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Phylogeny of the freshwater crayfish subfamily Cambarinae based on 16S rDNA gene analysis

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ABSTRACT

Freshwater crayfish have been a mainstay in biological experiments as a model species ever since Huxley's seminal publication *The Crayfish*. Crayfish have been used in research ranging from vision pigment studies to neural physiology. Non-native species have been introduced on four continents due to their immense economic value. Although crayfish taxonomy is reasonably well resolved at the highest levels, there are many problems at the levels of genus and species. New exploration, technology and methodology have led to the discovery of not only new species but to a phylogenetic complexity that would not have been imagined in Huxley's era. This complexity is caused by the conservatism of some morphological characters, high intraspecific diversity and convergence. The ambiguity of crayfish taxonomy is particularly evident for species native to South Georgia and North Florida, which are centers of crayfish diversity. Molecular phylogenetic analyses were employed to provide insight into three aspects of crayfish phylogeny. Using partial data from the 16S ribosomal gene, we determined: (a) the evolutionary relationships of a previously unanalyzed species, *Procambarus spiculifer*, (b) relationships within the genus *Procambarus*, and (c) the phylogeny of the entire subfamily Cambarinae. The resulting maximum likelihood tree produced phylogenies that were significantly different from the traditional systematic representation of relationships within the subfamily. Specifically, we show that the subfamily Cambarinae should not be divided into three distinct clades according to the genera *Procambarus*, *Cambarus*, and *Orconectes*. While most members of the genus *Procambarus* cluster within a single monophyletic clade, the genus *Orconectes* comprises a parayphyletic grouping that appears to also include members of the genus *Cambarus*.

KEYWORDS: *Procambarus spiculifer*, Cambaridae, 16s rDNA, astacidae, parastacidae, phylogenetics

INTRODUCTION

Since the late 1800s our knowledge of the origin, physiology, and taxonomy of freshwater crayfish has greatly expanded. New exploration, technology and methodology have led to the discovery of not only new species but also a phylogenetic complexity that would have not been imagined in Huxley's era [1]. Although crayfish taxonomy is reasonably well resolved at the highest levels using historically morphologically based approaches, there are many problems at the levels of genus and species. Much uncertainty among relationships is caused by high conservatism of some morphological characters, high intraspecific diversity in others and convergence among characters across habitat diversity [2]. The ambiguity of crayfish taxonomy is especially evident for species native to South Georgia and north Florida, which are near centers of crayfish diversity [3]. This study is intended to

provide some clarity to freshwater crayfish phylogeny.

In the past, phylogenetic trees of crayfish were almost wholly based on a wide variety of morphological characteristics that included body mass, shape of appendages, and color [4, 5]. An underlying problem with morphologically based taxonomy is its subjective nature often resulting in conflicting clades because of the use of different, arbitrarily chosen morphological features [5]. Morphological analyses are also susceptible to errors created by mistaking analogous features with those of homologous attributes [6].

The shortcomings of systematic classifications based on phenotypic analyses have led scientists to look for more reliable methodologies. Molecular techniques have become an important tool for estimating phylogenies. Decapods, as a whole, are an example of a group of organisms that were originally classified using morphology only, and are just now being reassessed using molecular phylogenetic procedures [7].

Within the crayfish, the subfamily Cambarinae does not have a group of characteristics that can be viewed as invariably indicative of each of its contained genera [4]. For example, one genus, *Procambarus*, is the most morphologically diverse of all freshwater crayfish genera [3]. Such diversity, coupled with a lack of clearly diagnostic characters, creates severe problems when using traditional phenotypic analyses for determining evolutionary relationships within the group. This study employed molecular phylogenetic analysis to provide insight into three aspects of crayfish phylogeny within the subfamily Cambarinae. Using data from the 16S ribosomal gene, we examined: (a) the evolutionary relationships of a previously unanalyzed species, *Procambarus spiculifer*, (b) the relationships among species within the genus *Procambarus*, and (c) the phylogeny of species from three genera in subfamily Cambarinae.

History of crayfish systematics

A brief introduction to decapod systematics is necessary to understand the basis of freshwater crayfish classifications. There are two schools of thought concerning the taxonomic status of decapods. One school, which is currently the most widely used, traditionally ignores the fossil record. The product of this approach is the division of decapods into two suborders, Natantia, which contains the Penaeids, Carideans, and Stenopodids, and the Reptantia, which contains the remaining decapods [8, 7]. The Reptantia (decapods including crabs, crayfish, and lobsters) are further divided into the traditional infra-orders of Astacura (crayfish), Palinura (spiny lobsters), Anomura (hermit crabs), and Brachyura (true crabs) [9].

The second approach utilizes new taxa, rather than traditional groupings and places a significant amount of importance upon the fossil record. The three clades created by this approach are the Nectochelida, Gastralida, and Anomocarida. Nectochelida consists of Stenopdidea (small shrimp-like decapods), Astacura (crayfish), and Dendrobranchiata (prawns). Polychelida, Achelata (infra-order formerly known as Palinura), and Brachyura (crabs) make up the Gastralida group. Anomocarida contains the infra-orders of Thalassinida (a small burrowing crayfish also known as the yabby or mud lobster), Paguridea (currently the super-family which contains hermit crabs), and Caridea (true shrimp) [7].

Despite the disagreements over relationships amongst the various decapod taxa produced by these two methodologies, the classification of decapods remained stagnant until the 1990's [7]. Scholtz and Richter [10] reinvigorated decapod systematics by applying the concepts of molecular cladistics to elucidate the relationships among Decapoda. Their research focused on Reptantia and created some interesting clades as well as new terms for groupings [7]. This research led to the reclassification of Decapoda by highlighting the inconsistencies between morphology-based and genetics-based phylogenies.

Freshwater crayfish systematics has undergone a unique history of reclassifications independent of the Decapoda as a whole. Although freshwater crayfish have been exploited for an assortment of scientific purposes, the use of molecular technology has rarely been used for their classification. Instead, most arrangements still rely on the works of Huxley [1] and Hobbs [3, 5] who created their

phylogenies using morphological, ecological, fossil and distributional data.

Initially Huxley [1] advocated two distinct origins of crayfish based on apparent radiations derived from regions in close proximity to currently extant centers of diversity. These current centers of diversity are in the southeastern United States and Victoria, Australia. This wide geographic separation was considered by Huxley to be reflective of the group's polyphyletic origins.

Current evidence supports an opposing hypothesis, first proposed by Ortman [11], that freshwater crayfish are monophyletic [12]. Crandall *et al*. [12] and Crandall and Fitzpatrick [13] provided evidence for this hypothesis and estimated the phylogenetic distances within the Cambaridae family of freshwater crayfish utilizing ribosomal DNA sequences. The firm establishment of freshwater crayfish as a monophyletic group allowed researchers to use a rigorous comparative approach when studying diverse questions in crayfish [12].

Freshwater crayfish systematics

Morphological characteristics have been the basis for almost all systematic examinations of freshwater crayfish since their initial classification. Secondary sexual structures are normally diverse and unique [14]. However, the diversity of secondary sexual structures has actually led to their exclusion in some systematic studies because the diversity becomes too excessive. Exclusion of secondary sexual structures eliminates a vital tool in freshwater crayfish systematics. Fortunately, the first pleopod, or swimmeret, of male crayfish can be used as a substitute for other excluded features and still allow for clearly defined systematic separations [14]. However, secondary sexual features appear to be one of the most reliable characteristics in the assessment of relationships within the genus *Procambarus*, with the male's morphology providing the clearest picture [4, 14].

Male freshwater crayfish are found in one of two stages, form I or form II. Form I are breeding males and are referred to as the adult stage of male crayfish. Generally, males molt after the breeding season and transform back to form II morphology [14]. Form II males do not have horny corneous projections on the distal ends of their first pleopod [14]. The pleopods are found under the abdomen and are considered to be the primary swimming legs of freshwater crayfish [1]. In form I males, the first one or two pairs of pleopods also serve as male reproductive organs for the transmission of the spermatophore to the female's annulus ventralis. Form I male crayfish can be used to distinguish between closely related subspecies by using the morphology of the first pleopods. The first pleopod has also been useful in differentiating species groups that have experienced convergent evolution and, therefore, have analogous physical features [14]. However, previous phylogenetic studies have shown that convergent evolution and the reversion of morphological features to primitive states can lead to the creation of morphologically based clades that inaccurately reflect evolutionary relationships [13, 15].

Based on characters such as those just described, freshwater crayfish are currently classified in the infra-order Astacidea, which includes the super-families Astacoidea (Northern Hemisphere crayfish), with over 350 species, Nephropoidea (clawed lobsters), and Parastacoidea (Southern Hemisphere crayfish) [5, 12, 16]. Parastacoidea contains only a single family, Parastacidae, which is comprised of 14 genera and incorporates around 180 known species. Australia is the home of nine of the 14 genera, with three other genera distributed in the southern region of South America. New Zealand and Madagascar both contain endemic genera belonging to this family as well [5].

Within the super-family Astacoidea, males of the family Astacidae, unlike males of other crayfish families, never exhibit cyclic dimorphism [5]. Their first pleopod's distal portion possesses a cylindrical form while the distal-most part is contracted to form a tube or simple spoon-like lobes. Also, the ischia of male periopods do not possess coital hooks. In contrast, males of the family Cambaridae do exhibit cyclic dimorphism, having both first and second form males. Their pleopods either bear a shallow sperm groove mesially or the distal portion is tightly folded, with the distal end of the sperm groove opening on one of 2-4 terminal elements, depending on the species [5].

The subfamily Cambarinae is currently comprised of ten genera. Of these, this study focuses on the three *Cambarus*, *Orconectes* and *Procambarus*. *Cambarus* species range from Minnesota and coastal New Brunswick into Texas and the panhandle of Florida. Thirty-three of the 70 species are known to occur in Georgia. One distinguishing feature of *Cambarus* is that males have an opposable margin of the dactyl of the chela without abrupt excision in the proximal half [14].

Orconectes is found in most of North America, with the notable exception of the eastern seaboard from South Carolina to Florida. Seventy-five species and subspecies are currently recognized as belonging to the genus. Only three species of *Orconectes* are known to occur in Georgia. *Orconectes* are recognizable by the straight first pleopod, with both terminal elements forming at least one-fifth of the total length of the entire pleopod [14].

The genus *Procambarus* is of particular interest to this study because of the vast number of diverse species placed within it which, coupled with the fact that most taxonomies have relied on traditional phenotypic data, has led to considerable uncertainty regarding freshwater crayfish evolutionary relationships. The range of this genus currently includes the whole of Central and North America. Twenty-eight of the 148 currently recognized species are found in Georgia [14]. Species within *Procambarus* are traditionally identified by possession of antennae that are free of conspicuous fringes on the mesial border. They are further identified by having the third maxilliped teeth resting on the mesial margin of the ischium [5, 14]. The first pleopods of Form I males are at times deeply withdrawn between the bases of the periopods, while those of Form II males are more fully exposed. Pleopods that are not deeply withdrawn in Form I males are partially concealed by setae that extend from the ventro-lateral margin of the sternum [14]. Females of the genus have an annulus ventralis that is freely movable, though in some species it may be partially covered ventrally by caudally projecting prominences. These caudal prominences originate from the most anterior cephalic sternal plate [5].

One particular member of *Procambarus* is *P. spiculifer*. There is very little known about this species outside of some aspects of its basic life history. Investigations into the phylogenetic position of *P. spiculifer*, as with other species of its genus, have mainly been confined to traditional morphological examinations [3, 17]. As mentioned above, the genus *Procambarus* has a history of uncertainty concerning its interrelationships. Hobbs [4] illustrated this with an example from his own work concerning the relationships between *P. spiculifer* and *Cambarellus montezumae*. If one only compared the number of terminal processes on the first pleopod of the Form I male, the two species would be considered identical because they both lacked the same process. However, when the arrangements, proportionate sizes, and other general conformations of the appendages were studied, it was obvious that the two species were only distantly related. With the advent of techniques such as DNA analysis, such problems can be bypassed, allowing reevaluation of traditional evolutionary relationships in light of these new data [2].

The lack of a comprehensive genetic analysis of *P. spiculifer* is a conspicuous hole in our understanding of relationships within the subfamily Cambarinae, and of decapods generally. This study seeks to elucidate some of the interrelationships within Cambarinae, specifically *P. spiculifer's* relationships with other *Procambarus* species.

Molecular genetic analyses in crayfish

More attention and resources are being focused on crayfish now due to their dwindling numbers and the imperiled status of more than half the known species [18]. Low population numbers, small geographic ranges and loss of habitat are some of the major causes for the endangerment of crayfish species [18]. Many national and international organizations, as well as local and state governments, are beginning to invest funds into research on this important aquatic organism. Although crayfish have been recognized as an important study animal in biology since the late 1800's it is only recently that these organisms have been examined from a genetic perspective [19, 20]. Genetic data may play an important role in the conservation of freshwater crayfish. However, there is a limited amount of genetic data available, and for a mere fraction of the world's described species [19].

Genetic data are needed to better address both applied and fundamental questions regarding crayfish. As discussed above, morphology has been an important tool for investigating past evolutionary history; however, many characters can be plastic or convergent [21, 19]. Our understanding of crayfish phylogenetics can be much improved by the incorporation of genetic data analyses into reconstructions of species relationships.

Procambarus spiculifer

Procambarus spiculifer [22] is distinguished morphologically by a rostrum that has marginal spines and lacks a carina, the raised bump in the median of the rostrum in certain species. The carapace is normally pale tan and has two pairs of cervical spines, although occasionally there may only be a single pair. The rostral margins are cephalolateral; there are also caudal margins [14]. The postorbital ridges of the carapace are normally dark brown. Males have hooks on the ischia of the third and fourth periopods. The hooks are asymmetrical and extend to the coxae of the third periopods [14].

The prominent and distinct color markings of this crayfish, found in the mandibular adductor region and the rostrum, may be species-specific. The mandibular adductor region possesses conspicuous reticulate dark brown patches, which are paired with dorsal longitudinal stripes extending from rostrum to cervical groove. The stripes become almost black just before merging with the caudomesial margins, which also show reticulate patches. The areola is usually straw brown and is darker along the branchiocardiac grooves [14].

The geographical range for *Procambarus spiculifer* is from the Altamaha River basin in Georgia, southward to the Saint Mary's and Suwannee River basins in Florida. It is also found in the majority of the Alabama River basin in Georgia, and throughout southern and eastern Alabama and the Florida panhandle [14]. *Procambarus spiculifer* is restricted to lotic waters and can be found in habitats that vary in size and flow rate. The largest populations have been found inhabiting riffle areas in which the rocks are partially buried by shifting sand. *Procambarus spiculifer* has been found in small first-order streams as well as larger, but swift, second-order streams that directly receive cool waters from brooks. It does not appear to have a preference for larger waters but rather exploits available habitats of varying size [14]. This crayfish also occurs rarely in sections of streams that flow over bare sand or bed-rock. It usually prefers habitats containing high densities of submerged plants with moderate to large sized rock substrates and tree detritus. *Procambarus spiculifer* constructs burrows in the streambed, preferring submerged portions of stream banks [14].

MATERIALS AND METHODS

Crayfish sampling

Procambarus spiculifer specimens were collected by hand utilizing dip nets and seines from the Alapaha and Withlacoochee Rivers, which are part of the Suwannee River System. The ten Alapaha River specimens were collected from a portion of the river that intersects Georgia Highway 37 just east of Lakeland, Lanier County, Georgia. Five specimens from the Withlacoochee River were collected from a section at Clyattville-Nankin Road, Lowndes County, Georgia. Some specimens were preserved at the collection sites in dry ice and others transported live and preserved at Valdosta State University. Visual inspection was utilized both at the site of collection and in the laboratory to verify genus and species of crayfish samples. All specimens were preserved whole for further analysis at -80°C.

Genomic DNA extraction

Genomic DNA extraction was carried out using the DNeasy Tissue Kit (Qiagen Group). Approximately 0.4-0.6 cm lengths of crayfish abdomen or cheliped were placed into 1.5 ml microcentrifuge tubes and mixed with 180 µl Buffer ATL. Proteinase K (20 µl), Spermidine (1 µl), and RNase (1 µl) were added to each centrifuge tube, mixed by vortexing, and incubated at 55°C overnight to ensure complete lysis. Following overnight incubation, the samples were vortexed for 15 s and 400 µl Buffer AL-ethanol mixture was added. The resulting solution was then mixed vigorously by vortexing to yield a homogenous solution. The mixture was pipetted

into DNeasy Mini Spin Columns (Qiagen Group), placed in new 2 ml collection tubes, then centrifuged at ≥6000 x *g* for 1 min. The flow-through, as well as the collection tubes, was discarded and the DNeasy Mini Spin Columns were placed in new 2 ml collection tubes. Buffer AW1 (500 µl) was added to the DNeasy Mini Spin Columns and centrifuged for 1 min at ≥6000 x *g*. Flow-through and collection tubes were once again discarded and the DNeasy Mini Spin Columns placed in new collection tubes. Buffer AW2 (500 µl) was added to the spin columns and centrifuged for 3 min at 20,000 x *g* to dry the DNeasy membrane. Flow-through and collection tubes were discarded. The DNeasy Mini Spin Columns were placed in clean 2 ml microcentrifuge tubes and Buffer AE (100 µl) was pipetted directly onto the DNeasy membrane. Samples were then incubated at room temperature for 1 min, and then centrifuged for 1 min at ≥6000 x *g* to elute genomic DNA. Elution was repeated once more to increase genomic DNA yield.

Polymerase chain reaction (PCR) was carried out using the procedure and 16S rDNA primers described in Crandall *et al*. (1996). Two microliters of extracted genomic DNA were used as a template for PCR. Mitochondrial DNA from the 16S ribosomal subunit was amplified using invertebrate mtDNA primers CCTGTTTANCAA AAACAT (forward) and AGATAGAAACCAA CCTGG (reverse) [23]. PCR was carried out in 50 µl reactions consisting of 2 µl of template DNA, 5 µl of primers (1mM), 5 µl of buffer, 5 µl 10 X reaction buffer containing $MgCl_2$, 5 µl (10 mM) of deoxynucleoside triphosphates, 1 µl of Taq DNA polymerase (2.5 units), and 32 µl of molecular grade H_2O. Template DNA was denatured at 92ºC for 30 seconds, annealed at 42ºC for 30 seconds, and elongated at 72ºC for 30 seconds in a PTC-100 Programmable Thermal Controller (MJ Research) for 35 cycles followed by 72ºC for 5 minutes [12].

Approximately 200 µl of PCR products from five crayfish specimens were sent via mail to the University of Georgia Laboratory of Genomics and Bioinformatics for sequencing. PCR products were sequenced and assembled utilizing a stand-alone ABI3730xl capillary sequencer/genotyper. The contig consensus sequence was downloaded from the University of Georgia Laboratory of Genomics and Bioinformatics website, and used as a BLASTn ([24]; version 2.2.23+) query sequence at the National Center for Biotechnology Information (NCBI) against all Astacidea sequences in the NR nucleic acid database (as of March, 2010). The most similar sequence to the *Procambarus spiculifer* sequence, from each species identified by BLASTn, up to those that included the American lobster, *Homarus americanus*, were then downloaded from NCBI. This produced a dataset of 81 unique sequences (Table 1) after redundancies were eliminated. These sequences included, but were not limited to, species in the families Parastacidae and Cambaridae (subfamily Cambarinae, 21 *Procambarus*, 17 *Orconectes*, and 12 *Cambarus*), plus the marine species *Homarus americanu*s (American lobster), *Homarus gammarus* (European Lobster), *Acanthacaris tenuimana* (Prickly deep-sea lobster), and *Enoplometopus debelius* (Debelius' Reef Lobster), which all served as the outgroup in the final phylogenetic tree. Interestingly, several of these sequences are deposited at NCBI in their reverse-complement orientation (noted in Table 1). Therefore, a naïve alignment of them all does not work - BLASTn identified those sequences that had to be reverse-complemented. Next, the dataset was loaded into the graphical multiple sequence editor SeaView ([25], version 1.3.1) where those identified sequences could be reverse-complemented, and a multiple sequence alignment could be prepared. The multiple sequence alignment package MAFFT ([26, 27] version 6.717) was used in its EINSI mode to prepare the alignment, and then it was manually refined, all within SeaView.

Phylogenetic analyses

The aligned data (81 taxa by 590 nucleotide base pair positions, see appendix) were analyzed by the maximum likelihood method [28], as implemented within RAxML ([29], version 7.0.4) using its rapid bootstrap approximation followed by a thorough maximum likelihood search strategy. The General Time Reversible (GTR) [30] DNA model of sequence evolution was used, along with a Gamma correction [31] for rate heterogeneity, using four discrete rate categories. All free parameters were

Table 1. 16S rDNA sequences used in the present study. The taxa name, sequence GenBank locus accession code, orientation as found in GenBank, BLAST E value relative to *Procambarus spiculifer*, and native location are all listed.

Taxa	Locus	Strand	E value	Location
Acanthacaris tenuimana	EU882872	forward	6e-71	Indian and Pacific Ocean (marine)
Astacoides betsileoensis	EU978458	forward	8e-70	Madagascar
Astacoides crosnieri	EU978461	forward	2e-75	Madagascar
Astacus astacus	AF235983	forward	4e-142	Europe
Austropotamobius italicus	AY611190	reverse	4e-117	Europe
Austropotamobius torrentium	AM181346	forward	3e-128	Central Europe
Barbicambarus cornutus	EU920913	forward	5e-121	Tennessee, Kentucky
Cambarellus shufeldtii	AF235986	forward	7e-155	South Central United States
Cambaroides dauricus	DQ666837	forward	2e-106	East Asia
Cambaroides japonicus	AB508253	forward	7e-125	Japan
Cambaroides schrenckii	DQ666835	forward	9e-104	Far East Russia
Cambaroides similis	DQ666842	forward	3e-108	Korea, China
Cambarus brachydactylus	DQ411732	reverse	3e-143	Tennessee
Cambarus friaufi	DQ411733	reverse	5e-156	Kentucky/Tennessee
Cambarus gentryi	AY853664	reverse	3e-148	Tennessee
Cambarus graysoni	AY853665	reverse	2e-155	South Central United States
Cambarus hamulatus	DQ411734	reverse	1e-152	Alabama/Tennessee (cave species)
Cambarus jonesi	EU433903	forward	5e-151	Alabama (cave species)
Cambarus maculatus	AF235988	forward	1e-131	Missouri
Cambarus monongalensis	AY590472	reverse	9e-154	East Central United States
Cambarus sp 1-JEB-2006	DQ411756	reverse	1e-147	South Central United States (cave species)
Cambarus sp 2-JEB-2006	EU433909	forward	1e-157	South Central United States (cave species)
Cambarus striatus	DQ087394	forward	3e-114	South Central United States
Cambarus tenebrosus	DQ087354	forward	2e-159	Central United States (cave species)
Cherax crassimanus	AF492805	forward	2e-71	Southwest Australia
Cherax destructor	AY191767	forward	2e-60	Australia
Cherax dispar	AY153860	forward	2e-71	Australia
Cherax glaber	AY211980	forward	6e-71	Australia
Cherax preissii	AF492807	forward	6e-71	Australia
Cherax quadricarinatus	EU244888	forward	1e-68	North/Northeast Australia, New Guinea
Cherax sp New Guinea DM-2003	AY191775	forward	1e-72	New Guinea
Engaewa subcoerulea	AF135983	forward	3e-138	Southwest Australia
Enoplometopus debelius	EU882869	forward	8e-70	Indian and Pacific Ocean (marine)

Table 1 continued..

Euastacus crassus	DQ006584	forward	1e-78	Southeast Australia
Euastacus fleckeri	DQ006595	forward	7e-80	Southeast Australia
Euastacus maidae	DQ006610	forward	1e-72	Southeast Australia
Euastacus robertsi	DQ006624	forward	2e-75	Southeast Australia
Euastacus spinifer	DQ006644	forward	7e-90	Southeast Australia
Homarus gammarus	EU882876	forward	4e-68	Northeast Atlantic Ocean (marine)
Homarus_americanus	EU882875	forward	4e-68	Northwest Atlantic Ocean (marine)
Orconectes australis	AY853624	reverse	7e-145	South Central United States (cave species)
Orconectes barri	AY853618	reverse	7e-150	South Central United States (cave species)
Orconectes compressus	EU433917	forward	9e-149	South Central United States
Orconectes deanae	EU442666	forward	2e-154	West Central United States
Orconectes erichsonianus	EU433918	forward	4e-147	South Central United States
Orconectes forceps	EU433919	forward	2e-150	South Central United States
Orconectes incomptus	AY853613	reverse	5e-146	Tennessee (cave species)
Orconectes limosus	EU442690	forward	1e-147	Eastern United States
Orconectes luteus	AF376486	reverse	2e-154	Central United States
Orconectes nais	EU442664	forward	5e-156	Central United States
Orconectes ozarkae	AY485443	reverse	9e-149	South Central United States
Orconectes packardi	AY853606	reverse	1e-142	Kentucky (cave species)
Orconectes pellucidus	EU433914	forward	1e-142	South Central United States (cave species)
Orconectes placidus	AY609334	reverse	9e-159	Central United States
Orconectes punctimanus	AY485442	reverse	9e-154	South Central United States
Orconectes rusticus	AY485441	reverse	2e-160	North America
Orconectes virilis	AY485437	reverse	9e-159	North America
Pacifastacus leniusculus	AF235985	forward	2e-139	Western North America
Paranephrops planifrons	DQ006669	forward	6e-91	New Zealand
Paranephrops zealandicus	DQ006671	forward	6e-91	New Zealand
Procambarus acutus	EF012354	forward	0.0	Eastern United States
Procambarus alleni	FJ619802	forward	0.0	Florida
Procambarus clarkii	EF012351	forward	0.0	Southeastern United States
Procambarus curdi	EF012344	forward	2e-165	South Central United States
Procambarus digueti	AY214435	reverse	2e-140	Central America, Mexico
Procambarus fallax	FJ619797	forward	0.0	Southeast United States
Procambarus gibbus	EU433916	forward	0.0	Georgia
Procambarus liberorum	EF012321	forward	1e-171	South Central United States
Procambarus nigrocinctus	EF012345	forward	7e-170	Texas

Table 1 continued..

Procambarus ouachitae	EF012356	forward	0.0	South Central United States
Procambarus pecki	EU433911	forward	7e-155	Central United States (cave species)
Procambarus reimeri	EF012343	forward	7e-170	Arkansas
Procambarus sp 1-JEB-2007	EF012337	forward	3e-168	United States
Procambarus sp 2-JEB-2007	EF012338	forward	1e-171	United States
Procambarus sp 3-JEB-2007	EF012334	forward	8e-174	United States
Procambarus sp *aguazarca*	AY214436	reverse	3e-178	Mexico
Procambarus sp *malagasy*	EU978457	forward	0.0	Madagascar (invasive/parthenogenetic)
Procambarus sp *platanos*	AY214437	reverse	3e-178	Mexico
Procambarus spiculifer	this study	forward	query	Southeast United States
Procambarus tenuis	EF012348	forward	4e-172	South Central United States
Procambarus toltecae	AY214438	reverse	7e-170	Mexico

optimized by RAxML. Tree robustness was assessed using the bootstrap procedure with 1000 replicates. As mentioned above, *H. americanus*, and the other three marine species, were designated the outgroup for the purpose of representing the final phylogenetic tree in order to estimate root placement and overall direction of evolution. This final tree representation (Figure 1) was drawn with the program FigTree ([32], version 1.3.1).

Analyses of all the sampled Cambarinae species provided a general overview of relationships among the three genera of interest, as well as the other genera included in the analysis. Although this study is limited in its sample size, and to only a portion of one gene, there is sufficient support for the conclusions made here. For example, Rosenberg and Kumar [33] found that sample size had a minimal effect when phylogenetically instructive character samples with high polymorphism were used. Phylogenetic interrelationships can be derived from data incorporating a limited number of species when using phylogenetically informative genetic sequences [23, 34, 35, 36]. The 16S rDNA sequence used in this study has proven to be consistently accurate in determining crayfish phylogeny [12, 13, 15].

RESULTS

Empirical nucleotide frequencies were pi(A): 0.337850, pi(C): 0.105359, pi(G): 0.202928, and pi(T): 0.353862. The GTR model parameters optimized at the following rates A⇆C: 0.522892, A⇆G: 7.924781, A⇆T: 1.436686, C⇆G: 0.336000, C⇆T: 3.978594 and G⇆T: 1.000000; with the Gamma alpha value 0.342488; for an overall tree length of 2.294079. The final, optimized maximum likelihood tree had a negative log likelihood of -8063.503349.

The family Cambaridae is strongly separated from the rest of our tree by a bootstrap value of 100%. However, the Astacidae are nested within it at the 84% bootstrap level as a sister clade (at 81% bootstrap value) to that clade containing Cambaroides (100% bootstrap value). The clade containing the remainder of the Cambaridae, largely from Eastern and Central North America, and Mesoamerica, is held together with a mediocre 63% bootstrap value.

Within this clade, the *Procambarus* genera are loosely held together in our tree (basal bootstrap value below 50%), with some distinct associations. Of those *P. fallax* and *P.* sp *malagasy* are related by a bootstrap value of 99%, and *P.* sp *platanos*, *P.* sp *aguazarca*, and *P. toltecae* are held together by a bootstrap value of 100%. The primary subject of the analysis, *P. spiculifer*, loosely associates with *P. alleni* and *P. clarkii*, but bootstrap values are below 50% at this node. Furthermore, *Cambarellus shufeldtii* is nested within this *Procambarus* grouping.

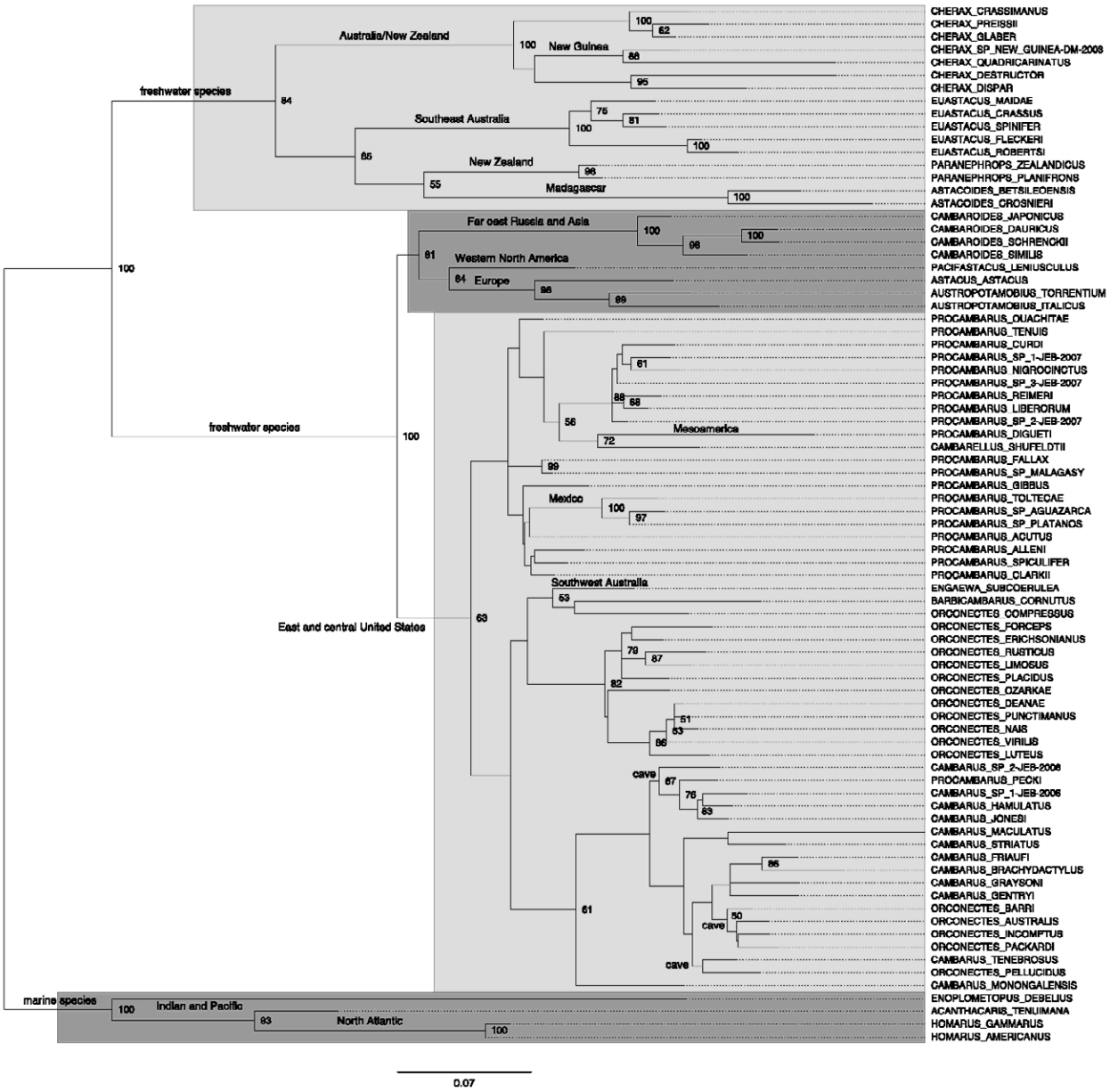

Figure 1. 16S rDNA phylogenetic tree of 81 Astacidea species most similar to *Procambarus spiculifer* based on BLAST similarity scores, excluding those sequences virtually identical to our query over the sequence length searched. The tree is based on maximum likelihood techniques as implemented in RAxML [29], run with 1000 bootstrap replicates. Bootstrap values greater than 50% are indicated at appropriate nodes, and geographic locations are annotated. Vertical distance is arbitrary, merely indicating lineage relations; horizontal distance is evolutionary distance in substitutions per site as shown by the scale bar at the bottom of the figure.

All of this *Procambarus* grouping in turn loosely associates (as noted above, bootstrap value of 63%) with another grouping containing the genus *Cambarus* as well as *Orconectes*. Most of these *Orconectes* taxa are held together in a clade by an adequate bootstrap value (82%) and contain many stream dwelling *Orconectes* species, such as *O. luteus, O. punctimanus, O. placidus, O. rusticus, and O. virilis*, but several other *Orconectes* intermingle with *Cambarus* taxa. This mixed *Cambarus* and *Orconectes* grouping is held together by a quite weak bootstrap value (61%).

The cave dwelling species *O. incomptus*, *O. australis* and *O. packardi* all occur in this mixed clade, and come out weakly associated with one another. Notable associations with strong bootstrap values in this mixed *Cambarus* and *Orconectes* part of our tree include *C.* sp 1-JEB-2006, *C. hamulatus* and *C. jonesi*, which cluster together at the 83% bootstrap value; and *C. friaufi* and *C. brachydactylus*, which cluster together at the 86% bootstrap value. Notably, *Procambarus pecki* is also nested within this mixed grouping.

The resulting tree differs from the traditional systematic representations of the subfamily Cambarinae in that the Cambarinae was not clearly divided into the three standard groupings of *Procambarus*, *Cambarus*, and *Orconectes*. Moreover, the interrelationships within these species is unlike customary phylogenies based on morphology. These inconsistencies with traditional, morphologically based taxonomies will be a primary focus of our discussion.

DISCUSSION

The primary purpose of this study was to determine the phylogenetic position of *P. spiculifer* within the Cambaridae, as well as its position within the genus *Procambarus*. Secondarily, this study also examined the phylogenetic relationships within the family Cambaridae, as well as relationships of the families, Astacidae and Parastacidae, for which 16S rDNA sequences homologous to ours existed at the National Center for Biotechnology Information's GenBank. Finally, based on works in the literature, we evaluated the robustness of using our 16S rDNA sequence for the development of phylogenetic trees within the Repentia.

Critical questions that can be asked are: (a) do our crayfish worldwide distributions correlate with the results of other studies, and (b) what is the strength of our data, since we have only used a partial sequence from only the 16S rDNA gene? Both questions are critical in understanding the strength of our findings and in providing insight into the value of such sequences compared to longer sequences and multiple genes. Crandall and Buhay [37] have published an analysis that looks at global distributions of crayfish in both the Northern and Southern Hemispheres, and have identified geographic regions within each hemisphere that correlate with the distribution of crayfish taxa. The ensuing discussion on taxonomic relationships correlates well with their work, yet raises additional questions.

Examining our primary objective of this study, *Procambarus spiculifer* associates with all other *Procambarus*, but forms a polytomy involving *P. alleni* and *P. clarkii*. This polytomy suggests a common ancestral origin in the eastern or southeastern United States, as all three species are found in the southeastern United States. *Procambarus alleni* is restricted to Florida east of the St Johns River and southern Florida into the Florida Keys [5, 38]. *Procambarus clarkii* has a distribution that covers the Southeastern U.S., extends into Mexico and up into the Midwest [5, 38]. *Procambarus spiculifer* has a more restricted distribution in southern Georgia and Alabama extending down into Florida [5, 38].

Examining our second objective within our phylogenetic tree, the Parastacidae breakout from the other two families with a strong bootstrap value (100%). However, the branch that includes the Cambaridae and the Astacidae is problematic in that associations based on genera do not follow current family groupings. The Cambaridae include the genera *Barbicambarus*, *Cambaroides*, *Cambarus*, *Cambarellus*, *Orconectes*, and *Procambarus*. The Astacidae include the genera *Astacus*, *Austropotamobius* and *Pacifastacus* [5, 38]. However, in our tree the *Cambaroides* are held together by a very strong bootstrap value (100%), and associate with the Astacidae rather than with the Cambaridae. The *Cambaroides* are held within the Astacidae by a moderate bootstrap value (81%). In their discussions on the global diversity and distribution of crayfish families, Sinclair *et al.* [2] and Crandall and Buhay [37] present a phylogenetic tree in which *Pacifastacus* lies within a grouping that includes genera in the Cambaridae while *Astacus* and *Austropotamobius* form a separate grouping. While the branching patterns differ between our study and theirs, the fact that the two families form overlapping groups raises important questions with respect to biogeographic origins and phylogenetic relationships as discussed by Crandall and Buhay [37].

Examining our phylogenetic tree by genera, subgenera and species within families provides insight into the strength of our 16S rDNA segment. The Cambaridae distributed throughout much of the Nearctic and northern reaches of the Neotropics (not including Cambaroides in the eastern Palaeartic) [37] is composed of two major clades in our analysis, although the bootstrap support is weak (63%). The first clade contains *Cambarellus shufeldtii* and all species within the genus *Procambarus* except for *P. pecki*, which emerges in the second clade along with the *Cambarus*. This second clade includes all *Cambarus* and *Orconectes*, *Barbicambarus cornutus*, *P. pecki* and *Engaewa subcoerula*, an Australian species, which is seen as an incongruity for the 16S sequence in GenBank, and that is most closely related to *E. similis*, another Australian species in the Parastacidae according to Crandall *et al*. [39]. Within the second clade, most *Orconectes* are held into a cohesive clade with a bootstrap value of 82%; however, a few are loosely associated within another clade (61% bootstrap value) along with *Cambarus*, *B. cornutus*, and *P. pecki*.

The genus *Orconectes* has proven problematic in the past, and is so in our phylogenetic tree as well. As mentioned above, the poorly supported clade that includes all the *Orconectes* splits into two subgroups on our tree, with one branch consisting of twelve *Orconectes* species as well as *E. subcoerulea*, previously identified as an incongruity, and *B. cornutus*. Of these twelve *Orconectes* species, *O. compressus* shows greater affinity to *B. cornutus* and *E. subcoerulea* than to the other eleven *Orconectes* species in this part of our tree, though the bootstrap support is quite poor. These eleven *Orconectes* species, however, are held together as a single clade with an adequate bootstrap value of 82%. *Orconectes compressus* is in the subgenus *Gremicambarus*, which also includes *O. nais* and *O. virilis*. However, *O. nais*, *O. virilis O. luteus*, and *O. punctimanus* (subgenera *Procericambarus*) and *O. deanae* (subgenera *Hespericambarus*) are held together as a single clade (bootstrap support 86%) on our tree. *Orconectes rusticus* and *O. limosus* also share a common branch (bootstrap value of 87%) in our tree, but are in the subgenera *Procericambarus* and *Faxonius* respectively. *Orconectes placidus*, *O. erichsonianus*, *O. forceps* and *O. ozarkae* are the other four species that occupy positions within this clade, but low bootstrap values prevent identifying any definitive relationships.

The other major clade of our tree containing species of *Orconectes* is potentially problematic in that it is separated from the previous clade and includes the genus *Cambarus*, as well as *P. pecki*. The five *Orconectes* species within this clade, *O. barri*, *O. australis*, *O. incomptus*, *O. packardi*, and *O. pellucidus*, are all within the subgenus *Orconectes*. Historically, *O. packardi* and *O. australis* were grouped together as a subspecies [15]. However, this same major branch includes all members of the genus *Cambarus* and *P. pecki*, which is embedded within the *Cambarus*. All other members of the genus *Procambarus* form a separate clade that breaks off from the clade currently under discussion. The assignment of *P. pecki* to *Procambarus* has been studied by Buhay and Crandall [40], and they note that an analysis of phylogenetic position using 16S rDNA and its zoogeographic position in the Highland Rim in Northwest Alabama argues that it is most closely related to the genus *Cambarus*. Thus they have proposed the renaming of it to *Cambarus pecki*, which our findings support. Fetzner [41] conducted a detailed phylogenetic and biogeographic analysis using 56 allozymes involving thirty *Orconectes* species along with selected outgroups. The work of Fetzner [41] generally reflects our findings on distribution patterns as discussed below. However, our data set contains fourteen species he did not examine and his contained twenty-five species not present in our data set. So while phylogenetic relationships are difficult to draw, it can be noted that the close relationships he found between *O. ozarkae* and *O. placidus*, and *O. nais* and *O. virilis* (his Groups 1 and 3, and Fig. 4) placing them in similar clades, matches our findings for these four species, as well as the fact that *O. australis* lies in its own separate clade.

Analyses of the genus *Cambarus* produces the following correlations, working on the assumption that *P. pecki* is more closely related to *Cambarus* than to *Orconectes* or *Procambarus* and should thus be assigned to *C. pecki*. *Cambarus pecki*, *C. hamulatus* and *C. jonesi* are loosely held together

in our tree (bootstrap support of 76%), and the latter two species are within the subgenus *Aviticambarus*. The fact that all three are cave species suggests an early ancestral cave species may have given rise to all three species. *Cambarus* (*Erebicambarus*) *maculatus* and *C.* (*Depressicambarus*) *striatus* form a sister clade, but the low bootstrap value prevents any definitive conclusions. *Cambarus* (*Depressicambarus*) *graysoni*, and *C.* (*Depressicambarus*) *striatus* are loosely associated with *C.* (*Jugicambarus) gentry*, but again low bootstrap values prevent definitive conclusions. *Cambarus* (*Jugicambarus) monongalensis* is the least derived of all the other species in our analysis of the *Cambarus*, which suggests early evolutionary divergence followed by possible morphological convergence. Taylor [42] in his introduction notes that *Cambarus*, and the subgenera within the genus, have been seen as phylogenetically problematic by Bouchard [43] and Hobbs [44]. Bootstrap values for this clade that holds all *Cambarus* along with the five *Orconectes* species mentioned above, and *P. pecki* is a mediocre 61%.

Another perspective that can be examined is the fact that within this poorly supported clade that includes all *Orconectes* and *Cambarus* are two subclades that further divide into yet additional subclades. One such subclade includes *Cambarus*, and they associate more closely to the five *Orconectes* species discussed earlier while the remaining species of Cambarus lie within the other subclade. This suggests that the morphological traits used to identify the species of *Orconectes* and *Cambarus* are not stable with respect to following strict phylogenetic lineages, but may be going through some combinations of convergent, divergent and parallel evolution. As such, these genera and species need to be critically examined in future studies. Erichson [45] first described *Cambarus*, and subsequently Hobbs [5, 38] retained the genus in his synopsis of North American crayfish families and genera and in his species checklist. An extensive listing of research references covering a wide range of disciplines employs the genus *Cambarus* suggesting strong adoption and morphological recognition of this genus. However, adoption of a specific epithet fails to answer questions as to whether or not such morphological traits are indicative of genus and/or species status or are more indicative of adaptations that have occurred in response to environmental and behavioral selection pressures. Numerous studies have examined such questions in reviews and analyses of various taxa, examples of some being West-Eberhard [46], De Queiroz and Wimberger [47], Schlulter [48], Ruber and Adams [49], and Stephens and Wiens [50].

Determining phylogenetic relationships within the *Procambarus* is problematic in that bootstrap values are often low in our analysis so that at best only polytomies can be ascertained for some groupings. Within that portion of our tree that contains all members of *Procambarus*; two groupings exist, although support for them is low. Within the first group are four members of the subgenus *Girardiella* involving *P. reimeri* and *P. liberorum*, forming a weakly supported sister clade, and *P. curdi* and *P. nigrocinctus*. Other species in the first grouping are *P* (*Tenicambarus*) *tenuis* and *P. (Ortmann) ouachitae* and *P. (Ortmann) digueti*. Unidentified species included *Procambarus* sp 1 JEB 2007 and *Procambarus* sp 3 JEB 2007. Within this first grouping is *C.* (*Dirigicambarus*) *shufeldtii*, which weakly forms a sister clade with *P. digueti*.

The second grouping containing *Procambarus* produces a polytomy involving *P. alleni*, *P. spiculifer*, and *P. clarkii* as discussed above. A strong clade involving *P. tolecae*, and *P.* sp *aguazarca* and *P.* sp *plantanos* (100% and 97%) is also found in this group. The third subclade consists of a single sister clade involving *P. fallax* and *P.* sp *malagasy* with a high bootstrap value (99%). This sister clade is of particular interest as *P.* sp *malagasy* is an invasive parthenogenic species reported from Madagascar [51]. Martin *et al*. [52] have identified it as a parthenogenic form of *P. fallax* using morphologic and molecular analyses. Jones *et al*. [51] have studied *P.* sp *malagasy* and shown it to be closely related to *P. alleni*. Finally the distribution of *P. fallax* overlaps that of *P. clarkii*, *P. alleni* and *P. spiculifer* further supporting a common ancestral origin.

A primary finding of the analyses on the Cambaridae and the subgenera within each genus was the fact that various species within different subgenera commonly group with each other rather

than with species of the same subgenus. This raises the question as to why such apparent groupings are occurring. An explanation is that the morphological traits used to determine genera, subgenera and species status fail to properly segregate populations or species within clades that represent individuals of common ancestral descent. This point can be seen as problematic or as a point of insight into evolutionary processes taking place within various taxa of crayfish. Brower *et al*. [53] note that some data sets employed for the understanding of phylogenetic relationships may lack sufficient strength as they do not reflect "organismal history". An example of this would be the polytomy involving *P. alleni* and *P. spiculifer* along with the closely related *P. clarkii*. If the assumption is made that the genetic differences that relate these species to one another, yet separate them from other *Procambarus* is valid, then morphological traits placing them in different subgenera represent an insufficient data set for making such assignments (perhaps due to convergence). *Procambarus spiculifer* is a lotic species inhabiting moderately flowing waters in streams and creeks, while *P. alleni* and *P. clarkii* are primarily lentic species found in backwaters, slow currents in rivers and creeks and the Everglades [54]. As such, each of these species must deal with different environmental conditions, different predators and possess different life history patterns [55, 56, 57]. Given these facts, it is reasonable to assume that very divergent morphologies may be possessed for some traits, and these morphologies may actually result in convergence with other species more similar in ecological and life history patterns, but less closely related.

Biogeographically, the phylogenetic tree in our data set matches reasonably well with the distributions of the species that makeup each clade or polytomy. The clade that includes *O. compresses*, *O. forceps*, *O. erichsonianus*, *O. rusticus*, *O. limosus* and *O. placidus* represents species with distributions found east of the Mississippi River from the Gulf Coast, up through the Highland Rim and Nashville Basin, and into the Midwest (primarily Illinois) as well as eastward into the Appalachians and coastal plains region [5, 39, 44]. Likewise the second clade containing *O. ozarkae*, *O. deanae*, *O. punctimanus*, *O. nais*, *O. virilis* and *O. luteus*, is found primarily west of the Mississippi River with the exception of *O. virilis* which possesses an extensive range throughout most of the central United States, into the Northeast and up into Canada [58, 59, 60]. However, the distribution and systematics of *O. virilis* is also problematic as discussed by Mathews *et al*. [61]. Using three genetic markers and the morphology of the male gonopodium, they found *O. virilis* to potentially represent a complex of species rather than a single species. Crandall and Fitzpatrick [13] looked at the *Procericambarus* using molecular and morphological traits and found that the monophyly of this subgenus was not supported, further raising concerns about phylogenetic relationships within the Cambaridae.

The clade that contains all the *Cambarus* taxa groups most closely with the *Orconectes* found east of the Mississippi River. As with the *Orconectes*, the *Cambarus* possess distributions that encompass Alabama, Tennessee, and Kentucky suggesting close ancestral relationships based on distributions. The distribution of *Procambarus pecki* in a select set of caves in northwest Alabama more closely associates it with the genus *Cambarus*. Thus the biogeographic position in combination with phylogenetic analyses of *P. pecki* further supports the contention that it be placed in the *Cambarus* [40]. Work by Fetzner [41] used several outgroups to examine the phylogenetic relationships of the *Orconectes*. In this work, he found that *O. australis* is in a clade most closely aligned with the *Cambarus*, which also holds for our data set.

Similarly, the clade containing *P. ouachitae*, *P. tenuis*, *P. curdi*, *P. nigrocinctus*, *P. reimeri*, *P. liberorum*, *P. digueti* and *C. shufeldtii* represents species found predominately west of the Mississippi River. *Procambarus digueti* is found in Mexico along the Pacific Coast [3]. The occurrence of *P. digueti* on the Pacific coast along with the fact that it forms a sister clade with *C. shufeldtii*, as pointed out above, raises two key points: (a) why the close association with *C. shufeldtii*, and (b) what can account for the seemingly disjunct distribution of *P. digueti*? The association of the two species as a sister clade

suggest that one species or the other is misplaced as to genus; and as discussed below, this misplacement may result from a combination of convergent and/or divergent evolutionary events involving morphological features. This supposition is supported by a moderate bootstrap value (72%) that suggests a common ancestral origin. *Cambarellus shufeldtii* possesses an extensive range from Illinois down to Tennessee and into Arkansas, Louisiana and Texas [5, 38]. While the ranges of the two species are disjunct, it is reasonable to hypothesize that an extinct ancestor possessed a much broader range that extended into Mexico. The range of *P. clarkii* [5, 38], which covers much of the eastern United States and extends into northern Mexico, further supports such an argument.

The other subclade possessing *Procambarus* includes *P. fallax*, *P.* sp *malagasy*, *P. gibbus*, *P. toltecae*, *P.* sp *aguazarca*, *P.* sp *platanos*, *P. acutus*, *P. alleni*, *P. spiculifer* and *P. clarkii*. The collective distribution of these species is quite broad ranging from central Mexico through Texas and into the Southeastern and Midwest United States. With the exception of *P.* sp *malagasy*, which is a parthenogenic invasive species in Madagascar, the species in this subclade with the widest distribution is *P. clarkii*, which is found throughout much of the Western Hemisphere described above [3].

An examination of other clades in our phylogenetic tree show congruence for the Astacidae, Parastacidae and the Asian branch of the Cambaridae, genus *Cambaroides*, in that they form groupings that correlate by geographic location. The Astacidae include species in Europe, western Asia and western North America, which are in the Palaeartic and Neartic regions discussed by Crandall and Buhay [37] and form a clade (bootstrap value of 84%) along with the sister clade containing the Cambaridae genus *Cambaroides* (bootstrap support of 100%). These two sister clades are held together with a relatively strong bootstrap value of 81%. In turn these two sister clades are strongly associated with the North American Cambaridae clade (bootstrap value of 100%) discussed previously. Within the Astacidae clade the two European species *Austropotamobius italicus* and *A. torrentium* are tightly held together with a robust bootstrap value of 99%, along with *Astacus astacus*, which extends well into Asia (bootstrap value of 96%). *Pasifastacus leniusculus*, which is found west of the Rocky Mountains in North America, is basal within this Astacidae clade with a reasonably strong bootstrap value of 84%. The *Cambaroides* from eastern Asia form a very robust clade (bootstrap value of 100%), and as mentioned just above, come out as a sister clade with reasonably strong support (81%) to the Astacidae clade containing *Austropotamobius*, *A. astacus*, and *Pasifastacus leniusculus*. *Cambaroides dauricus* (China) and *C. schrenckii* (Russia) are tightly held together (bootstrap value of 100%), with *C. similis* (Korea) basal to them (bootstrap value of 98%), and *C. japonicas* basal to all three (bootstrap value of 100%).

Within the Parastacidae our GenBank search found a limited number of species from Australia, New Zealand, New Guinea and Madagascar, but no South American species. Most likely no 16S sequencing had been done in these species as of the date of our initial BLAST search. Crayfish genera that were found showed reasonably strong congruence with other studies. The entire clade is held together at the 84% bootstrap level. Munasinghe *et al*. [62] and Shull *et al*. [63], looking at *Cherax* and *Euastacus*, have produced phylogenetic trees with similar branching patterns. Their inclusion of other species results in the differences between their trees and ours. *Paranephrops zealandicus* and *P. planifrons* are the only two species in New Zealand [64] and form a sister clade in our tree as would be expected, and the close association to the *Euastacus* matches the tree of Crandall *et al*. [65]. The Madagascar species *Astacoides betsileoensis* and *A. crosnieri* form a sister clade weakly associated with the New Zealand *Paranephrops*, an association supported by the biogeographic analysis of McDowall [64] and the phylogenetic tree of Sinclair *et al*. [2].

Sinclair *et al*. [2] have developed a strong argument for the need for a comprehensive phylogenetic revision of crayfish worldwide and have identified a cohort of researchers that are currently involved in this phylogenetic revision. Their arguments involve robust analyses as to why such a study should be completed to include

a set of five standardized genetic loci, public domains such as GenBank and a crayfish homepage. This and similar work such as that done on gnathostome fishes [66] should provide standards by which all such phylogenetic analyses should be carried out, and are critical if the biology of various species is to be clearly understood. The identification of five genetic loci is of particular importance as seen in this study. Our single locus of insufficient sequence length has produced a phylogentic tree in which families with limited numbers of species are similar in cladistic patterns to what others have found; however, discrepancies exist within the Cambaridae indicating that a single locus, especially of such restricted length, has limits with respect to differentiating cladistic relationships. Questions have been raised by others [50, 67, 68, 69] as to validity of earlier morphologically based phylogenetic trees. By comparing morphologically and phylogenetically based trees, individuals studying crayfish biology may be better able to sort out evolutionary processes such as the influence of environmental factors involving convergence and divergence that reflect multiple aspects of crayfish biology, as discussed above in our analysis of the cladistic relationships between *P. spiculifer*, *P. alleni* and *P. clarkii* that show phylogenetic relatedness, but inhabit vary different habitats.

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