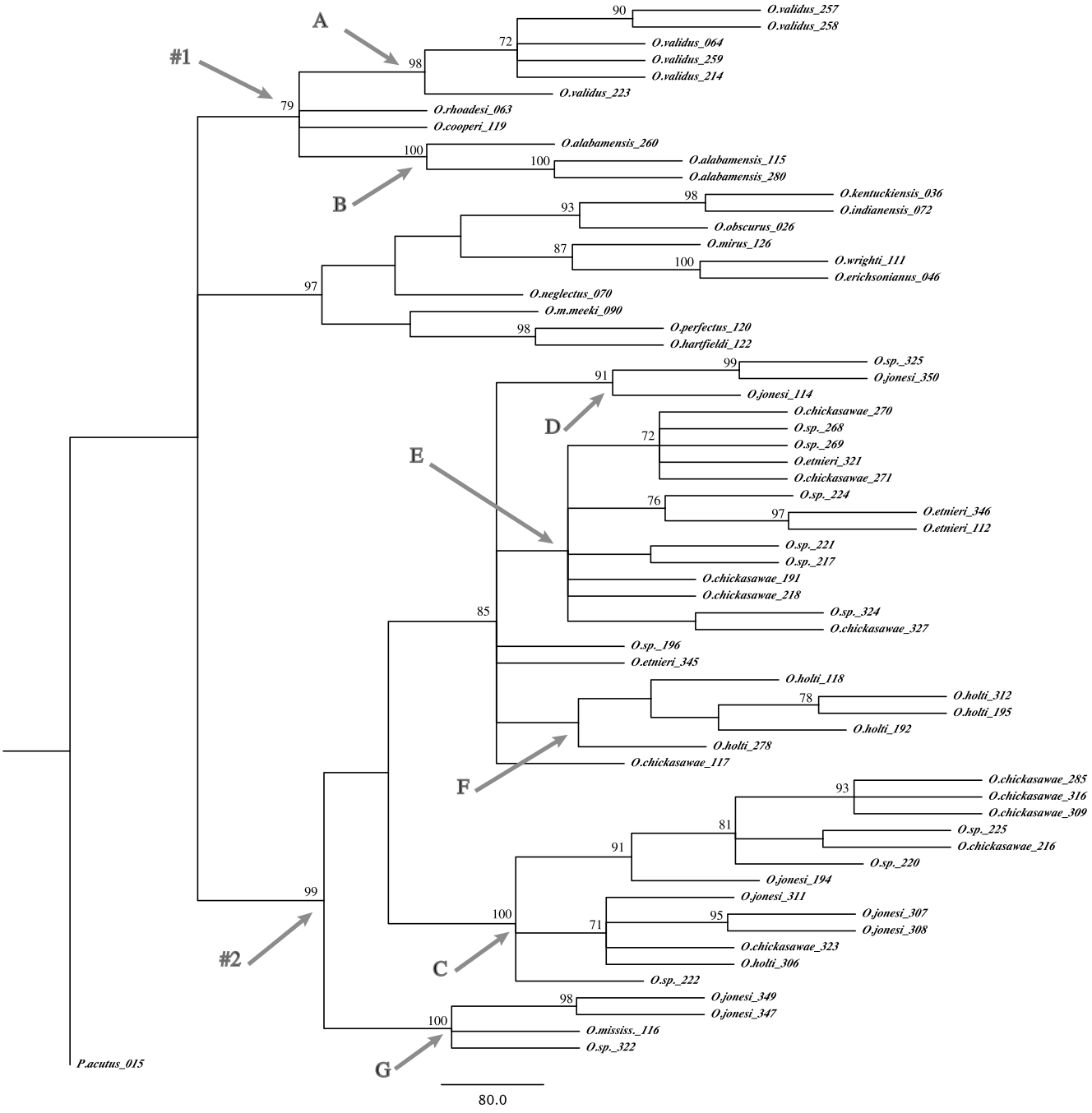

Fig. S3. 50% Majority rule consensus tree of 1248 most parsimonious trees from analysis of combined 16S and COI sequence data. Numbers indicate bootstrap support values from 100 000 pseudoreplicates. Clade labels as described in Fig. 1.