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Molecular phylogenetics of American snapping shrimps allied to *Alpheus floridanus* Kingsley, 1878 (Crustacea: Decapoda: Alpheidae)

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Abstract

Widely distributed populations of the snapping shrimp, *Alpheus floridanus* Kinglsey, 1878, from the eastern Pacific and western and eastern Atlantic Ocean have long been suspected to represent different species due to extreme morphological variation among conspecifics. A companion study (Bracken-Grissom & Felder 2014) contains redescriptions of two western Atlantic species (*A. floridanus* Kingsley 1878 *sensu stricto* and *A. platycheirus* Boone, 1927), assignments of the original syntypes for *A. floridanus*, descriptions of three new species from the Atlantic and eastern Pacific (*A. hephaestus* Bracken-Grissom & Felder, 2014; *A. roblesi* Bracken-Grissom & Felder, 2014; *A. ulalae* Bracken-Grissom & Felder, 2014), and discussion of the relationship of the eastern Atlantic *A. floridanus africanus* Balss, 1916. The present study underpins all these findings by application of molecular phylogenetic techniques. Analysis of partial sequences of the 16S, 12S, and COI mitochondrial genes separate species throughout the eastern Pacific and the eastern and western Atlantic. Morphological comparisons suggested that the two syntypes of *A. floridanus* belong to different species, and molecular results in this study confirm this separation. Genetic data suggest a strong affinity between the western Atlantic *A. platycheirus* and the eastern Pacific *A. hephaestus.* Close relationships are evident between trans-Atlantic species, *A. floridanus africanus* and *A. floridanus,* a pattern also seen for other cryptic and pseudocryptic species of *Alpheus*. *Alpheus roblesi* and *A. ulalae* represent early-branching lineages within the complex. In some cases, molecular phylogenetic relationships between members of the *A. floridanus* complex can be reconciled with postulated biogeographic history.

Key words: *Alpheus*, Alpheidae, species complex, snapping shrimp, 16S, 12S, COI, mitochondrial genes, phylogenetic analysis

Introduction

Genetic data can provide tools for defining and describing taxa within cryptic and pseudocryptic species complexes. Here we define a "species complex" to be a group of species that are morphologically similar but genetically distinct ("pseudo-sibling species" of Knowlton 1993). Williams *et al.* (2001) demonstrated the usefulness of molecular data in studies of species complexes while conducting the first large-scale phylogenetic study of the genus *Alpheus* based on both mitochondrial and nuclear genes and including several transisthmian pairs. Williams *et al.* (2001) reported evidence of at least seven cryptic species among American species of *Alpheus*, revealing a need for future studies in this genus. With the application of both morphological examinations and DNA analyses, several American complexes of *Alpheus* have been recently revised: *A. formosus* Gibbes, 1850, *A. cristulifrons* Rathbun, 1900, *A. nuttingi* (Schmitt, 1924), *A. websteri* Kingsley, 1880, and *A. armillatus* H. Milne Edwards, 1837 (Anker 2012; Anker *et al.* 2007; 2008a; 2008b; 2008c; Mathews & Anker 2009). *Alpheus floridanus* Kingsley, 1878, which was revised based on morphology and color patterns (Bracken-Grissom & Felder 2014), represents yet another species complex, with five species and one subspecies in the eastern Pacific and tropical eastern and western Atlantic. In the present study, we present the underpinning molecular phylogenetic

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analyses of the *A. floridanus* complex, combining sequences of three ribosomal and protein-coding mitochondrial genes (16S, 12S, and COI). We examine evolutionary relationships among the five species and one subspecies and discuss patterns of biogeography across the complex.

Materials and Methods

Specimens of the *Alpheus floridanus* complex used in this study were collected from the northern Gulf of Mexico (NGMx), eastern Gulf of Mexico (EGMx), southwestern Gulf of Mexico (SWGMx), southeastern Gulf of Mexico (SEGMx), eastern Pacific (EP) off the coast of Costa Rica and Panama, western Atlantic off the coast of eastern Florida (EF) and Brazil (BRZ), eastern Atlantic (EA) off the coast of West Africa, southern Caribbean Sea (COL) off the coast of Colombia (Table 1). Most specimens were collected during cruise and field expeditions and archived in the University of Louisiana at Lafayette Zoological Collection (ULLZ) or were available among pre-existing holdings of ULLZ, the National Museum of Natural History, Smithsonian Institution, Washington DC (USNM), and Naturalis – National Museum of Natural History, Leiden (RMNH). The syntypes (1 male, 1 female) of *A. floridanus* were obtained on loan from the Museum of Comparative Zoology, Harvard University (MCZ), along with special permission for their use in minimally invasive molecular analyses (given pre-existing damage that exposed abdominal muscle in both types). Similar permission was granted for the holotype of *A. platycheirus* Boone, 1927 by the Peabody Museum of Natural History, Yale University, New Haven (YPM). Freshly collected specimens were taken during research cruises in trawls and dredges or excavated by yabby pump from burrows in shallow waters along shorelines. Fresh specimens were either frozen in glycerol at -80ºC and later transferred to 80% ethyl alcohol (ETOH) or placed directly into 80% ETOH. Two species of the genus *Alpheus* (*A. carlae* Anker, 2012*, A.* nr. *amblyonyx* Chace, 1972) were used as outgroups.

TABLE 1. Shrimp species used for phylogeny reconstruction, showing catalog number, collection locality, and GenBank accession numbers for partial sequences of 16S, 12S, and COI respectively [for museum abbreviations see Material and Methods] * = female and male syntypes of *A. floridanus* Kingsley, 1878; #= holotype of *A. platycheirus* Boone, 1927. Catalog number indicates number for both morphological voucher specimen (listed first) and tissue sample (listed second). In some instances the catalog number is the same for both.

Taxon	Catalog No.	Collection Locality	16S	12S	COI
Alpheidae Rafinesque, 1815					
Alpheus floridanus africanus Balss, 1916	RMNH 9312/ ULLZ 9122	Sierra Leone, western Africa, Atlantic Ocean	KP100618	KP100579	N/A
Alpheus roblesi Bracken-Grissom & Felder, this volume	ULLZ 7197B	southwest Gulf of Mexico, Atlantic Ocean	KP100608	KP100582	KP100627
Alpheus roblesi Bracken-Grissom & Felder, this volume	ULLZ 7197C	southwest Gulf of Mexico, Atlantic Ocean	KP100609	KP100583	KP100628
Alpheus roblesi Bracken-Grissom & Felder, this volume	ULLZ 6623	southwest Gulf of Mexico, Atlantic Ocean	KP100610	KP100584	N/A
Alpheus roblesi Bracken-Grissom & Felder, this volume	ULLZ 6549	southwest Gulf of Mexico, Atlantic Ocean	KP100611	KP100585	KP100636
Alpheus roblesi Bracken-Grissom & Felder, this volume	ULLZ 12208/ KC 6870	southeastern Florida, United States, Atlantic Ocean	KP100612	KP100586	N/A
Alpheus ulalae Bracken-Grissom & Felder, this volume	USNM 1265092/ ULLZ 6815	eastern Gulf of Mexico, Atlantic Ocean	KP100619	KP100591	KP100632
Alpheus hephaestus Bracken-Grissom & Felder, this volume	ULLZ 6204	Costa Rica, eastern Pacific Ocean	KP100613	KP100576	KP100629

.....continued on the next page

TABLE 1. (Continued)

Taxon	Catalog No.	Collection Locality	16S	12S	COI
Alpheus hephaestus Bracken-Grissom & Felder, this volume	USNM 1265093/ ULLZ 6205	Costa Rica, eastern Pacific Ocean	KP100615	KP100577	KP100630
Alpheus hephaestus Bracken-Grissom & Felder, this volume	ULLZ 6206	Costa Rica, eastern Pacific Ocean	KP100614	KP100578	KP100631
Alpheus floridanus Kingsley, 1878	MCZ 4987* (female)/ ULLZ 9140	southeast Gulf of Mexico, Atlantic Ocean	KP100598	KP100575	N/A
Alpheus floridanus Kingsley, 1878	ULLZ 6208	southeastern Florida, United States, Atlantic Ocean	KP100597	KP100574	KP100634
Alpheus floridanus Kingsley, 1878	ULLZ 5854	southeastern Florida, United States, Atlantic Ocean	KP100594	KP100571	KP100622
Alpheus floridanus Kingsley, 1878	ULLZ 6207	southeastern Florida, United States, Atlantic Ocean	KP100595	KP100572	KP100633
Alpheus floridanus Kingsley, 1878	ULLZ 6203	southwest Gulf of Mexico, Atlantic Ocean	KP100596	KP100573	KP100635
Alpheus platycheirus Boone, 1927	YPM 6628#/ ULLZ 8630	Siguanea Bay, Isle of Pines, Cuba	KP100617	N/A	N/A
Alpheus platycheirus Boone, 1927	MCZ 4987* (male)/ ULLZ 9139	southeast Gulf of Mexico, Atlantic Ocean	KP100616	N/A	N/A
Alpheus platycheirus Boone, 1927	USNM 1071493/ ULLZ 8627	Gulf of Morrosquillo, Colombia, Caribbean Sea	KP100606	KP100580	N/A
Alpheus platycheirus Boone, 1927	USNM 1071493/ ULLZ 9258	Gulf of Morrosquillo, Colombia, Caribbean Sea	KP100607	KP100581	N/A
Alpheus platycheirus Boone, 1927	ULLZ 6819	northern Gulf of Mexico, Atlantic Ocean	KP100600	N/A	KP100624
Alpheus platycheirus Boone, 1927	USNM 1265090/ ULLZ 5855	northern Gulf of Mexico, Atlantic Ocean	KP100599	N/A	KP100623
Alpheus platycheirus Boone, 1927	ULLZ 10599	northern Gulf of Mexico, Atlantic Ocean	KP100604	KP100589	KP100638
Alpheus platycheirus Boone, 1927	ULLZ 11939	northern Gulf of Mexico, Atlantic Ocean	KP100602	KP100590	N/A
Alpheus platycheirus Boone, 1927	ULLZ 11937	northern Gulf of Mexico, Atlantic Ocean	KP100603	KP100587	KP100637
Alpheus platycheirus Boone, 1927	ULLZ 11936	northern Gulf of Mexico, Atlantic Ocean	KP100601	KP100588	KP100639
Alpheus platycheirus Boone, 1927	CCDB 3888	eastern Brazil, Atlantic Ocean	KP100605	N/A	N/A
Outgroups					
Alpheus carlae Anker, 2012	ULLZ 6631	southeastern Florida, United States, Atlantic Ocean	KP100620	KP100592	KP100626
Alpheus nr. *amblyonyx*	ULLZ 6618	northern Gulf of Mexico, Atlantic Ocean	KP100621	KP100593	KP100625

Molecular analyses

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from the abdomen or eggs under 1 of 3 different extraction protocols. Extraction kits included the Genomic DNA Extraction Kit for Arthropods (Cartagen Cat. No. 20810-050) and Qiagen DNeasy® Blood and Tissue Kit (Cat. No. 69504). For some extractions, we used an isopropanol-

precipitation as follows: Muscle was ground and then incubated for 12h in 600µl of lysis buffer (100mM EDTA, 10mM tris pH 7.5, 1% SDS) at 65ºC; protein was separated by the addition of 200µl of 7.5M ammonium acetate and subsequent centrifugation. DNA was precipitated by the addition of 600µl of cold isopropanol followed by overnight refrigeration (4ºC) and later centrifugation (10-30 min at 14,000 rpm); the resulting pellet was rinsed in 70% ETOH, dried in a speed vacuum system (DNA110 Speed Vac®) and resuspended in 10-50µl of nanopure water (Robles *et al.* 2007).

Three partial mitochondrial genes were selected due to their utility in resolving phylogenetic relationships at low taxonomic levels (Harrison 2004; Lefebure *et al.* 2007; Mantelatto *et al.* 2007; Robles *et al.* 2007; Schubart *et al.* 2000; Stillman & Reeb 2001; Yang *et al.* 2006). These included the 16S large ribosomal subunit of ~550 basepairs (bps), 12S small ribosomal subunit of ~430 bps, and protein coding gene cytochrome oxidase I of ~610 bps (Table 2). Targeted gene regions were amplified by means of the polymerase chain reaction (PCR). The large ribosomal subunit (16S) was amplified with the primers 16SL2, 16S-ar and 1472 to create 1 overlapping region of approximately 550 bps in length (Crandall & Fitzpatrick 1996; Palumbi *et al.* 1991; Schubart *et al.* 2002). The small ribosomal unit (12S) was amplified using the primers 12Sf and 12S1R to create 1 overlapping region of approximately 430 bps in length (Buhay *et al.* 2007) and cytochrome oxidase I was amplified using the primers COI10 and COIf to create 1 overlapping region of approximately 610 bps in length (Baldwin *et al.* 1998; Kessing *et al.* 1989). Additionally, internal "species-specific" 16S and 12S primers were designed to help amplify old material that we could not amplify with the abovementioned primers (Table 2). These primers produced several overlapping regions ranging from approximately 100-200 bps in length each (all primers listed in Table 2). Reactions were performed in 25µl volumes containing 0.5µM forward and reverse primer for each gene, 200µM each dNTP, PCR buffer, magnesium chloride, 5M betaine, 1 unit AmpliTaqGOLD® polymerase, and 30-50ng extracted DNA. The thermal cycling profile conformed to the following parameters: Initial denaturation for 10 min at 94ºC followed by 40 cycles of 1 min at 94ºC, 1.5 min at 46-58ºC, 1.5 min at 72ºC and a final extension of 10 min at 72ºC. PCR products were purified using filters (Microcon-100® Millipore Corp., Billerica, MA, USA or EPOCH GenCatch PCR Clean-up Kit Cat. No. 13-60250) and sequenced with ABI BigDye® terminator mix (Applied Biosystems, Foster City, CA, USA). A Robocycler 96 cycler was used in all PCR and cycle sequencing reactions. Forward and reverse sequencing products were run on a 3100 Applied Biosystems automated sequencer at University of Louisiana at Lafayette or ABI 3730xl DNA Analyzer 96-capillary automated sequencer in the Brigham Young University (BYU) sequencing center.

TABLE 2: 16S, 12S, and COI primers used in this study. References: (1) Palumbi *et al.* 1991, (2) Schubart *et al.* 2002, (3) Crandall & Fitzpatrick 1996, (4) Buhay *et al.* 2007, (5) Kessing *et al.* 1989, (6) Baldwin *et al.* 1998.

Gene	Primer	Primer Pair	Sequence 5'à 3'	Ref.
16S	16S–ar	1472	CGC CTG TTT ATC AAA AAC AT	(1)
16S	16S–L2	1472	TGC CTG TTT ATC AAA AAC AT	(2)
16S	1472	16Sar/16SL2	AGA TAG AAA CCA ACC TGG	(3)
16S	16SHB1	16SHB3	TGA GGT CTG GCC TGC CCA CTG A	New
16S	16SHB3	16SHB1	AAT GCC TTT TCA CAC AAA AGT TAA AT	New
16S	16SHB4	16SHB5	GCG AAG GTA GCA TAG TCA ATA GTC	New
16S	16SHB5	16SHB4	CAA GTT TTA TAG GGT CTT ATC GTC	New
16S	16SHB6	1472	GAA TTT AAC TTT TGT GTG AA	New
12S	12Sf	12S1R	GAA ACC AGG ATT AGA TAC CC	(4)
12S	12S1R	12Sf	AGC GAC GGG CGA TAT GTA C	(4)
12S	12S1FOR	12S1R	TTT GTA TAC CGC CAT TAT TAG	New
12S	12S1REV	12Sf	TAT CTA ATA ATG GCG GTA T	New
COI	COIF	COI10	CCA GCT GGA GGA GGA GAY CC	(5)
COI	COI10	COIF	TAA GCG TCT GGG TAG TCT GAR TAK CG	(6)

Phylogenetic analyses

A phylogenetic approach was selected over alternative species delimitation methods (Ence & Carstens 2011; Masters *et al.* 2011; Yang & Rannala 2010) due to the limited availability of molecular grade materials for inclusion in the analysis (molecular grade material limited to 1 individual each of *A. ulalae* Bracken-Grissom & Felder, 2014 and *A. floridanus africanus* Balss, 1916). Sequences were assembled using the computer program Sequencher 4.7 (GeneCodes, Ann Arbor, MI, USA). Once assembled, sequences were aligned using MUSCLE (multiple sequence comparison by log-expectation) or MAFTT which are found to be more accurate and faster than other alignment algorithms (Edgar 2004). Missing data were designated as a "?" in our alignment. To identify pseudogenes, we followed suggestions by Song *et al.* (2008) which included translating protein-coding sequences (COI) to check for in/dels (insertions/deletions) and stop codons and comparing sequences among conspecifics. In addition, individual gene trees were constructed to compare 16S, 12S, and COI topologies. When multiple genes are combined in phylogenetic analyses, resolution increases; these approaches have gained popularity over single gene studies (Ahyong & O' Meally 2004; Page *et al.* 2008; Porter *et al.* 2005; Robles *et al.* 2007). For these reasons, we concatenated our 16S, 12S, and COI data sets into a single alignment consisting of 1578 bps and 23 ingroup sequences. We conducted a partition test of heterogeneity or incongruence length difference (ILD) test (Bull *et al.* 1993), as implemented in PAUP (Swofford 2003), and results indicated that the 3 gene regions could be combined.

With the development of short internal primers, we were able to successfully amplify a partial 16S sequence for the male syntype of *A. floridanus* and the holotype of *A. platycheirus*. 16S gene sequence was also collected for a single specimen from Brazil. Amplifications of other gene regions (i.e. 12S, COI) for the male syntype of *A. floridanus*, type of *A. platycheirus* and Brazilian material were unsuccessful. To confirm our previous morphological placements of these individuals (Bracken-Grissom & Felder 2014), we present the 16S single gene phylogeny along with the concatenated analysis. The female syntype of *A. floridanus* is included in both the 16S single gene phylogeny and the combined phylogeny since we were able to amplify multiple gene regions.

The model of evolution that best fit the individual data sets (16S, 12S, and COI) was determined by MODELTEST 3.06 (Posada & Crandall 1998) before conducting phylogenetic analyses. Independent models of evolution and parameters were partitioned in the Bayesian (BAY) concatenated analysis. The ML analyses were conducted using RAxML (Randomized Axelerated Maximum Likelihood, Stamatakis *et al.* 2005) with computations preformed on the high performance computing cluster at Florida International University. Likelihood settings followed the General Time Reversible Model (GTR) with a gamma distribution and invariable sites and RAxML estimated all free parameters. Confidence in the resulting topologies was assessed using non-parametric bootstrap estimates (Felsenstein 1985) with 1000 replicates. The BAY analyses were preformed using parameters selected by MODELTEST and conducted in MrBayes v3.2 (Huelsenbeck & Ronquist 2001). A Markov chain Montecarlo (MCMC) algorithm ran for 10,000,000 generations, sampling 1 tree every 1000 generations. Three independent BI runs were performed. Convergence of the Bayesian runs was assessed using Tracer v1.5 (Rambaut & Drummond 2007). Observation of the likelihood (-LnL) scores allowed us to determine burn-ins and stationary distributions for the data. Once the split frequency in each analysis was < 1%, a 50% majority-rule consensus tree was obtained from the remaining saved trees. Posterior probabilities (pP) for clades were compared for congruence and then combined between individual analyses. Bootstrap values >70 for ML and posterior probabilities >90 for BAY are presented on the BAY phylograms (shown as percentages, Figs. 1, 2).

Genetic distances were calculated with the Kimura-2-Parameter distance (K2P) method using Sequencher 4.7 (GeneCodes, Ann Arbor, MI, USA). These distances were used to estimate percent sequence divergence between closely related species. Previous studies on alpheids estimated a 1.5% sequence divergence rate per million years for COI gene sequences (Anker *et al.* 2007).

Results

Concatenated data set (16S, 12S, and COI)

Our combined analysis represented 23 terminals from the *Alpheus floridanus* complex and two outgroups, *A. carlae* and *A.* nr. *amblyonyx*. Initially, we included several outgroups from the genus *Alpheus* and other genera within Alpheidae but ultimately they were removed to preserve the integrity of our alignment. Removal of those outgroups did not affect internal relationships. In total we generated 28 new 16S sequences (~550 bps), 23 new

12S sequences (~430 bps), and 18 new COI sequences (~610 bps). Despite multiple attempts, we were unable to obtain COI sequences for 7 individuals used in the combined molecular analysis. Likewise, we could not obtain 12S sequences for 2 specimens. 16S sequences were obtained for every individual presented in the molecular analysis (Table 1).

The ILD test showed no significant incongruence (P = 0.48) between data sets, so the 16S, 12S, and COI alignments were combined. The optimal model of evolution selected in MODELTEST was as follows: transversion model (TVM) (16S) with gamma-distributed among-site rate heterogeneity and invariant sites; Tamura and Nei model (TrN) (12s) with gamma-distributed among-site rate heterogeneity; Tamura and Nei model (TrN) (COI) with gamma-distributed among-site rate heterogeneity and invariant sites.

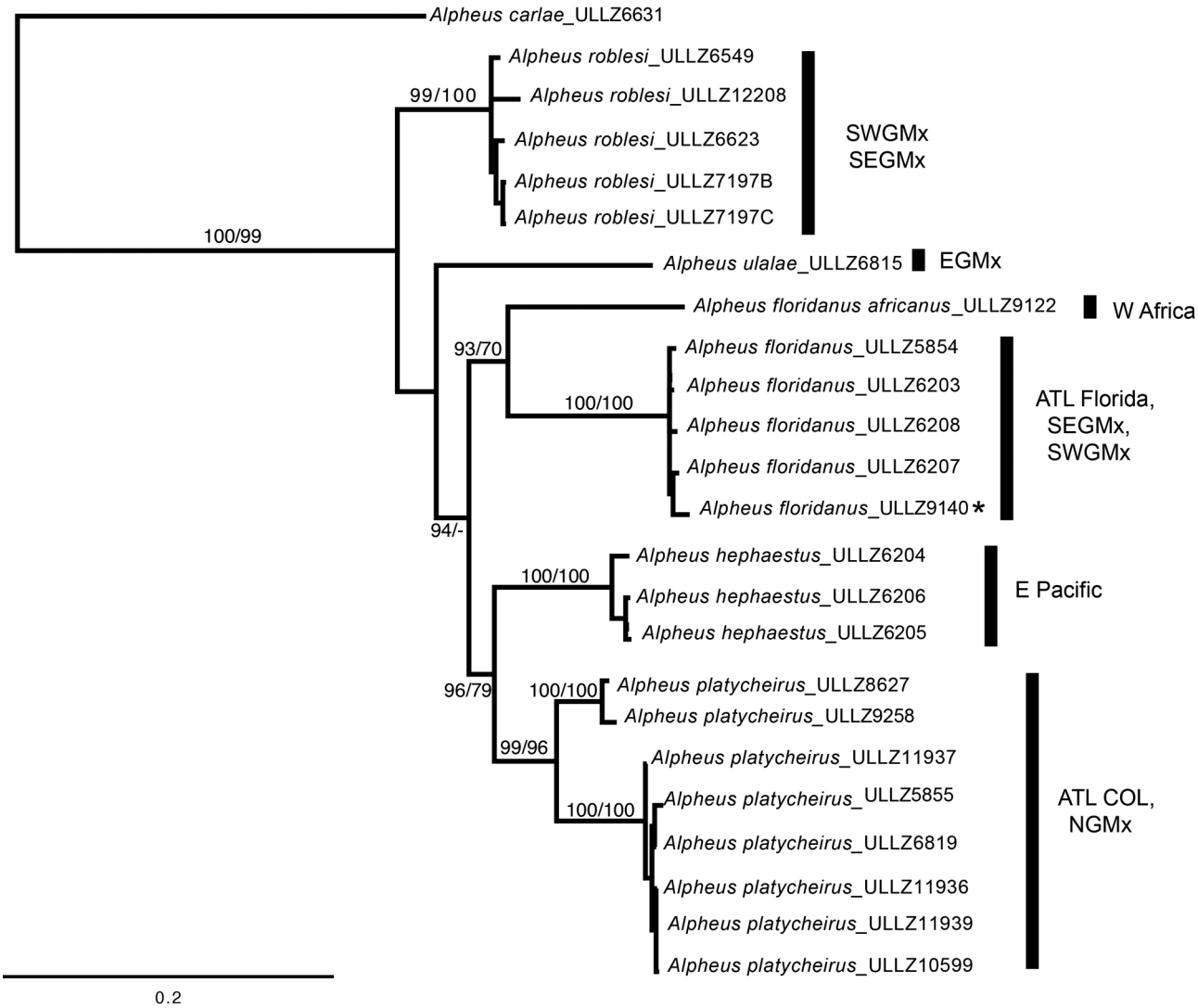

FIGURE 1. Bayesian (BAY) phylogram for *Alpheus floridanus* complex (n = 23) based on a 16S, 12S, and COI concatenated data set. BAY posterior probabilities and Maximum likelihood (ML) bootstrap values noted above branches (BAY/ML). Values >0.7 for ML and >90% for BAY are shown and represented by percentages. Vertical black bars represent the collection locality. NGMx = northern Gulf of Mexico, ATL = Atlantic Ocean, SEGMx = southeastern Gulf of Mexico, SWGMx = southwestern Gulf of Mexico, EGMx = eastern Gulf of Mexico, E Pacific = eastern Pacific, W Africa = western Africa. * = female syntype of *A. floridanus*, Kingsley 1878. One outgroups omitted due to long branch lengths. All catalog numbers represent tissue voucher numbers (ULLZ).

Results strongly supported the formation of four distinct clades and two unique lineages, which we interpreted to represent five species and one subspecies of the *Alpheus floridanus* complex (Fig. 1). Our tree suggested the western Atlantic *A. platycheirus* to be sister to the eastern Pacific *A. hephaestus* Bracken-Grissom & Felder, 2014, in a clade with strong statistical support (96/79). While the combined analyses did not include the Cuban holotype of *A. platycheirus* (for which 12S and COI sequence data were lacking), the two Colombian specimens of *A.*

platycheirus formed a strongly supported subclade that was separated from other members of this species from the northern Gulf of Mexico, thus suggesting geographic population structure within *A. platycheirus*. The clade that included the female syntype of *A. floridanus* included populations ranging from southeastern Florida to the southwestern Gulf of Mexico. The one representative sequenced from western Africa, *A. floridanus africanus* showed a strong affinity with *A. floridanus* (93/70) suggesting a close trans-Atlantic relationship between these two species. Finally, *A. roblesi* Bracken-Grissom & Felder, 2014 and *A. ulalae* Bracken-Grissom & Felder, 2014 were found to be more distantly related to the afore-mentioned species and appear to represent early-branching lineages within the complex.

16S data set

We obtained 16S sequences for all ingroup and outgroup taxa (Table 1, Fig. 2), though we were able to successfully amplify only 170 bps for the male syntype of *A. floridanus* and 228 base pairs for the holotype of *A. platycheirus*. However, these incomplete sequences were included in the 16S single-gene tree phylogeny (Fig. 2), with missing bps designated as a "?" in our alignment. In addition, we successfully obtained a full-length 16S sequence for a Brazilian representative of *A. playtcheirus*, also included in the single gene (16S) phylogeny (Fig. 2).

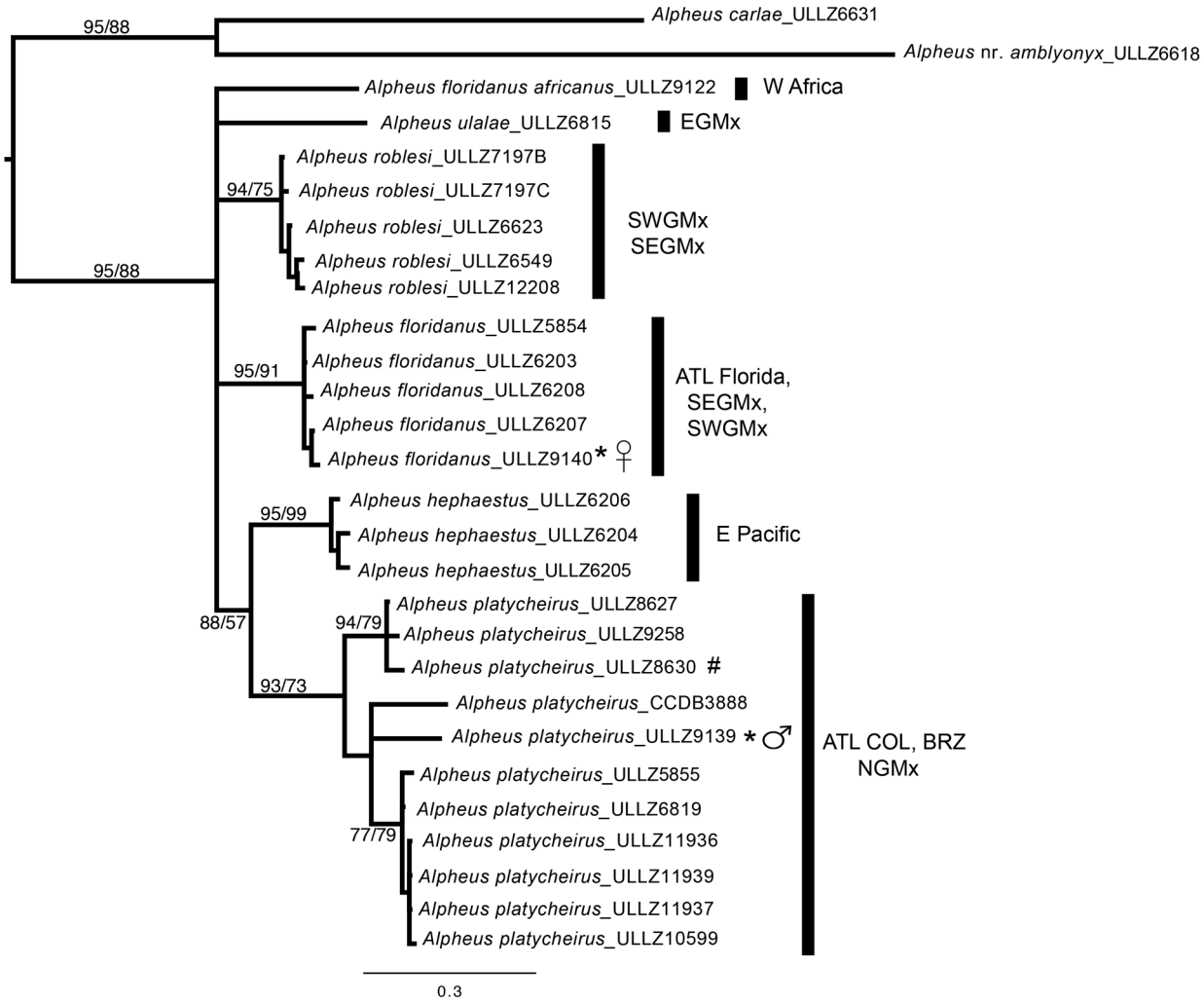

FIGURE 2. Bayesian (BAY) phylogram for *Alpheus floridanus* complex (n = 26) based on 16S sequences. BAY posterior probabilities and ML bootstrap values and are noted above or below branches (BAY/ML). Values >0.5 for ML and >70% for BAY are presented as percentages. Vertical black bars represent the collection locality. NGMx = northern Gulf of Mexico, ATL = Atlantic Ocean, SEGMx = southeastern Gulf of Mexico, SWGMx = southwestern Gulf of Mexico, EGMx = eastern Gulf of Mexico, E Pacific = eastern Pacific, W Africa = western Africa. * = female and male syntypes of *A. floridanus* Kingsley, 1878; #= holotype of *A. platycheirus* Boone, 1927. All catalog numbers represent tissue voucher numbers (ULLZ)

Although our 16S tree was less resolved at deeper nodes, all species-level groupings within the *A. floridanus* complex were strongly supported by bootstrap values and/or posterior probabilities (i.e., those represented by more than one individual), as seen in the combined analysis (Figs. 1, 2). Many of the interspecific relationships were unresolved due to the limited power of using a single gene to resolve deeper relationships. With the addition of more genes, deep relationships become apparent (Fig. 1). Positioning of the male syntype of *A. floridanus* within the *A. platycheirus* clade, which also includes the holotype of *A. platycheirus* from Cuba, is supported (93/73, Fig. 2). Partial 16S sequence data placed the holotype of *A. platycheirus* (YPM 6628/ tissue voucher ULLZ 8630) closer to the Colombian population than to the northern Gulf of Mexico population (Fig. 2).

Discussion

Type material

Our study re-emphasizes the importance of careful examination of type material and the benefit of acquiring sequence data from type specimens, when this can be accomplished non-destructively. In accord with morphological results (Bracken-Grissom & Felder 2014), molecular evidence suggests that Kingsley's syntypes MCZ 4987 represent two different species. Although we have successfully sequenced only 170 bps (16S) for the male syntype, both molecular evidence and morphological characters affiliate it with *A. platycheirus* (Fig. 2). This further compels our designation of the female syntype as the lectotype of *A. floridanus sensu stricto* (Bracken-Grissom & Felder 2014). Molecular evidence allies the lectotype of *A. floridanus* to individuals from the southwestern Gulf of Mexico and southeastern Florida, while morphological evidence suggests a wide distribution of *A. floridanus* throughout the subtropical and tropical western Atlantic (Bracken-Grissom & Felder 2014).

In addition to Kingsley's material, we were able to successfully amplify 228 base pairs of 16S for Boone's holotype of *A. platycheirus* from Isla de la Juventud (= Isle of Pines), Cuba. Based on this sequence, *A. platycheirus* forms a monophyletic clade, which also includes other representatives from Colombia, Brazil, and the Gulf of Mexico. On the basis of its partial 16S sequence, the Cuban type specimen is most closely related to our sequenced Colombian material of this species, which suggests gene flow between populations in Brazil, Colombia, Cuba, and the northern Gulf of Mexico, possibly facilitated by the Loop Current. Our concatenated analysis does suggest two distinct subclades within *A. platycheirus* for populations collected off Colombia and the northern Gulf of Mexico, respectively. A robust morphological examination revealed no diagnostic characters to separate these two subclades, and comparisons of the color patterns was not possible due to the lack of color photographs for the Colombian individuals. Because the known distribution of *A. platycheirus* now appears to extend throughout the western Atlantic Ocean, increased future molecular sampling across the geographic range would allow us to investigate whether or not population structure correlates with biogeographical patterns.

Morphological and molecular comparisons, with geographical considerations

Williams *et al.* (2001) proposed a phylogeny for the genus *Alpheus* wherein the *Alpheus floridanus* species complex was represented by eight terminals from the Caribbean Sea and eastern Pacific Ocean. Examination of available COI data using a combination of BLAST (Basic Local Alignment and Search Tool) and phylogenetic methods (single gene COI tree) was performed to help affiliate specimens sampled in the analyses by Williams *et al.* (2001). Both methods confirmed that two individuals (GenBank nos. AF308987, AF309901, *A. floridanus* 'A') should be identified as *A. hephaestus* (maximum identity ~ 97% in BLAST). Likewise, two individuals (GenBank nos. AF309897, AF309900, *A. floridanus* 'B') were most closely related to *A. platycheirus* (maximum identity ~ 92% in BLAST). Unfortunately, the GenBank sequences are labeled differently (missing Caribbean and Pacific designations in GenBank) than in Williams *et al.* (2001), so a direct correlation cannot be made. Of the remaining four individuals, two (AF309898 and AF309902, *A. floridanus* 'B') show a very weak affinity with *A. ulalae* and two (AF308993, AF309899, *A. floridanus* 'A') cluster with the *A. hephaestus* clade, all with low support. The lack of resolution may be due to missing COI sequences in our data set (see Table 1). The inclusion of additional genes (16S, 12S) would confirm the placement of the Williams *et al.* (2001) specimens, however multiple attempts to locate this material proved unproductive. It is also possible that additional new species exist within the *A. floridanus* complex even though we saw no evidence for this among our available collections.

Our molecular phylogeny proposes a close relationship (sister position) between *A. hephaestus* and *A. platycheirus* (Figs. 1, 2). These two species are similar ecologically, burrowing in offshore muddy bottoms at

depths ranging from 12 to 122 m, where they are collected with trawls, box corers, or dredges. These species also share similarities in morphological traits, such as the length and shape of the antennal scale, structure of the mandible, and elongate major and minor chelae (Bracken-Grissom & Felder 2014; Chace 1972). Applying a molecular clock calculated in previous studies for alpheids (1.5% divergence per million years in COI sequences; (Anker *et al.* 2007; Knowlton & Weigt 1998; Knowlton *et al.* 1993), suggests that the species pair *A. hephaestus / A. platycheirus** diverged many years prior to the closing of the Panamanian Isthmus, approximately 9.1 mya.

While the length and shape of the scaphocerite could suggest an affinity among *A. hephaestus*, *A. platycheirus,* and *A. floridanus africanus*, molecular data instead place *A. floridanus africanus* as the sister species to *A. floridanus.* Although these two species are geographically separated, their genetic affinity is not surprising given a number of closely related decapod species that reside on either side of the Atlantic Ocean (Bracken-Grissom & Felder 2014; Chace & Manning 1972; Wirtz 2004). These often have broad distribution ranges and the potential for interbreeding, perhaps facilitated by the strong westward equatorial currents of the Atlantic Ocean. Many trans-Atlantic sister species pairs are known in mollusks (Lapegue *et al.* 2002), crabs (Weinberg *et al.* 2003), and other groups of snapping shrimps (Anker *et al.* 2008a; 2008c).

The distribution range of *A. floridanus* and *A. platycheirus* in the western Atlantic may be at least in part determined by the Loop Current, which travels northward, entering the Gulf of Mexico through the Yucatan Channel and exiting through the Florida Straits. This current plays a major role in the circulation and transport of water throughout the southeastern Gulf of Mexico and has been suggested to influence the distribution of other species in the region (Bangma & Haedrich 2008; Williams & Williams 1981). One species known so far from only the eastern Gulf of Mexico, *A. ulalae*, represents a distinct lineage in our concatenated molecular analysis (Fig. 1). Undoubtedly, *A. ulalae* is genetically distinct from all other species of *Alpheus* within the *A. floridanus* complex, and morphological differences have been documented (Bracken-Grissom & Felder 2014). The amphi-Atlantic *A. roblesi* is morphologically the most distinctive species within the *A. floridanus* complex and also the earliest branching lineage within the group (Fig. 1), being characterized by a sinuous shape to the scaphocerite margin, presence of conspicuous spines along the propodus of the fifth pereopod (rarely lacking), and less elongate chelae than in the compared species (Bracken-Grissom & Felder 2014).

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