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Identifying lineages of *Alpheus angulosus* McClure, 2002 (Caridea: Alpheidae) in South Carolina, USA

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ABSTRACT

The *Alpheus armillatus* H. Milne Edwards, 1837 species complex contains several cryptic species of snapping shrimps including *Alpheus angulosus* McClure, 2002. A previous study of the phylogeography and coloration of the *A. armillatus* species complex found three distinct lineages of *A. angulosus* distributed throughout the Gulf of Mexico, Caribbean, and Western Atlantic, with the Atlantic coastline of North America less thoroughly studied. We sequenced a portion of the mitochondrial cytochrome oxidase I gene of 20 individuals of *A. angulosus* from Charleston, South Carolina, USA to determine the lineages present. We found two of the three lineages of *A. angulosus*; nineteen specimens were found to be within a subtropical clade, whereas one was within a clade previously described as having only a Caribbean distribution. We found that body coloration was not consistent with biogeographical patterns observed in previous studies, which suggests that coloration should only be used in conjunction with genetics and other morphological characters when determining identities of cryptic species.

Key Words: biogeography, cryptic species, phylogeography, phylogenetics, snapping shrimps

Cryptic species are common throughout metazoan taxa (Bickford *et al.*, 2007; Pfenninger & Schwenk, 2007), including many lineages of alpheid snapping shrimps (Knowlton & Keller, 1983; Almeida *et al.*, 2014). New cryptic species within the *Alpheus armillatus* H. Milne Edwards, 1837 species complex have been identified based on both morphological and genetic data (Anker, 2012). These species identifications stemmed from a study by Mathews & Anker (2009), which used mitochondrial 16S (mt16S) and cytochrome oxidase I (mtCOI) gene markers and color pattern to better understand the phylogeography of members of the species complex. They found that the *Alpheus* lineages within this complex represent many divergence events within multiple independent lineages across the Isthmus of Panama (Mathews & Anker, 2009).

One member of this complex is *Alpheus angulosus* McClure, 2002, an intertidal and subtidal species distributed throughout the Caribbean and Gulf of Mexico, extending southward to the Atlantic coast of northern South America and northward along the Atlantic coast of North America (McClure, 1995, 2002; Mathews & Anker, 2009; Anker, 2012). Previous phylogenetic analyses revealed that *A. angulosus* occupied three clades in a larger paraphyletic clade of the *A. armillatus* species complex (Mathews & Anker, 2009). Each clade corresponded to specimens from different geographic locations: a subtropical clade from the northern Gulf of Mexico to Florida and presumed to extend to North Carolina (Anker, 2012), a tropical clade from southern Florida to the Caribbean Sea (Costa Rica, Panama, Cuba, Jamaica, and Puerto Rico), and a Brazilian clade (Mathews & Anker, 2009; Anker, 2012), with only a small number of specimens examined from each clade (N = 5, 11, 2, respectively). All specimens of the species shared the same general coloration pattern, described as brown to green or yellowish with bluish antennae (McClure, 1995; Mathews & Anker, 2009; Anker, 2012), with different variations of these colors found in different locations (Anker, 2012).

Only one previous phylogeographic study included a single specimen of *A. angulosus* north of Florida, USA (Beaufort, North Carolina; McClure & Greenbaum, 1999). In order to determine the lineages of *A. angulosus* present in South Carolina, we used genetic tools, which allow for the elucidation of cryptic species when morphological characters are not sufficient. We collected male and female specimens from Charleston Harbor, Charleston, South Carolina, and examined the phylogenetic relationships among the South Carolina specimens and those from previous studies using mtCOI sequencing data, in addition to examining body coloration.

Snapping shrimp (11 males and 9 females) morphologically identified as *A. angulosus* (McClure, 1995, 2002) were collected in June 2015 from an intertidal mudflat in Charleston Harbor, South Carolina (32.7524°N, 79.8975°W). Shrimp were collected at low tide by flipping over substrate and captured with a net. The specimens were brought back to Grice Marine Laboratory where body coloration was recorded and photographs were taken. The shrimp were preserved in 95% ethanol and refrigerated until DNA extraction.

Genomic DNA was extracted from pereiopod tissue from ethanol-preserved shrimp using a NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. Primers COIa (5′-AGTATAAGCGTCTGGGTAGTC-3′) and COIf (5′-CCTGCAGGAGGAGGAGAYCC-3′) (Palumbi *et al.*, 1991) were used to amplify a portion of the mtCOI gene. PCR reactions were performed in 20 µl volumes that included 1x Promega GoTaq buffer (Madison, WI, USA), 3.0 mM $MgCl_2$, 1.0 µl bovine serum albumin, 0.2 mM dNTPs, 0.2 µM of each primer, 0.5 U Promega GoTaq DNA polymerase, and 2.0 µl genomic DNA. PCR conditions included an initial denaturation of 2 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 60 s, and a final extension for 10 min at 72 °C. Amplified products were then treated with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) following manufacturer's instructions, and products were sent to Eurofins MWG Operon LLC (Louisville, KY, USA) for direct, bi-directional sequencing using the same primers as above.

Forward and reverse complementary sequences were assembled to form consensus sequences when both directions were successfully sequenced using Sequencher 5.3 (Gene Codes Corporation, Ann Arbor, MI, USA); all sequences were subsequently compared to their chromatograms and edited accordingly. mtCOI sequences from Mathews & Anker (2009) were retrieved from GenBank and aligned with sequences from the present study. Based on a preliminary neighbor-joining tree, the following sequences were selected to be included with our sequences for further phylogenetic analyses: all sequences from clade 2 (*Alpheus angulosus*: FJ528493–FJ528510; *Alpheus carlae* Anker, 2012: FJ528492, FJ528511–FJ528515; *Alpheus martini* Kim & Abele, 1988: FJ528491); two sequences each from clade 1 (*Alpheus amarillo* Anker, 2012: FJ528485–FJ528486), clade 3 (*Alpheus mathewsae* Anker, 2012: FJ528520–FJ528521), clade 4 (*Alpheus cf. tenuis*: FJ528531–FJ528532), clade 5 (*Alpheus armillatus*: FJ528542–FJ528543), and clade 6 (*Alpheus lancirostris* Rankin, 1900: FJ528546–FJ528547); and a pair of outgroup sequences (*Alpheus viridari* (Armstrong, 1949): FJ528483–FJ528484). The species names used throughout the study reflect the ones used in Anker (2012), a study that reclassified some species in the complex and described new species. Sequences were aligned using MUSCLE (Edgar, 2004) in MEGA6 (Tamura *et al.*, 2013) using default parameters and trimmed so that sequences were of equal length. jModelTest 2.1.7 (Guindon & Gascuel, 2003; Darriba *et al.*, 2012) was used to determine that the Tamura-Nei plus gamma model (gamma shape = 0.1300; Tamura & Nei, 1993) was the best nucleotide substitution model for the data. Both Bayesian and maximum-likelihood (ML) methods were used for phylogenetic analyses of the mtCOI sequencing data using the Tamura-Nei plus gamma model. ML analysis was performed using PhyML 3.0 (Guindon *et al.*, 2010) with 1000 bootstrap replicates. For the Bayesian analyses, MrBayes 3.2.4 (Huelsenbeck & Ronquist, 2001; Ronquist *et al.*, 2012) was run for 10 million generations with sampling every 1000 generations, and the first 25% of the trees were discarded as burn-in. Pairwise distances within and between phylogenetic clades were conducted in MEGA6 (Tamura *et al.*, 2013).

Putative *A. angulosus* mtCOI sequences were generated for all 20 specimens; 8 of 20 were successfully sequenced in both directions (specimens 2, 5, 8, 9, 12, 14, 19, 20), whereas the remaining specimens yielded unidirectional sequence. Sequences were deposited into GenBank (accession numbers KX587512, KX687135–KX687153). Nine of the specimens shared an identical sequence in the 401 base-pair alignment (specimens 2–10; Fig. 1). Bayesian and ML analyses revealed that all 20 specimens from our study were within the same clade as those in what Mathews & Anker (2009) called clade 2 (98 poster probability (PP), 91% bootstrap support; Fig. 1). Our Bayesian analysis revealed only four strongly supported clades within clade 2 instead of five, with no distinct 2b (Fig. 1). When examining pairwise distances, 19 specimens were more similar to clade 2b specimens from Mathews & Anker (2009) than they were to specimens from the other clades. Pairwise distances ranged from 0.000–0.027 among the specimens in the present study and clade 2b specimens from Mathews & Anker (2009), which was lower than the pairwise distances between specimens from clade 2b (and our specimens) and the specimens within other clades (Table 1). We referred to this group of specimens as "2b" (Fig. 1, Table 1). A single male specimen formed a monophyletic clade with clade 2d specimens from Mathews & Anker (2009) (99 PP, 70% bootstrap support; Fig. 1).

A. angulosus from South Carolina had a variety of light and dark body colors ranging from green to blue to brown, similar to the colors previously described. These specimens, however, had greenish-brown/orange antennae, which differed from the bluish antennae previously described in Mathews & Anker (2009) and Anker (2012).

The phylogenies generated using partial mtCOI sequences confirm the presence of two lineages of *A. angulosus* from the *A. armillatus* species complex in South Carolina. While most of the specimens from the present study were similar to specimens from subtropical regions (clade 2b), one specimen was within a clade of *A. angulosus* from tropical locations (clade 2d). Previous specimens in this tropical 2d clade were primarily collected from the Caribbean; however, one member of this clade was from the same Atlantic coast location in Florida as members of the subtropical 2b clade (Mathews & Anker, 2009: fig. 4). The presence of two lineages in two locations (Florida and South Carolina) suggests that mitochondrial polymorphism was maintained within each population. It could have been difficult for Mathews and Anker (2009) to detect such within-population polymorphism because of their small sample sizes for each clade of *A. angulosus*.

While the divergence between sister clades 2b-e and subsequent range expansion led to the apparent geographic structuring between *A. angulosus* clades 2b and 2d and the Brazilian clade 2e (Mathews & Anker, 2009), the presence of clade 2b and 2d specimens in the same locations both in South Carolina and the Atlantic coast of Florida suggests that geographic isolation is no longer a factor contributing to the divergence between clades 2b and 2d of *A. angulosus*. While it is possible that the two populations were historically separated, and are now making secondary contact (Anker, 2012), it is equally plausible that these clades reflect no reproductive isolation and reflect within-population polymorphism.

While the body colorations of the shrimp were a variety of shades of green, blue, and brown, most of the shrimp had pale red pereiopods with occasional blue/grey sections and greenish-brown/orange antennae. These body and pereiopod colors were consistent with those described previously (McClure, 1995; Mathews & Anker, 2009; Anker, 2012), but the antennae of our specimens differed from the bluish antennae found by Mathews & Anker (2009) and Anker (2012). A range of both light and dark shades of green to brown colored shrimp were found in one location, with all of the darker shrimp being females. This differs from the hypothesis that darker (dark-green to brown-green) body colorations are found from Florida to North Carolina and northern Gulf of Mexico, with lighter colors found from south Florida to the Caribbean (Anker, 2012). It thus appears that overall body coloration is variable and is not a reliable characteristic for discriminating shrimp from different locations or lineages and may also vary between sexes.

We have confirmed the presence of two lineages of *A. angulosus* in South Carolina, but more extensive sampling throughout the geographic range of *A. angulosus* could reveal whether these lineages represent reproductively-isolated populations or within-population polymorphism. We also found that *A. angulosus* individuals from South Carolina have a range of body colorations, suggesting that coloration should only be used in conjunction with genetics and other morphological characters when determining identities of cryptic species.

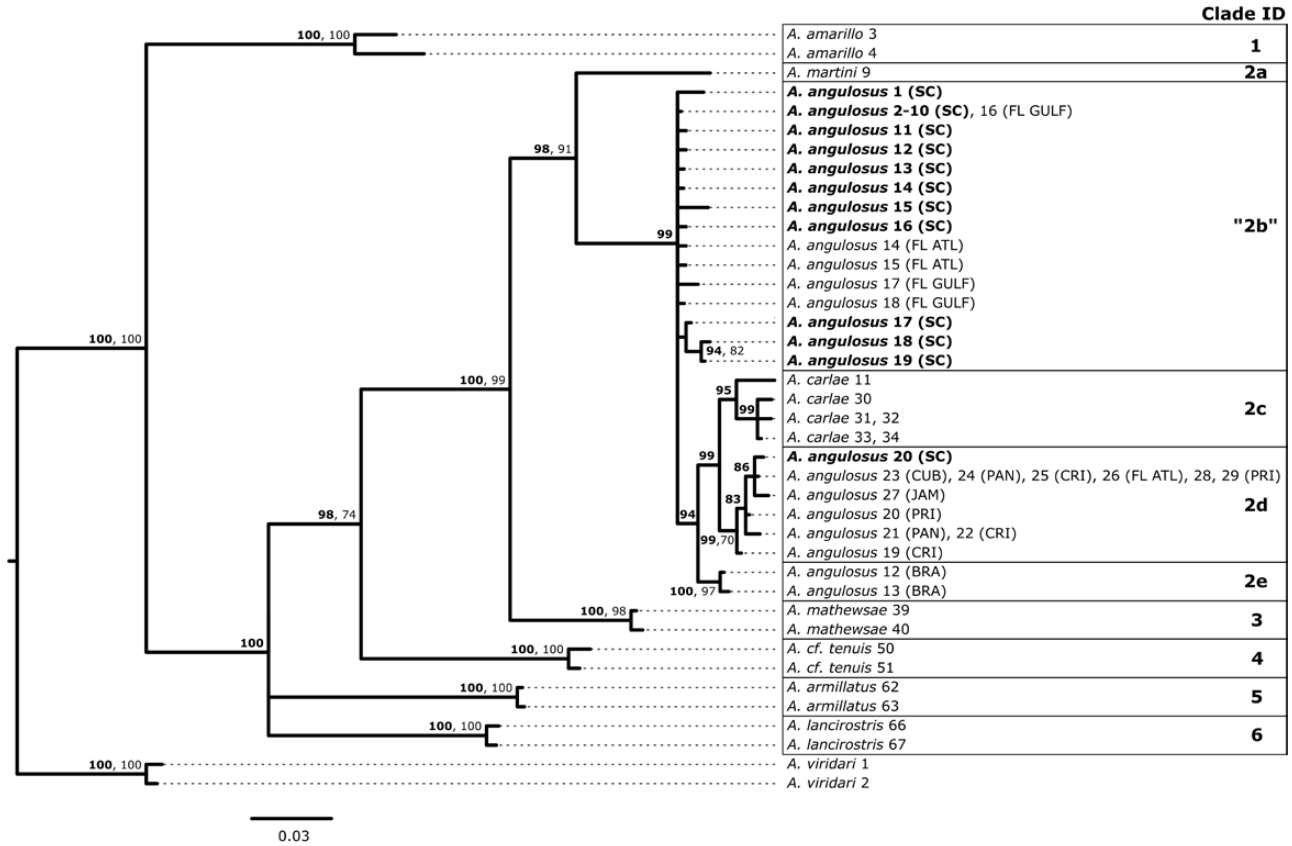

Figure 1. Bayesian phylogram generated from a 401 base-pair alignment of partial cytochrome oxidase I sequences from 20 specimens of *Alpheus angulosus* from the present study and 37 specimens within the *Alpheus armillatus* species complex retrieved from GenBank. *Alpheus viridari* was selected as the outgroup. Clade identifications come from Mathews & Anker (2009), with "2b" referring to the group including clade 2b specimens from Mathews & Anker (2009), and 19 specimens from the present study that were most similar to clade 2b. Bolded taxa indicate sequences generated in the present study. Numbers that follow each taxon refer to the specimen numbers either in Mathews & Anker (2009) or the present study. Collection locations are also listed for *A. angulosus* specimens: BRA: northern coast of Brazil; CRI: Caribbean Sea coast of Costa Rica; CUB: Caribbean Sea coast of Cuba; FL ATL: Atlantic coast of Florida, USA; FL GULF: Gulf coast of Florida, USA: JAM: western coast of Jamaica; PAN: Caribbean Sea coast of Panama; PRI: Atlantic coast of Puerto Rico; and SC: South Carolina. Bolded numbers next to the nodes indicate Bayesian posterior probabilities and unbolded numbers indicate maximum likelihood bootstrap support values; only values ≥ 70 are shown.

Table 1. Pairwise mean genetic distances between clades within clade 2 of the *Alpheus armillatus* McClure, 2002 species complex computed using default parameters in MEGA6 (Tamura *et al.*, 2013). There were a total of 28 sequences and 401 positions in the final dataset that included sequences from the present study and those retrieved from GenBank from Mathews & Anker (2009). Pairwise mean genetic distances are shown below the diagonal and standard error estimates are shown above the diagonal. Clade identifications follow Mathews & Anker (2009), with clade "2b" referring to the group including clade 2b specimens from Mathews & Anker (2009) and 19 specimens from the present study that were most similar to clade 2b.

	2a	"2b"	2c	2d	2e
2a		0.203	0.266	0.337	0.230
"2b"	0.327		0.015	0.013	0.011
2c	0.402	0.048		0.011	0.015
2d	0.465	0.040	0.033		0.012
2e	0.364	0.028	0.046	0.033	

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